The paradox of the retinoic acid receptor beta 2 in cancer

by

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ABSTRACT

INTRODUCTION: Recent lines of evidence suggest that retinoids[1,2] and RARβ2[3] may have both beneficial and harmful effects in cancer. Little is known regarding the specifics of RARβ2 promoter silencing in cancer, or about the role that RARβ2 plays in RARβ2-expressing cancer cells. **OBJECTIVES**: To analyze the patterns and heritability of RARβ2 promoter methylation in cancer and determine whether it is subject to allelic bias; and to analyze the effects of RARβ2 knockdown on growth and mRNA expression profiles in cancer. **METHODS**: RARβ2 promoter methylation was analyzed in 20 parental cancer cell lines and 5 subcloned lines using bisulfite genomic sequencing (>150 sequencings); the proportion of methylated alleles was estimated using methylation-sensitive restriction enzyme digestion followed by PCR and single nucleotide polymorphism identification; 18 antisense oligonucleotides against RARβ2 were tested in various cancer cell lines using RT-PCR, cell counting and annexin V staining; and gene expression profiles were compared following knockdown versus *all trans* retinoic acid (ATRA) stimulation using cDNA microarray technology (>14,000 genes), SOURCE and GOMINER databases. **RESULTS**: Hypo- and hypermethylated alleles frequently co-exist in lines in which RARβ2 is inactivated (5/11, 45%); divergent methylation is heritable in the majority of subclones analyzed (6/8, 75%); and methylation is subject to allelic bias at a ratio of ~2:1 (3/3 CpG sites, 100%). Cellular proliferation is correlated with RARβ2 expression levels (p=0.0003); the most effective oligo reduces cellular proliferation by up to 80% in cancer cell lines in which RARβ2 expression has been retained (3/3, 100%), but has no apparent effects in lines in which it has been lost (3/3, 100%); reduction in cell growth following oligo treatment is at least partially due to activation of programmed cell death; and over a dozen pro-carcinogenesis genes are downregulated following RARβ2 knockdown, whereas half are upregulated following ATRA stimulation. **CONCLUSIONS**: This work is the first to report the co-existence and heritability of hypo- and hypermethylated allelic copies of a gene completely inactivated in cancer; that hypermethylation in cancer is subject to allelic bias; that a promoter methylation-independent *cis*-acting silencing mechanism is involved in RARβ2 inactivation; and that RARβ2 expression may be necessary for growth and oncogenic phenotype of certain RARβ2-expressing cancer cells. These findings help explain the paradoxical effects of retinoids and RARβ2 in cancer.

[1]Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, et al. Risk factors for lung cancer and for intervention effects in CARET, the Beta-Carotene and Retinol Efficacy Trial. J Natl Cancer Inst 1996;88:1550-9.

[2]Anon., The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. N Engl J Med 1994;330:1029-35.

[3]Khuri FR, Wu H, Lee JJ, Kemp BL, Lotan R, Lippman SM, et al. Cyclooxygenase-2 overexpression is a marker of poor prognosis in stage I non-small cell lung cancer. Clin Cancer Res 2001;7:861-7.

SOMMAIRE

INTRODUCTION : Des études récentes suggèrent que les rétinoïdes[1, 2] et RARβ2[3] ont des effets à la fois bénéfiques et néfastes dans le cancer. Peu est connu vis-à-vis des mécanismes d'inactivation du promoteur de RARβ2 dans le cancer, et vis-à-vis du rôle de RARβ2 dans les cellules cancéreuses ayant gardé l'expression de RARβ2. **OBJECTIFS** : Analyser les patrons de méthylation du promoteur de RARβ2 et leur transmission mitotique ; déterminer si cette méthylation est affectée par un biais allélique; et analyser les effets de la diminution de l'expression de RARβ2 sur la croissance et le profil d'expression ARNm dans le cancer. **MÉTHODES** : Le patron de méthylation du promoteur de RARβ2 a été analysé dans 20 lignées cellulaires cancéreuses parentales et 5 lignées sous-clonées utilisant le séquençage génomique aux bisulfites (>150 séquençages); la proportion d'allèles méthylés a été estimée utilisant la digestion par enzymes sensibles à la méthylation suivie par PCR et identification par polymorphisme nucléotidique simple. 18 oligonucléotides antisens contre RARβ2 ont été testés utilisant le RT-PCR, le décompte cellulaire et la coloration contre l'annexine V; et les profils d'expression suivant l'inhibition de RARβ2 versus la stimulation par l'acide rétinoïque *all-trans* (ATRA) ont étés comparés par la technologie des biopuces ADNc (>14 000 gènes), et les bases de données SOURCE et GOMINER. **RÉSULTATS** : Des allèles hypo- et hyperméthylés co-existent fréquemment dans les lignées inactivées (5/11, 45 %) ; cette divergence de méthylation est conservée dans la majorité des sous-clones analysés (6/8, 75 %) ; et cette divergence reflète une préférence allélique ~2:1 (3/3 sites, 100 %). La prolifération est corrélée avec l'expression de RARβ2 (p=0.0003) ; l'oligonucléotide le plus puissant réduit la prolifération cellulaire jusqu'a 80 % dans les lignées cellulaires exprimant RARβ2 (3/3, 100 %), mais n'a aucun effet apparent dans les lignées inactivées (3/3, 100 %) ; l'expression d'une douzaine de gènes impliqués positivement dans la carcinogenèse est diminuée suite au traitement par l'oligonucléotide antisens, tandis que l'expression de la moitié de ceux-çi est augmentée suite à la stimulation par ATRA. **CONCLUSIONS** : Ces études sont les premières à documenter la co-existence et la transmission d'allèles hypo- et hyperméthylés d'un gène complètement inactivé dans le cancer ; que l'hyperméthylation dans le cancer est influencée par une préférence allélique ; qu'un nouveau mécanisme d'inactivation indépendant de la méthylation et agissant en *cis* est impliqué dans la répression de RARβ2 ; et que le potentiel d'expression de RARβ2 est nécessaire pour la croissance ainsi que le phénotype oncogénique de certains cancers. Ces découvertes offrent quelques explications sur pourquoi les effets des rétinoïdes et de RARβ2 dans le cancer sont si paradoxaux.

[1]Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, et al. Risk factors for lung cancer and for intervention effects in CARET, the Beta-Carotene and Retinol Efficacy Trial. J Natl Cancer Inst 1996;88:1550-9.

[2]Anon., The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. N Engl J Med 1994;330:1029-35.

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ABBREVIATIONS

AF-1	activator function-1
ALDH-1	aldehyde dehydrogenase-1
AP-1	activator protein-1
APC	antigen-presenting cell
ATRA	all-trans-retinoic acid
BGS	bisulfite genomic sequencing
brdU	bromodeoxyuridine
cDNA	complementary DNA
CGH	comparative genomic hybridization
CK	cytokine
CNS	central nervous system
Cox-2	cyclooxygenase-2/phospholipase A_2
CpG	cytosine-guanine dinucleotide
CRABP	cellular retinoic acid binding protein
CRBP	cellular retinol binding protein
CRE	cAMP response element
CREB	cAMP response element binding protein
Cyp24	cytochrome protein 24/vitamin D3 hydroxylase
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DR	direct repeat
EGFP	enhanced green fluorescence protein
ET-1	endothelin-1
FGF-4	fibroblast growth factor receptor-4
FISH	fluorescence *in situ* hybridization
HDAC	histone deacetylase
HPLC	high performance liquid chromatography
HSC	hepatic stellate cells
ICAM-1	intercellular adhesion molecule-1
IGFBP-3	insulin-like growth factor binding protein-3
IRES	internal ribosomal entry site
LOH	loss of heterozygosity
MAGI	methylation-associated gene inactivation
MeCP	methyl-CpG-binding protein
MHC I	major histocompatibility complex class I

mRNA	messenger ribonucleic acid
MSP	methylation-specific PCR
N-CoR	nuclear repressor corepressor
NSCLC	non-small cell lung carcinoma
Oligo	oligodeoxynucleotide
PBMC	peripheral blood mononuclear cells
P/CAF	p300/CBP-associated factor
PCR	polymerase chain reaction
Pu	purine
Py	pyrimidine
RA	retinoic acid
RAE	retinol activity equivalent
RAR	retinoic acid receptor
RARβ2	RAR isoform β2
RARE	retinoic acid response element
RBP	retinol binding protein
RNA	ribonucleic acid
ROH	retention of heterozygosity
ROS	reactive oxygen species
RXR	retinoid x receptor
SAM	s-adenosylmethionine
SCD	short-chain dehydrogenases
SCLC	small cell lung carcinoma
SKY	spectral karyotyping
SMRT	silencing mediator for retinoid and thyroid receptors
SNP	single nucleotide polymorphism
TAF	TBP-associated factor
TF	transcription factor
TSA	trichostatin A
TSG	tumor suppressor gene
THRβ2	thyroid hormone receptor isoform β2
TIF	transcription intermediary factor
TUBA3	tubulin α3
VAD	vitamin A deficiency
VDRE	vitamin D responsive element
5'-azaCdR	5'-aza, 2'-deoxycytidine
5'UTR	5' untranslated region

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF MANUSCRIPTS/PUBLICATIONS HEREIN

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CONTRIBUTIONS OF CO-AUTHORS

Mark Basik, M.D. (Lady Davis Institute, Jewish General Hospital, Montreal, Canada) performed the cDNA microarray experiments and some of the data mining using GoMiner[4] and SOURCE[5] databases, and reviewed the antisense manuscript (Chapter 3).

W.E.C. Bradley, Ph.D. (Institut du cancer de Montréal, Hôpital Notre-Dame, Montreal, Canada) generated the MDA-MB-231 and LS-180 subclones (Chapter 2), helped with microscopy and cell counting (Chapter 3), and reviewed the manuscripts.

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OTHER ASSISTANCE

Marc Dumont, Ph.D. (Hôpital Notre-Dame, Montreal, Canada) performed the statistical analysis shown in Fig. 29, p. 181 (Chapter 3).

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[4]Zeeberg, B. R., Feng, W., Wang, G., Wang, M. D., Fojo, A. T., Sunshine, M., Narasimhan, S., Kane, D. W., Reinhold, W. C., Lababidi, S., Bussey, K. J., Riss, J., Barrett, J. C. & Weinstein, J. N. (2003) *Genome Biology* 4, R28. (http://discover.nci.nih.gov/gominer/)

[5]Diehn, M., Sherlock, G., Binkley, G., Jin, H., Matese, J. C., Hernandez-Boussard, T., Rees, C. A., Cherry, J. M., Botstein, D., Brown, P. O. & Alizadeh, A. A. (2003) *Nucl. Acids. Res.* **31**, 219-223. (http://source.stanford.edu/cgi-bin/source/sourceSearch)

To H.S.A. and M. A.

FOREWORD

My aspiration as I began this project was to explore the depths of lung cancer biology and the roles of retinoid receptors, especially RARβ2, in carcinogenesis. This is what I advance: the works herein are the first to report the *co-existence* and *heritability* of hypo- and hypermethylated allelic copies of a gene that is completely inactivated in cancer; that a novel promoter methylation-independent mechanism is responsible for silencing the hypomethylated allele; that hypermethylation in cancer is subject to allelic bias, akin to mechanisms of *de novo* imprinting; and that RARβ2 expression, thought to be solely beneficial, may be necessary for the growth and oncogenic phenotype of certain RARβ2-expressing cancer cells. Together, these findings suggest that DNA methylation, a reversible process, may be a mechanism that allows RARβ2 reactivation at alternate points along the carcinogenic pathway, and alternation of this kind has not been reported before. This elucidation of duality of function may have wide implications in cancer research since it helps explain one potential source from which the inconsistent findings regarding the paradoxical effects, beneficial and harmful, even deadly, of retinoids/carotenoids and RARβ2 in cancer may come. My aspiration as I end this project is that these works will form practical building blocks that may be utilized in the continued efforts toward improving cancer detection and care.

This thesis was written in accordance with the Faculty of Graduate and Postdoctoral Studies "Guidelines for Thesis Preparation and Submission" (http://www.mcgill.ca/gps/programs/thesis/guidelines/) as they pertain to manuscript-based theses.

CHAPTER 1

INTRODUCTION

1 Lung cancer epidemiology and pathology

Current lung cancer statistics show that there has never been a more dire need for progress in cancer research - lung cancer in particular - whether in Canada, the United States, or Worldwide. The present section offers an outline of up-to-date lung cancer epidemiological and pathological findings in light of the impact that certain discoveries have had or might have on the guidelines used in clinical pathology and oncology.

1.1 Lung cancer epidemiology

1.1.1 Lung cancer incidence and mortality in North America

Cancer (all types) is currently the second leading cause of death (Table I) after heart disease in North America (*1,2*), and it is generally accepted that smoking is the leading cause of lung cancer [for excellent reviews see references (*3,4*)]. It is of interest to note that tobacco smoke is also correlated with several other leading causes of death, including both heart and cerebrovascular diseases. In addition, ambient air pollution, which consists of ultrafine particulate matter (i.e. <10 nm aerodynamic diameter; PM10) and other particulate matters are presently gaining popularity in the field of lung cancer epidemiology, and have been found to be associated with other leading causes of death as well, including heart attacks, strokes, and chronic obstructive pulmonary disorder (5).

TABLE I. Causes of mortality in Canada and the U.S. in 2000. Based on references (*1,2*). *Correlated with tobacco smoke; #Correlated with pollution.

Rank	Disease	Mortality (%) Canada	U.S.
1	Heart disease*#	30	30
2	Cancer*	23	23
3	Cerebrovascular disease*#	7	7
4	Chronic lower respiratory disease*#	5	5
5	Accidents (Involuntary death)	4	4
6	Diabetes mellitus (Type II) *	3	3
7	Influenza/Pneumonia	3	3
8	Alzheimer disease	2	2
9	Nephritis	2	2
10	Septicemia	1	1

Cancer of the lung is currently the leading cause of cancer-related deaths (Table II) and has maintained this rank since the 1950's in men and the 1980's in women. It is the second leading type of newly diagnosed cancer (15.1%) after breast cancer (15.2%). Its incidence in men reached its peak in 1984, nearing 100 new cases per 100,000 inhabitants, whereas its incidence in women is still rising at an alarmingly rapid rate, reaching 45 new cases per 100,000 in 2003. The number of mortalities due to lung cancer has been steadily decreasing for men since the early 1980's, but is rising in women. The precise reasons for these differences are still unclear; however studies concerned with sex and gender differences are currently underway. Lifestyle and environment likely play prominent roles as both Canadian and American societies, which share highly similar environmental factors, also share highly similar incidence and mortality rates.

TABLE II. Rates of cancer deaths at the four leading anatomical sites in Canada and the U.S. in 2003. Based on references (*1,2*).

Cancer site	Death rate (%)			
	Men		Women	
	Canada	U.S.	Canada	U.S.
Lung & bronchus	31	31	25	25
Prostate or breast	12	10	17	15
Colon and rectum	12	10	12	11
Pancreas	4	5	5	6

1.1.2 Lung cancer incidence and mortality worldwide

Lung cancer is currently the ninth leading cause of worldwide death (Table III) after heart disease, cerebrovascular disease, various types of childhood and adult infective diseases, and chronic obstructive pulmonary disease (COPD). It is estimated to cause the death of more than 1.2 million people per year (6).

Tobacco smoking is most strongly associated with the development of a cancer in the lung; it has been estimated that 85-90% of all lung cancers are due to smoking (*7*). In addition, the International Agency for Research in Cancer (IARC) has estimated that there is sufficient evidence to establish a positive correlation between tobacco exposure

and cancer at fifteen other sites, including the oral and nasal cavities, paranasal sinuses, nasopharynx, oropharynx, hypopharynx, larynx, esophagus, stomach, pancreas, liver, kidney, urinary tract, uterine cervix, and myeloid blood cells (*8*). The trend of tobacco consumption began and peaked in the first half of the 20th century and this rise is thought to be the main cause of the marked increases in the incidences of lung and other cancers.

Epidemiologic and mutational researches converge in support of a causal relationship between smoking and lung cancer, however, this has not been decisively proven. First, the period of time between the onset of smoking behavior and the diagnosis of lung cancer has usually been approximately 20 years (*9*), which is the estimated period of time required for the bronchial epithelium to acquire the carcinogen-induced DNA aberrations associated with lung cancer. In addition, this 20-year lag has been described in two different sub-populations (males and females) and at two different points in time (the 1930s and 1960s, respectively). Second, tobacco smoke contains thousands (>3,500, minimum) of chemicals of which at least 20 are known pulmonary carcinogens (*10*), and of all sources of carcinogens (cigarette-derived or other), cigarette smoke is the most greatly inhaled (*11*). Third, epithelia from current and former smokers have a high rate of cellular proliferation (as reflected by ki-67 expression assays) and it is correlated with the index of metaplasia (*12*). Fourth, genomic instability and clonal outgrowth have been demonstrated in the epithelia of smokers but not non-smokers (*13*). Fifth, many of the genetic and epigenetic aberrations known to be involved in lung cancer are more frequent in current and former smokers than non-smokers. For example, k-ras mutations are more frequent in lung adenocarcinoma biopsies from smokers than non-smokers (*14*), and increases in the incidence of adenocarcinoma are more strongly correlated with changes in smoking behavior and the use of filter tips than the development of novel diagnostic techniques (*15*), for example. Such genetic aberrations are sometimes present in the non-malignant epithelium of smokers and are known to persist (*16*). Sixth, p53 mutations, which are found in the majority of lung cancers (90%) and are thought to be essential in the dysregulation of the cell cycle and subsequent carcinogenesis, are correlated with tobacco smoke exposure. Seventh, epigenetic aberrations, such as the hypermethylation of key tumor suppressor genes (TSGs), are more frequent in biopsies from smokers than non-smokers. For example, methylation of p16/Ink4a is known be highly correlated with squamous cell carcinoma of

the lung (*17*), and to occur in the non-malignant epithelium of smokers but not non-smokers (*18*). Methylation of p16/Ink4a and DAPK may be found in non-malignant tissues from former smokers as well as current smokers (*19*).

Not surprisingly, developing countries where tobacco consumption is very frequent, such as China, have lung cancer incidence and mortality rates that are even higher than those in N. America. The frequency of male smokers in China, for example, is over 2.5 times higher than that in the U.S. [67% versus 25.7%; (*20*)]. Cigarette smoking is more preponderant in developing nations than in industrialized nations (*20*).

TABLE III. Leading causes of mortality throughout the world in 2001. Note that cancers of the trachea, bronchus and lung ranked as 9th most common cause of death worldwide. Also, tobacco-smoking is associated with many other diseases, including ischemic heart disease, cerebrovascular disease, and COPD, but correlations are highest for cancers of the larynx, trachea, lung and bronchus, lip, oral cavity and pharynx, and emphysema and chronic airway obstructive diseases (CDC, 1997). Taken from reference (*6*).

Rank	Cause	Deaths (thousands)	%Total
1	Ischemic heart disease	7,181	12.7
2	Cerebrovascular disease	5,454	9.6
3	Lower respiratory tract infections	3,871	6.8
4	Acquired Immunodeficiency Syndrome	2,866	5.1
5	Chronic obstructive pulmonary disease	2,672	4.7
6	Diarrheal diseases	2,001	3.5
7	Tuberculosis	1,644	2.9
8	Childhood diseases	1,318	2.3
9	Cancer of trachea/bronchus/lung	1,213	2.1
10	Road traffic accidents	1,194	2.1
11	Malaria	1,124	2.0
12	Hypertensive heart disease	874	1.5
13	Accidents	874	1.5
14	Stomach cancer	850	1.5
15	Self-inflicted	849	1.5
16	Cirrhosis of the liver	796	1.4
17	Measles	745	1.3
18	Nephritis/nephrosis	625	1.1
19	Liver cancer	616	1.1
20	Colon/rectum cancer	615	1.1

1.1.3 Lung cancer survival rates in North America

The five-year survival rate for pulmonary cancer is <15%, among the three worst, after pancreatic (<4%) and esophageal (<9%) cancers. This survival rate has not improved in over 50 years even though there has been much progress in cancer research during this period. In addition, though the rate of new cases has remained constant for over 5 years, the rate of death has increased by approximately 2.3% for men and 1.6% for women ($p=0.01$) between 1991 and 1999 (1). The significant decline in the tobacco smoking trend, as well as recent advances in the biological sciences and in biotechnology, such as the sequencing of the human genome (*21*) and consequent emerging technologies, including comparative genomic hybridization (CGH), RNA microarrays, and proteomics, are too recent to affect current incidence or mortality statistics, but will likely cause their decreases in the future.

1.1.4 Lung cancer is the leading cause of potential years of life lost

The average age of people diagnosed with lung cancer is 60, and lung cancer is rare in people under 40 (*1,2*). Lung cancer is not only the greatest cause of cancer deaths, it is also the greatest cause of potential years of life lost (PYLL). It has been estimated that lung cancer usurps approximately 252,000 potential years of life annually, which is more than breast (91,000), prostate (32,000), colon (105,000) and pancreas (43,000) combined. In addition, the treatment modalities for lung cancer, which include surgery, chemotherapy and radiation, have in essence remained unchanged in several decades.

1.1.5 Major types of lung pathologies including cancer may have immune origins

Diseases of the lung can be categorized into seven main classes according to their etiology and pathology: (1) atelectasic; (2) restrictive; (3) obstructive; (4) pneumoconiotic; (5) vascular; (6) infective; and (7) tumoral (*22*). It is interesting to note that many of the major examples of diseases in 3/7 categories are immune-related (i.e. obstructive, restrictive and pneumoconiotic diseases), because a current peripheral hypothesis in lung cancer research is that inflammatory reactions in the lung likely expose the bronchial epithelium to oxidative stress and other stresses capable of inducing DNA damage and consequently play a role in lung carcinogenesis [for a brief

review, see reference (*23*)]. Thus, in certain instances and in the context of certain genetic predispositions, repeated immune-related insults to the respiratory epithelium may cause a micro-environment favorable for lung cancer growth.

1.2 Lung cancer pathology

1.2.1 Lung cancer symptomatology and correlation with prognosis

Over 90% of patients are symptomatic at presentation (*24*), but symptoms (Table IV) are usually manifested at late, metastatic and mostly incurable stages, explaining the excessively low 5-year survival rate (<15%). Also, most patients present with non-specific systemic symptoms, such as anorexia, fatigue and weight loss (*24*).

Specific lung cancer symptoms stem from four sources: (1) the primary lung tumor; (2) intrathoracic spread from direct or lymphatic metastases; (3) extrathoracic spread from distant metastases; and (4) paraneoplastic syndromes. Since each of these categories affects different organs, each comprises a different set of possible symptoms.

The primary tumor is generally associated with cough, dyspnea, hemoptysis, and chest discomfort.

Intrathoracic spread tends to be associated with recurrent laryngeal nerve palsy (hoarseness), phrenic nerve paralysis (elevation of the hemidiaphragm and/or breathlessness), pancoast tumor (causing cervical or thoracic spine pain, muscle wasting, and changes in skin temperature), Horner syndrome (facial changes such as small pupils or ipsilateral lack of sweating), chest wall and pulmonary pleural pain, and heart and esophageal pain/dysfunction.

Extrathoracic spread usually reaches the bones, liver, adrenal glands, intra-abdominal lymph nodes, brain, spinal cord, or skin.

Paraneoplastic syndromes, which affect approximately 10% of patients, and are more common in small cell lung carcinoma (SCLC) patients than non-SCLC (NSCLC), consist of signs and symptoms physically unrelated to the tumor or its metastases; they are related to the various substances that the tumor secretes, or substances that other

organs secrete in response to the tumor. Examples include neuropeptides, hormones, growth factors, immunoglobulins, prostaglandins, and cytokines. SCLC tumors tend to secrete large amounts of neuropeptides, such as adenocorticotrophic hormone (ACTH), gastrin-releasing peptide (GRP), antidiuretic hormone (ADH), cholecystokinin (CCK), bombesin and vasopressin.

Cough is the most common symptom, present in over half of the patients who are diagnosed with lung cancer (*24*). Cough accompanied by hemoptysis (typically only a faint smear of blood in the sputum) in the absence of any other airway disease (such as viral disease) is frequently the first suspicious set of symptoms. Recently, Beckles *et al.* suggested that patients presenting with hemoptysis accompanied with COPD who are also smokers or ex-smokers >40 years of age, should be carefully observed and tested using sputum cytology, bronchoscopy or chest computerized tomography (CT), even when their radiological findings are unremarkable (*24*). This emphasizes the great need for new methodologies that will allow the earlier detection of lung carcinogenesis.

TABLE IV. Major symptoms of lung cancer and their incidences in SCLC and NSCLC. Primary symptoms are due to local tumor growth and intrathoracic spread; secondary symptoms are manifestations of systemic effects. SVC = superior vena cava. Based on Table 88.4, in reference (*25*).

Symptom class	Symptoms	Frequency (%)	
		SCLC	NSCLC
Primary	Cough	50-76	40
	Dyspnea	34-40	30-40
	Chest pain	35-36	25-40
	Hemoptysis	15-23	15-35
	Pneumonitis	21-25	13-24
	Vocal cord paralysis	15	Uncommon
	SVC syndrome	12	<10
	Pleural effusion	10-15	15
	Pancoast's syndrome	Rare	3
	Pericardial effusion	Uncommon	Rare
	Hoarseness	N/A	N/A
	Swollen lymph nodes	N/A	N/A
	Pallor	N/A	N/A
	Muscle weakness	N/A	N/A
	Facial swelling	N/A	N/A
	Droopy eyelids	N/A	N/A
	Speech difficulties	N/A	N/A
Secondary	Weight loss ≥ 10 lbs	35-52	40-52
	Anorexia	30	30
	Fatigue	23-42	35
	Fever	11-15	7-16
	Anemia	11-15	16-20

1.2.2 Histological classification of lung cancers

Approximately 95% of primary lung cancers originate from the bronchial epithelium and are thus frequently referred to as bronchiogenic carcinomas. The remaining 5% comprise soft tissue, mesothelial and miscellaneous tumors, lymphoproliferative diseases, and tumor-like lesions originating from cell types other than bronchial epithelial cells.

An international consensus for the histological classification of lung cancers of bronchiogenic origin was first established in 1967 (*26*). Three classes, squamous cell

carcinomas, adenocarcinomas and large cell carcinomas, were grouped together as the NSCLCs because they share many histological and clinical characteristics, and this group is distinct from the fourth group, SCLC.

Diagnostic algorithms, such as the one illustrated below (Fig. 1) are used to identify the histological classification of suspicious specimens, which are retrieved via biopsy, bronchial washing, bronchial brushing, or thoracotomy.

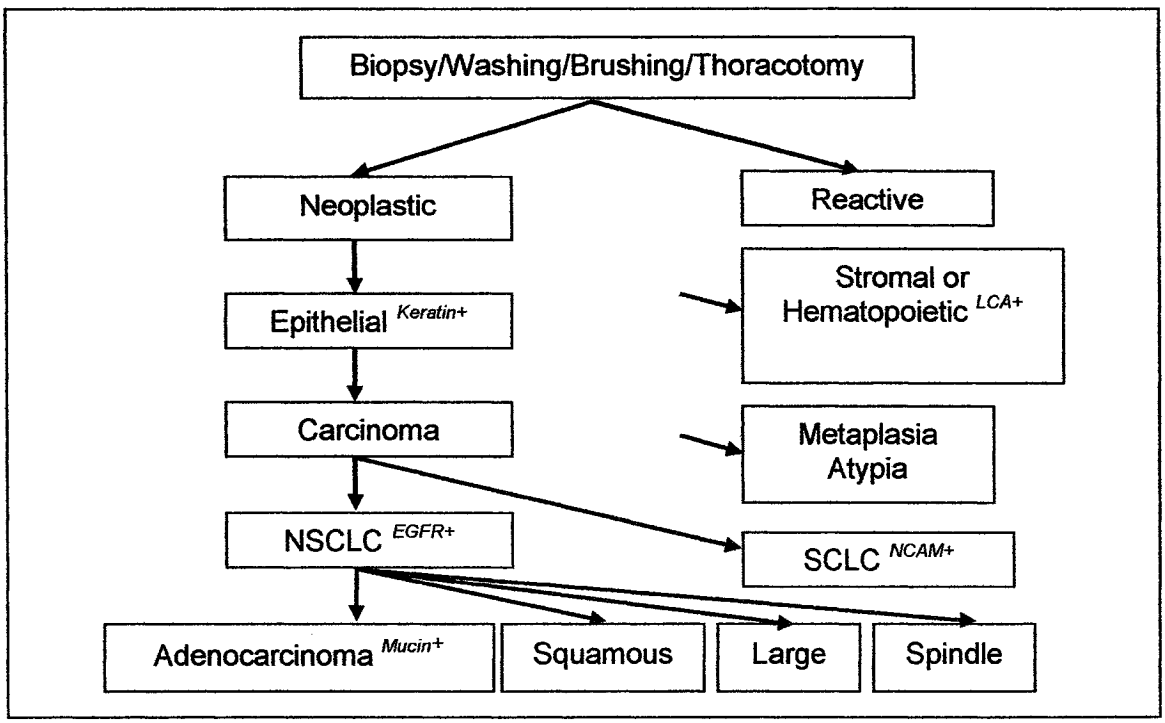

FIGURE 1. Algorithm used in the differential diagnosis of the main classes of lung cancer. These are adeno-, squamous cell, large cell, small cell (SCLC), and spindle cell carcinomas. Note that keratin, leukocyte common antigen (LCA), neural cell adhesion molecule (NCAM), mucin and epidermal growth factor (EGF) are among the key immunohistochemical stains, and many other stains, such as carcinoembryonic antigen (CEA) and MOC-31, are used to diagnose difficult specimens (*27*). Modified from reference (*28*).

Following sample harvest and fixation/preservation, the methods of chemical staining, microscopic observation of cellular morphology, and immunohistochemical staining are used in combination. There are five key molecules presently used in the differential diagnosis of lung cancer classes: (1) keratin, (2) leukocyte common antigen (LCA), (3) neural adhesion molecule (NCAM), (4) mucin, and (5) epidermal growth factor (EGF). Diagnosis is not as straightforward as this algorithm appears to suggest.

Many specimens are difficult to diagnose, mainly because of their inherent heterogeneity of morphological and staining characteristics. In order to classify a difficult specimen, which may not have typical morphological features or which may present features characteristic of more than one class of lung cancer, other molecules such as carcinoembryonic antigen (CEA), epithelial membrane antigen (EMA), proliferation-related Ki antigen (Ki-67), proliferating cell nuclear antigen (PCNA), chromogranin and folate receptor are tested for their level of expression using immunohistochemistry, a laboratory technique that uses commercially available specific antibodies to detect cognate antigens in clinical specimens (Table V). This highlights the intra-tumoral heterogeneity of lung cancers.

TABLE V. Common immunohistochemical stains used to differentially diagnose lung cancer classes. List is non-exhaustive. CEA=carcinoembryonic antigen; EMA=epithelial membrane antigen; Ki-67= proliferation-related ki antigen, NEC=neuroendocrine; PCNA=proliferating cell nuclear antigen; +=positive; -=negative; L=low; I=intermediate; H=high; n/a=not applicable. Modified from reference (*28*).

Lung cancer type	Keratin, CEA, EMA, (...)	Ki-67, PCNA	NCAM	Chromo-granin	EGFR	Folate Receptor	LCA
Adenocarcinoma	+	L to I	-	-	+	+	-
Bronchioalveolar	+	L	-	-	+/-	+	-
Squamous	+	I to H	-	-	+	-	-
Large cell	+	I to H	-	-	+	-	-
Carcinoid, typical	+	L	+	+	-	+/-	-
Carcinoid, atypical	+	I to H	+	+	n/a	n/a	-
Large cell, NEC	+	I to H	+	-	+/-	+/-	-
Small cell	+	H	+	-	-	+/-	-
Lymphoma	-	L to H	+/-	-	-	n/a	+

1.2.2.1 The 1999 revision of the WHO histological typing of lung tumors

In 1999, the World Health Organization (WHO) updated the protocol for histological lung tumor typing. It consists of guidelines used around the world for diagnosis, prognosis and treatment determination (*29,30*). The primary reason for revision was that, as mentioned above, lung tumors are very heterogeneous and thus difficult to diagnose. They frequently contain cells belonging to >1 histological classification, which may lead to error or sub-optimal diagnosis. Even when three clinical laboratory methods, such as light microscopic observation, histological staining and immunohistochemical staining,

are combined to characterize a tumor specimen, the potential for a difference of opinions between pathologists remains. The reasons are numerous. The tumor specimen may be highly heterogeneous, too small to process, or the classification system is not sufficiently specific. The goal of the 1999 revision was to improve histopathological classification criteria, in order to increase the consistency and reproducibility of diagnosis. Improving individual diagnoses and consequently fine-tuning treatment was hoped to improve both patient survival and interpretation/comparison of international research studies upon meta-analysis. Conclusions concerning these issues have not yet been made.

Numerous changes were brought to the new guidelines. Briefly, there are now three times as many classifications as the 1981 edition (*31*), for a total of 47 sub-classifications of lung tumors (35 malignant invasive, 3 preinvasive, and 9 benign), versus 15 in the previous edition (10 malignant, 1 dysplastic and 4 benign). Though benign and pre-malignant classes were modified, the "frank cancers" is the class that was most modified. The three classes of NSCLCs (squamous, adenocarcinoma and large cell carcinoma) and the SCLC class remain, but 5 new categories were added (Table VI b). These include the adenosquamous mixed histology, the carcinoid-tumors, the sarcomatous-tumors, the salivary gland-like tumors, and the "others". There is also a new sub-category for lymphoproliferative diseases.

The availability of the flexible bronchoscope, which allows access to smaller and consequently more peripheral airways than its contemporary version, the abundance of new specific immunoreactive reagents for immunohistochemical tests, the advances made in histopathology, and the ability to rapidly share information over the Web, are no doubt the components that have made these advances possible.

Increasing the specificity of diagnosis may have very important effects on treatment options and prognosis. For example, bronchioalveolar carcinoma, an adenocarcinoma subtype, has a better prognosis than adenocarcinoma, and is frequently confused with non-lung metastases to the lung (*32,33*). Two publications in the November 20th, 2001 issue of the Proceedings of the National Academy of Sciences U.S.A. have shown that adenocarcinoma subclasses are distinguishable using mRNA microarray technology (*34,35*) respectively). In general, they show that the use of microarray expression

profiling to generate "mRNA taxonomy" (*35*) of lung cancers essentially recapitulates the 1999 WHO revision of the histological principles, and provides a larger database of lung cancer molecular markers.

Another level of appreciation for the complexity of lung cancer is seen in the use of attributes like "Clara cell/type II pneumocyte type" and "goblet cell type" to differentially classify invasive malignant lung tumors (Table VI b). This represents an attempt to synthesize current knowledge on the origins of these cancers.

1.2.2.1.1 Squamous cell carcinoma

Squamous cell carcinomas (Fig. 2A) presently account for 30% of all new lung cancers (*36*). They usually arise in the primary bronchi, which are in the central region, and measure 4 cm or more in diameter (*37*). Squamous cell carcinomas may consist of poorly, moderately or well differentiated epithelial cells. Well differentiated squamous cell carcinomas present keratin pearls, whereas poorly differentiated squamous cell carcinomas have epithelioid sheets (*38*). Intercellular bridges and clear or eosinophilic cytoplasm, and small or shrunken nuclei are other common microscopic features. Squamous cell carcinomas grow relatively slowly and can be detected in their earliest forms from cytological examination of bronchial washings (bronchioalveolar lavage; BAL). For these reasons, squamous cell carcinomas tend to have the best prognoses of all lung cancers. The 1981 WHO edition of the histological classification of lung tumors accounted for only one variant type, the spindle variant, whereas the 1999 edition accounts for four: papillary, small cell, clear cell, and basaloid variants (Table VI b).

1.2.2.1.2 Adenocarcinoma

Adenocarcinomas (31%) and bronchioalveolar carcinomas (10%) together presently account for approximately 41% of all lung cancers (*36*). They usually arise from the alveolar epithelium or the bronchial mucosal glands and measure 4 cm or less in diameter (*37*). Unlike squamous cell carcinomas, they usually develop in the periphery of the lungs. These cancers form highly characteristic glandular structures or acinar structures (Fig. 2B), that may or may not secrete mucin. They may also have a growth pattern that is accompanied by alveolar destruction (*38*). Adenocarcinomas stain positively for carcinoembryonic antigen (CEA) and keratin. They have a worse

prognosis than squamous cell carcinomas. The 1981 WHO edition of the histological classification of lung tumors accounted for four subclasses of adenocarcinoma, whereas the 1999 edition accounts for six, with the greatest changes being the increased specifications about bronchioalveolar carcinoma, and its cellular appearance, goblet cell, Clara cell, or Type II pneumocyte (Table VI b).

1.2.2.1.3 Large cell carcinoma

Large cell carcinomas account for 9% of all lung cancers. As their name indicates, large cell carcinomas (Fig. 2C) consist of cells that are larger than the cells of squamous cell and adenocarcinomas, and have characteristically large nuclei and nucleoli, with coarse chromatin. They do not have intercellular bridges or keratin pearls, characteristic of squamous cell carcinomas, or glandular structures, characteristic of adenocarcinomas (*38*). Cells measure approximately 30 to 50 μm in diameter. Large cell carcinomas usually arise in the peripheral lungs, and measure well over 4 cm in diameter. These tumors have a worse prognosis than adenocarcinomas and squamous cell carcinomas. The 1981 WHO edition of the histological classification of lung tumors accounted for only two subtypes, giant and clear cell large cell carcinoma, whereas the 1999 edition describes five variants, large cell neuroendocrine, basaloid, lymphoepithelioma-like, clear cell and large cell with rhabdoid phenotype (Table VI b).

1.2.2.1.4 Small cell lung carcinoma

Small cell lung carcinomas (SCLCs; Fig. 2D) have several characteristic features, such as uniformly small cell size (less than three leukocyte diameters), large, round nuclei, "salt & pepper" textured chromatin (*38*), high nucleus/cytoplasm ratio, frequent mitoses, and argentophilic neuroendocrine granules. They may store and secrete several different neuroendocrine factors, such as adenocorticotrophic hormone (ACTH), gastrin-releasing peptide (GRP), antidiuretic hormone (ADH), cholecystokinin (CCK), bombesin and vasopressin. Though they respond well to early chemotherapeutic and radiological treatments, they grow rapidly and metastasize early. Thus, they are often diagnosed late in their progression and are characterized by very poor prognoses. The 1981 WHO edition of the histological classification of lung tumors accounted for three subclasses, oat cell, intermediate cell type, and combined oat cell, whereas the 1999 edition lists only the latter variant as a SCLC (Table VI b).

It is of interest to note that neuroendocrine markers, previously thought to be present only in SCLC, are now known to be present in several lung cancer subclasses and variants. As previously cited in the literature, expression of neuroendocrine markers is not taken into account in current treatment protocols (*38*).

For further details on the less common lung cancer classes, such as adenosquamous carcinoma, carcinomas with pleomorphic, sarcomatoid, or sarcomatous elements, carcinoid tumors, carcinomas of salivary gland type, and unclassified carcinoma, please refer to references (*39,40*).

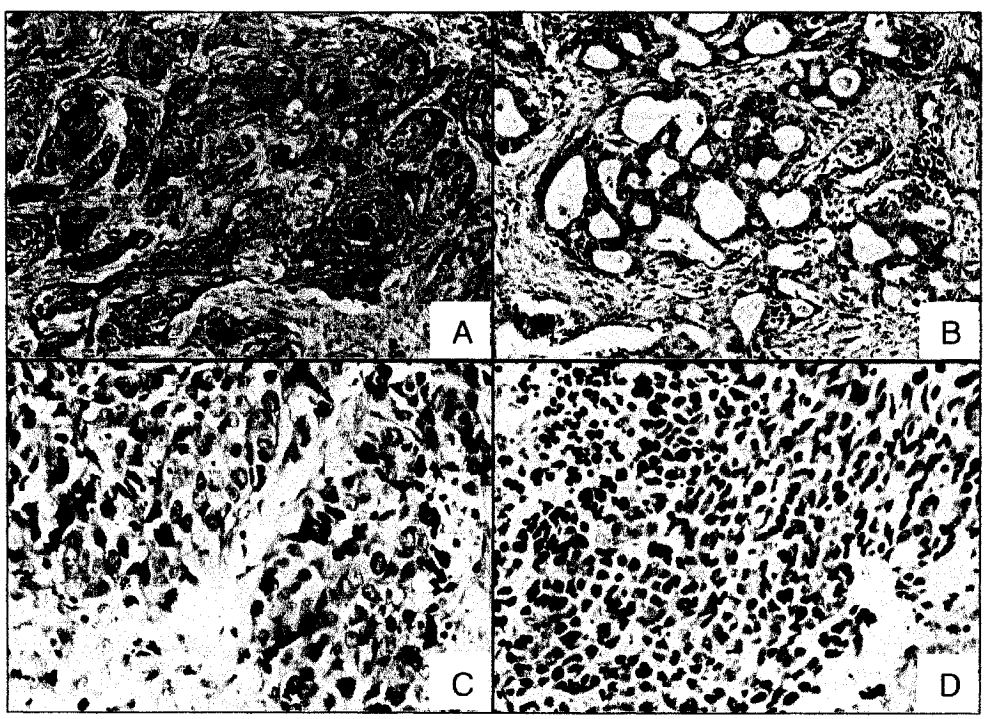

FIGURE 2. Photomicrographs representing histological examples of the four main classifications of lung cancer. **A**: Squamous cell carcinoma (moderately well differentiated); **B**: Adenocarcinoma (papillary). **C**: Large cell carcinoma; **D**: Small cell carcinoma. Light micrographs of cross-sections stained with hematoxylin and eosin, magnification of 180x each, except (d), x360, Taken from reference (*39*).

TABLE VI. Current (1999-present) and previous (1981-1999) histological classifications of lung tumors. A. Benign and pre-malignant lung tumors. B. Malignant and invasive lung tumors. Uses: diagnostic, prognostic and treatment assessments. Note that there are now 12 classifications rather than 5. Based on references (*31,41*)

Benign lung tumors	
1. Papilloma 1.1 Squamous cell papilloma 1.1.1 Exophytic 1.1.2 Inverted 1.2 Glandular papilloma 1.3 Mixed squamous cell and glandular papilloma **2. Adenoma** 2.1 Alveolar adenoma 2.2 Papillary adenoma 2.3 Adenomas of salivary gland type 2.3.1 Mucous gland adenoma 2.3.2 Pleomorphic adenoma 2.4 Mucinous cystadenoma	Current
1. Papilloma 1.1 Squamous cell papilloma 1.2 Transitional papilloma **2. Adenoma** 2.1 Pleomorphic adenoma 2.2 Monomorphis adenoma	Previous
Premalignant lung tumors	
1. Preinvasive lesion 1.1 Squamous dysplasia/carcinoma *in situ* 1.2 Atypical adenomatous hyperplasia 1.3 Diffuse idiopathic pulmonary neuroendocrine cell hyperplasia	Current
1. Dysplastic lesion 1.1 Carcinoma *in situ*	Previous

TABLE VI b. Current (1999-present) and previous (1981-1999) histological classifications of malignant and invasive malignant lung tumors. Note that the NSCLCs and the SCLCs remain as separate classes, but there are now 35 subclassifications rather than 10. Also note the frequent use of comparison to epithelial cell types, such as "Clara cell/type II pneumocyte type" to describe and "philogenize" the various cancer classes. Based on references (*31,41*), respectively.

Malignant	
1. Squamous Variant: 1.1 Spindle cell carcinoma (squamous cell carcinoma) **2. Adenocarcinoma** 2.1 Acinar adenocarcinoma 2.2 Papillary adenocarcinoma 2.3 Bronchio-alveolar adenocarcinoma 2.4 Solid carcinoma with mucus formation **3. Large cell** 3.1 Giant cell carcinoma 3.2 Clear cell carcinoma **4. Small cell carcinoma** 4.1 Small cell (oat cell) carcinoma 4.2 Intermediate cell type carcinoma 4.3 Combined oat cell carcinoma	Previous

Invasive malignant

1. Squamous cell carcinoma
- Variants:
- 1.1 Papillary
- 1.2 Clear Cell
- 1.3 Small cell
- 1.4 Basaloid

2. Small cell carcinoma
- Variant:
- 2.1 Combined small cell carcinoma

3. Adenocarcinoma
- 3.1 Acinar
- 3.2 Papillary
- 3.3 Bronchioloalveolar carcinoma
  - 3.3.1 Non-mucinous (Clara cell/type II pneumocyte type)
  - 3.3.2 Mucinous (goblet cell type)
  - 3.3.3 Mixed mucinous and non-mucinous (Clara cell/type II pneumocyte goblet cell type) or indeterminate
- 3.4 Solid adenocarcinoma with mucin formation
- 3.5 Mixed
- 3.6 Variants:
  - 3.6.1 Well differentiated fetal adenocarcinoma
  - 3.6.2 Mucinous ("colloid")
  - 3.6.3 Mucinous cystadenocarcinoma
  - 3.6.4 Signet ring, Clear cell
  - 6.6.5 Clear cell

3.4 Large cell carcinoma
- Variants:
- 3.4.1 Large cell neuroendocrine carcinoma
  - 3.4.1.1 Combined large cell neuroendocrine carcinoma
- 3.4.2 Basaloid carcinoma
- 3.4.3 Lymphoepithelioma-like carcinoma
- 3.4.4 Clear cell carcinoma
- 3.4.5 Large cell carcinoma with rhabdoid phenotype

3.5 Adenosquamous carcinoma

3.6 Carcinomas with pleomorphic, sarcomatoid, or sarcomatous elements
- 3.6.1 Carcinomas with spindle and/or giant cells
  - 3.6.1.1 Pleomorphic carcinoma
  - 3.6.1.2 Spindle cell carcinoma
  - 3.6.1.3 Giant cell carcinoma
- 3.6.2 Carcinosarcoma
- 3.6.3 Blastoma (pulmonary blastoma)
- 3.6.4 Others

3.7 Carcinoid tumors
- 3.7.1 Typical carcinoid
- 3.7.2 Atypical carcinoid

3.8 Carcinomas of salivary gland type
- 3.8.1 Mucoepidermoid carcinoma
- 3.8.2 Adenoid cystic carcinoma
- 3.8.3 Others

3.9 Unclassified carcinoma

Current

1.2.2.2 The frequency of adenocarcinoma is on the rise

Trends in lung cancer incidence according to histological classification and sex have been changing dramatically. The main change is that adenocarcinoma, which used to be less frequent than squamous cell carcinoma and which was not correlated to tobacco smoking, is now the most preponderant lung cancer class (40%; Table VII), and appears to be associated with tobacco smoke (*36*). Adenocarcinoma of the lung has been increasing since the 1950's both in males and in females, in Canada (*42*) and the U.S. (*15*). The rise in the incidence of adenocarcinoma of the lung is so important that it nearly counterbalances the falls observed in all three other classes combined. Evidence shows that trends in tobacco smoking or in the type of cigarette smoked likely play a role (*15*). For instance, it is thought that changes in the manufacturing of cigarette filters have resulted in the decreased size of particulate matter emanating from the cigarette mouthpiece and accessing the lung, gaining entrance to the smaller and more distal (peripheral) airways. Other factors, such as the increased capacity to biopsy small lesions using flexible bronchoscopes may also play an important role, but this is an issue that remains to be resolved.

TABLE VII. Major lung cancer classifications and pertinent characteristics. (Incidence based on reference (*36*); anatomical distribution within the lung and bronchi based on references (*40,43*). Assoc./smok.=Degree of association to cigarette smoking.

Group	Class	Incidence	Distribution	Assoc/smok.
NSCLC	Squamous	30%	Central	Very high
	Adenocarcinoma	41%	Peripheral	High
	Large	9%	Peripheral	Low
SCLC	Small	18%	Central	Very high

A recent study that considered sex, age, and ethnicity, as well as histological tumor classification, found that lung cancer incidence and mortality rates in the U.S. between young white women and young white men are converging rapidly for adenocarcinoma, large cell and SCLC, but not as rapidly for squamous cell lung carcinoma (*44*). This convergence is less important between young black women and young black men (Fig. 3). Overall, the study showed that the patterns of change observed in these cancers of

converging incidence paralleled the societal changes in smoking behavior, rather than our increased capacity to biopsy small lesions.

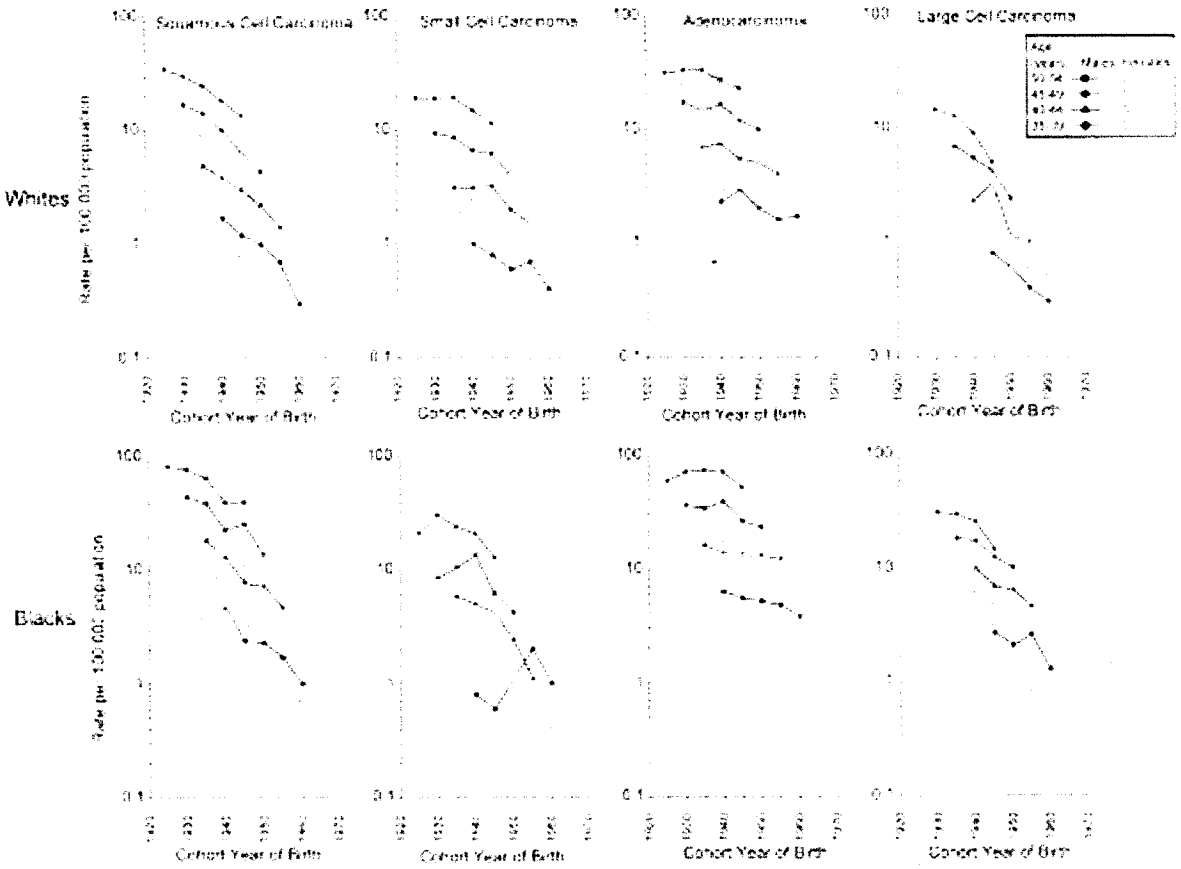

FIGURE 3. Graphs representing the incidence of lung cancer in Black and White females and males by birth cohort from 1925 to 1960. For squamous cell, adeno-, large cell and small cell carcinomas, SEER Program, showing the converging incidences between young white women and young white men. Taken from reference (*44*).

1.3 Conclusion

In conclusion, the epidemiology and pathology of lung cancer demonstrate high degrees of complexity, which have only come to the fore relatively recently. This complexity demonstrates a need for new paradigms in lung cancer research, with a special emphasis on developing strategies to detect and target lung cancer at earlier stages. The first step may lie in the molecular understanding of the origin of lung cancer.

2. Retinoids

Retinoids are vitamin A-related compounds that act as cellular messengers. In particular, some retinoids have been shown to block proliferation, induce differentiation, modulate immune responses and/or trigger apoptosis. Hence, retinoids have long been studied for their potential beneficial uses in the prevention and treatment of cancer. To date, over 30,000 scientific papers have been published on retinoid biology, including over 8,500 regarding their role in cancer alone (*45*).

2.1 Vitamin A and retinoids

Vitamin A is metabolized into various related compounds known as retinoids. These share certain common structural and functional characteristics. Vitamin A is an essential nutrient that is crucial for all stages of life. During embryogenesis, retinoids act upon anterior-posterior axial specification, limb bud development and organogenesis. During development, retinoids are crucial in the formation and maturation of the epidermal, pulmonary and reproductive epithelia, as well as the eyes, bones and teeth. Finally, during adult life, retinoids act upon mechanisms of homeostasis on these very same tissues.

Vitamin A is mainly obtained either directly, through the ingestion of retinol (vitamin A in its true form) or indirectly, through the ingestion and metabolism of provitamins A, such as carotenoids, the most common being the well known β-carotene.

In the developing world, vitamin A deficiency (VAD), which is mainly due to malnourishment, has played a predominant role in the causation of blindness, whose onset is marked by xerophthalmia and keratomalacia, underdevelopment, infection and death. Other common and major symptoms include rickets (the shunted development of bones and teeth in the developing years), osteomalacia (the demineralization and consequent softening of the bones, which is accompanied by pain and weakness), and the inability to gain weight. VAD is presently estimated to affect >1 million children worldwide, of which >250,000 will develop blindness and >125,000 die, annually.

Research originally aimed at preventing VAD at the dawn of the last century provided the first evidence suggesting that vitamin A plays a role both in immunity and cancer

prevention. In particular, vitamin A was discovered in 1913 and shown to be crucial for growth and survival in rats at this time (*46,47*). It was later shown to be essential for the proper development of the eye and the prevention of xerophthalmia and keratomalacia in children in 1919 (*48*). Interestingly, it was at this time that a correlation between low vitamin A intake and decreased immunity (and increased occurrence of infection) was also first observed (*48*). The initial experimental evidence that vitamin A plays a role in cancer came in 1925, when studies showed that VAD rats developed widespread squamous hyperplasia and metaplasia (*49*), which are reversible pre-neoplastic conditions.

Other vitamin A-related ailments include hypervitaminosis A, a condition that may occur due to an acute vitamin A over-dosage or the chronic ingestion of vitamin A equivalents, which is extremely toxic and may result in death. The effects of retinoids are so potent that hypervitaminosis A in pregnant women is known to have teratogenic effects on the unborn embryo. Thus, retinoic acid (RA) levels are tightly regulated both intra- and extracellularly, most likely due to their potent and rapid effects on the cellular processes mentioned above.

2.2 Chemical structure

Retinoid molecules mainly consist of fat-soluble terpenes ($C_{10}H_{16}$) that are conjugated to alcohol- or aldehyde-groups and are generally comprised of three variable moieties (Fig. 4): (1) an aromatic (cyclic) group; (2) a polyene chain; and (3) a polar group, such as a hydroxyl (-OH), an oxy (=O), an alcohol (-OH) or an aldehyde (=O-H) group. However, recent generations of synthetic retinoids may have unique differences, such as the lack of a polar group, for example. The retinoid family includes dozens of naturally occurring compounds and thousands of chemically synthesized and tested substances. In addition, the advent of computer-based screening methods, such as *Virtual Library Screening Modules*, has increased the speed and reduced the cost associated with the testing of retinoids by allowing the analyses - ultimately countless – of virtual retinoid and retinoid-like molecules *in silico* (*50*), using software such as Molsoft ICM (*51*), for example.

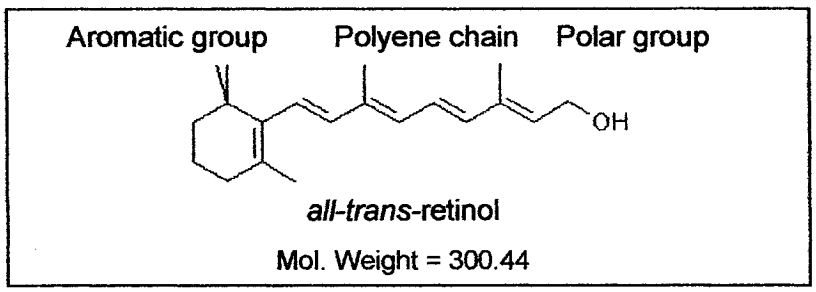

FIGURE 4: Chemical structure of all-trans-retinol representing the three moieties common to most retinoids.

2.3 Classification

Retinoids have been classified as steroid-like molecules because they resemble the steroid hormones, including estradiol, testosterone, progesterone, cortisol, aldosterone, and mineralocorticoid, and they also resemble the amino acid-derived hormones, including triiodothyronine and dihydroxyvitamin D_3. Structure-based similarities include: (1) low molecular weight (~300); (2) lipophilicity; (3) spontaneous diffusion across the cellular membrane, abrogating the need for membrane receptors and multi-messenger signal transduction cascades, which are common to water-soluble peptide hormones and growth factors; and (4) binding and activation of corresponding cognate receptor(s) in the cytoplasm and nucleus. Functional similarities include; (1) pleiotropy; (2) redundancy; (3) differential control of gene expression; and (4) involvement in developmental regulation.

2.4 Dietary sources

Vitamin A (*all-trans*-retinol) is obtained and metabolized from two main sources: (1) carotenoids, which are provitamins mainly found in plant sources, but which are also contained, albeit in lesser amounts, in animal sources; and (2) retinyl esters, which are only found in animal sources, but are more bioavailable than carotenoids. Table VIII consists of a selection of common foods high in retinol activity equivalents (RAE; see section 2.5). Carotenoids are found at high concentrations in most yellow and orange fruits and vegetables; retinyl esters are found at high concentrations in animal fats, fish oils, fish meats, milk, eggs and butter.

TABLE VIII: List of Western foods containing high amounts of RAE. Selection made from among the 999 highest in RAE, both common and less/not common, based on 100g serving portions, as per search performed through reference (*52*). RAE = Retinol activity equivalent.

Category	Type	RAE (ug)	Category	Type	RAE (ug)
Meat	Calf-liver, pan-fried	20,100	Vegetable	Sweet potato, baked	961
	Lamb liver, pan-fried	7,780		Carrots, boiled	860
	Beef liver, pan-fried	7,740		Kale, boiled	681
	Pork liver, braised	5,400		Carrots, raw	602
	Chicken liver, pan-fried	4,300		Winter squash, baked	558
Fish	Tuna, dry-heat	757	Fruit	Cantaloupe, raw	169
	Herring, Atlantic, pickled	258		Apricots, raw	96
	King mackerel, dry-heat	252		Persimmons, raw	81
	Salmon, Chinook, dry-heat	148		Sour red cherries, raw	64
	Salmon, Sockeye, canned	53		Papayas	55
Dairy/ Egg	Butter	684	Oils/Fats	Cod liver oil	30,000
	Cheese, goat, hard	486		Fat-free margarine	993
	Cream cheese	366		Corn margarine	819
	Milk, non-fat	338		Soybean margarine	819
	Whole egg, fried	198		Cotton-seed margarine	819
	Milk, calcium-fortified non-fat	61		Vegetable oil margarine	819

2.5 Retinol activity equivalency

Since retinol is available from several dietary sources, the U.S. Institute of Medicine (UIM) has devised the "retinol activity equivalent" (RAE), which is simply based on the efficiency of bioconversion of each compound, and this depends both on chemical structure and biologic efficiency of conversion (i.e. bioefficacy). The latter factor frequently depends on the food matrix. Thus, equivalencies may vary according to the foodstuff in which they are contained. For example, though 1 μg RAE would be expected to be extracted from 2 μg β-carotene based on chemical structure alone, it has recently been estimated that 12 μg β-carotene are actually normally required. In addition, when emulsified in certain oils such as corn oil for example, only 2 μg β-

carotene are required for 1 μg RAE. Based on these findings, the UIM recently modified the formulae to correct for the previous over-estimations of RAEs in β-carotene, α-carotene, and β-cryptoxanthin (*53*) (Fig. 5). These new bioconversions impact the interpretation of past studies and the design of future ones.

1 RAE (3.33 IU)	=	1 μg	retinol
	=	2 μg	β-carotene in oil
	=	12 μg	β-carotene in mixed foods
	=	24 μg	α-carotene
	=	24 μg	β-cryptoxanthin

FIGURE 5: Retinol activity equivalency for certain pro-vitamin A compounds. Based on reference (*54*). IU = International unit; RAE = Retinol activity equivalent.

It is also important to clarify that hydrocarbon carotenoids, such as α-, β- and γ-carotenes, are considered pro-vitamins A, but polar carotenoids such as lycopene, which mainly act as potent anti-oxidants and which have recently gained much interest for their beneficial effects in prostate and other cancers, are not. For a review on lycopene in cancer see reference (*55*).

2.6 Retinyl ester and β-carotene metabolism

Dietary retinyl esters are hydrolyzed by pancreatic triglyceride lipase (PTL) in the intestine and phospholipase B at the brush border of the small intestine. On the other hand, β-carotene is oxidatively cleaved at the centric C-15,15' bond into two molecules of retinol (*all-trans*-retinol, an alcohol conjugate) in the intestinal mucosa and possibly also in the liver (*56*). However, it may also be rarely cleaved eccentrically, such as at the C-9'-10', 11'-12' and 13'-14 bonds (*57*). The effects of these eccentric cleavage products have not been intensively studied.

Two thirds of the world's population is estimated to follow a plant-based regimen (*58*), and ½ of the world's population is estimated to be malnourished (*59*). In addition, approximately 2.5% and 4.0% of adults in the U.S. and Canada, respectively, are estimated to follow a vegetarian diet (excluding meat, fish and poultry), or a vegan diet

(excluding dairy products in addition to meat, fish and poultry), and these percentages are thought to be increasing (*58*). Hence, the effects of plant-based diets and increasing trends toward plant-based diets on the levels of vitamin A ingestion may have effects on the development of VAD-related diseases, including cancer, which remains a hypothetical and controversial link. To date, little is known with regard to these issues. Though vegetarianism appears to be associated with lower rates of colon cancer (*60*), no differences have been observed in lung, breast, prostate or stomach cancer (*61*).

2.7 Bioavailability

The bioavailability, cellular distribution and content, and efficient usage of retinoids in tissues such as the bronchial epithelium, for example, depends on at least eight known factors: (1) the level of hepatic reserves; (2) the rate of retinoid metabolism; (3) the level of ingestion of retinol activity equivalents (RAEs); (4) the level of intracellular retinoid storage; (5) the histological classification of the tissue being studied; (6) the developmental stage; (7) age; and (8) health status - of the organism being studied. At present, there is more literature regarding the first four than the latter four factors.

2.7.1 Hepatic reserves

The majority of the vitamin A absorbed from the diet (60-80%) is stored in the liver. Retinyl esters and retinol are transported from the gut to the liver via circulating chylomicrons remnants, the lipoproteins responsible for directing all dietary lipids from the gut to the liver. Adequate bile salt production is required for optimal absorption, but absorption is generally thought to be very poor at best. Long chain retinyl esters are contained in the liposomes of hepatic stellate cells (HSCs), however, a minority is also stored in kidney cells.

Retinol is esterified and thereby rendered inactive by various enzymes having esterase activity located in different tissues. Lecithin:retinol acyltransferase (LRAT) and acylCoA:retinol acyltransferase esterify retinol bound or not by cellular retinol binding protein 2 (CRBP2; section 2.7.4), respectively. Aberrant LRAT activity and reduced retinol esterification have recently been associated with various forms of cancer, including oral (*62*), breast (*62,63*), kidney (*53*) and skin (*62,64*).

Upon tissue requirement, retinol is directed from the HSCs to the plasma, where it re-circulates as part of protein complexes comprising vitamin A, retinol binding protein (RBP), and transthyretin (at a 1:1:1 ratio).

The transport of carotenoids is essentially similar to that of retinol and retinyl esters [for a description see reference (*65*)].

2.7.2 Retinoid metabolism

The understanding of retinoid metabolism remains highly incomplete, however, several recent advances have helped clarify and contrast the roles of various enzymes.

All-trans-retinol, the parent compound of the main bioactive retinoids, yields several metabolic products, including *all-trans*-retinal, *all-trans*-retinoic acid (*ATRA*), *all-trans*-3,4,-didehydroRA, 9-*cis*-RA, and several other polar forms of RA (Fig. 6), which are water soluble and ultimately excreted through the urine.

2.7.2.1 The bioactive retinoids ATRA and 9-cis-RA

The most bio-active naturally occurring retinoids are ATRA and 9-cis-RA. Bioactivity is manifested as cell signaling through the trans-activation of the retinoid receptors, including the RA receptors (RARs) and the retinoid X receptors (RXRs). However, as discussed later, RARs, including RARβ2, bind both ATRA and 9-cis-RA with high affinity, whereas RXRs only bind 9-*cis*-RA. Interestingly, the level of ATRA in mouse embryo tissues is relatively high (30-90 nM), whereas the level of 9-*cis*-RA is not detectable (*66*). Little is known with regard to the tissue distribution of ATRA versus 9-*cis* and 13-*cis*-RA in humans. Their concentrations are thought to be approximately 40-50 nM.

2.7.2.2 Retinol conversion to ATRA and 9-cis-RA

It may be said that there are three important steps in retinol conversion to ATRA: Step 1, retinol oxidation into retinaldehyde; Step 2, retinaldehyde dehydrogenation into bioactive metabolites; and Step 3, *all-trans*-RA degradation. Steps 1 and 2 insure the formation of the bioactive retinoids, ATRA and 9-cis-RA; step 3 ensures their elimination. Multiple

enzymes, which are subject to variant polymorphisms, are responsible for each of these steps, and this may thus contribute to differences in the bioavailability and distribution of RA in different individuals.

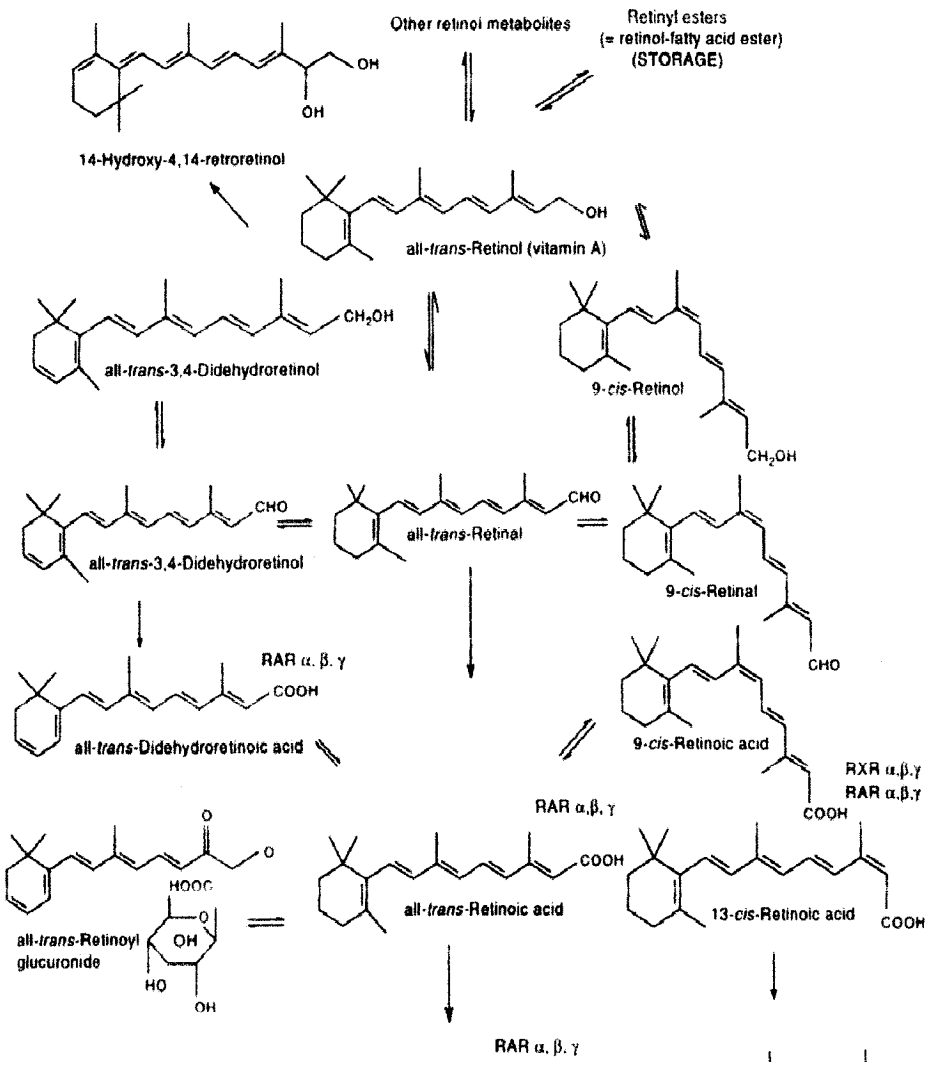

FIGURE 6. Hypothetical scheme of all-trans-retinol catabolism. Taken from reference (*30*).

2.7.2.2.1 Step 1: Retinol oxidation

The oxidative conversion of *all-trans*-retinol to *all-trans*-retinal (retinaldehyde) is a reversible reaction that is catalyzed by one of several genes, the cytosolic or microsomal alcohol dehydrogenases (ADHs) (*67*) or the short-chain dehydrogenases (SDRs). The most efficient enzyme is thought to be ADH4 (*68*). These enzymes are classified into different groups depending on their structural and kinetic characteristics, but may also present unique substrate and/or tissue specificities.

2.7.2.2.2 Step 2: Retinaldehyde dehydrogenation

Less is known about the irreversible and rate-limiting step of the oxidative conversion of *all-trans*-retinal to ATRA. This step is crucial for RAR signaling since, as mentioned above, ATRA selectively activates the RARs. Three enzymes have been identified, ALDH1a1, 2 and 3. Bhat *et al.* showed that ALDH1a1 is equally efficient at oxidizing ATRA, 9-cis and 13-cis-RA, but has an affinity more than twofold greater for ATRA (K_m = 2.2, 5.5, 4.6 uM, respectively) (*69*), and that ATRA is a competitive inhibitor of ALDH1.

2.7.2.2.3 Step 3: ATRA degradation

This step is essential in converting the highly potent ATRA into inactive and excretable polar catabolites. The Cyp26 family of enzymes has been shown to be responsible for ATRA and 9-*cis*-RA catabolism into more polar and less bioactive metabolites (*70*). In particular, CYP26A1 and B1 are ATRA and 9-cis-RA-inducible (*70*). Neiderreither *et al.* recently demonstrated - through the crossing of cyp26-/-xAldh1a2 mice - that the main function of cyp26 is the degradation of ATRA (*71*). Certain individuals, including those subjected to ATRA therapy, catabolize ATRA extraordinarily rapidly, and it is thought that polymorphisms in genes such as cyp26 (*72*) may be responsible for these differences.

2.7.3 Ingestion of retinol activity equivalents

There are at least five situations known to cause vitamin deficiencies that may affect vitamin A specifically: (1) poor intake (e.g. parenterel nutrition); (2) abnormal losses (e.g.

hemodialysis); (3) abnormal metabolism (e.g. alcoholism); (4) veganism; and (5) increasing age (*73*).

RAE ingestion is usually determined indirectly via high performance liquid chromatography (HPLC)-measured concentrations of seral retinoids. A combination of factors, such as the low levels of circulating RAs (nM range) and the protein-carrier-associated transport of retinol and certain RAs (including RBP and albumin), cause this method to be somewhat inadequate. In fact, serum concentrations may not at all reflect the level of ingestion. For example, Willet *et al.* found that serum concentrations did not correlate with ingestion even when subjects were supplemented with very large doses of retinyl palmitate (25,000 IU, or 7.5 mg) daily for a prolonged period (four months) (*74*). Other studies have shown that the levels of circulating retinoids are not reflective of dietary intake unless the hepatic stores have been severely depleted.

2.7.3.1 The recommended daily allowance

The current recommended daily allowances (RDAs) of vitamin A for healthy women and men are 700 μg (i.e. 2,330 IU) and 900 μg RAE (i.e. 3,000 IU), respectively, and 760 μg and 1300 μg for pregnant and lactating women, respectively (*54*). These values have decreased substantially over the last few years.

2.7.3.1.1 β-carotene supplementation

Many substances have gained much interest both by the scientific community and the public for their potential protective effects in multiple diseases such as cancer and cardiovascular disease. These include vitamins A, C and E, as well as the trace mineral selenium (Se), an essential inorganic anti-oxidant co-factor. However, last year, the Food and Nutrition Board of the National Academy of Sciences decided not to revise the RDA for β–carotene and carotenoids because of insufficient research to support the postulate that increased levels are safe. In fact, they recommended that pro-vitamin A intake be reduced, since RAE formulae were revised, as mentioned earlier, thereby attributing greater importance to carotenes as sources of RAEs (*75*). Also, since hypervitaminosis A is toxic in the adult and teratogenic in the embryo, the Institute of Medicine stated that β-carotene supplements are *not advisable* to the general public

even though sub-clinical deficiencies may exist. The upper tolerable limit in healthy people was thus set at 10,000 IU for both men and women, but the upper limit for carotenoids has not yet been established.

β-carotene and isotretinoin have indeed been shown to have potentially harmful effects in healthy smokers at risk for lung cancer and patients with stage I NSCLC, respectively, in two large randomized and placebo-controlled studies (*76,77*). The former consisted of 30 mg β-carotene and 25,000 IU retinyl palmitate/day; the latter consisted of 30 mg isotretinoin/day. Evidently, these doses (mg range) are thousands of times greater than the RDA (μg range). However, it is of the opinion of the author that warnings about the potential harmful effects of vitamin A/RAEs should be made increasingly public, especially in light of the potential for vitamin self-administration that exists among adults. For instance, 50% of American adults regularly take vitamins, minerals and/or other supplements (*78*), and the main motivation of approximately 1/10 of these people is the desire to prevent chronic diseases including cancer (*79*).

2.7.4 Intracellular retinoid storage

Several forms of non-specific and specific retinoid binding proteins (BPs) sequester retinoids in various compartments, such as plasma, hepatocytes, and cells in some other tissues, such as the epithelia, for example. In the liver, retinoids are packaged in chylomicrons. In the other cells such as pulmonary epithelial cells, cellular retinol BPs 1 and 2 (CRBP1 and 2) and cellular retinoic acid BPs 1 and 2 (CRABP1 and 2), bind and sequester retinol and RA, respectively (Table IX). The levels of expression of CRBPs and CRABPs have been shown to be temporally and spatially regulated. In fact, the expressions of CRBP1 and 2 are essential for post-natal alveologenesis in murine models (*80*). CRABP1 is widely expressed in adult mouse tissues, whereas CRABP2 is heavily restricted, being expressed only in a few tissues such as the eye (*81*), the skin and testis (*82*), and monocytes and macrophages (*83*). Since studies have mainly focused on mouse and rat lungs, much less is known about their human counterparts. In the normal human epidermis, CRBP1 and CRABP1 are co-expressed and exist in increasing quantities from the basal layer to the most superficial layer (*84*). F9 cells, which normally respond to ATRA via upregulation of several differentiation genes (including RARβ2, laminin B1 and collagen type IV) exhibit a decrease in upregulation by

up to 90% upon low concentration ATRA treatment when stably transfected with a CRABP1 construct, demonstrating that CRABP1 sequesters RA in the cytosol, disallowing differentiation (*85*). Conversely, antisense cDNA constructs against CRABP1 result in increased ATRA sensitivity, where the rate of RA metabolism to 4-oxo-RA (a polar and thus water-soluble catabolite) is correlated with the level of CRABP1 (*86*). It is therefore thought that CRABPs facilitate the storage and timely catabolism of retinoids for usage upon requirement, and help protect the cell from their potent effects. Genetic polymorphisms as well as methylation-associated gene inactivation (MAGI) have been shown to alter the expression of retinoid BPs, such as CRBPI, for example, whose mRNA expression is frequently lost in breast cancer cells and the mechanism of inactivation is thought to occur through DNA methylation (*87*).

TABLE IX. Dissociation constants of the retinoid binding proteins. Taken from Table 1, reference (*88*). BP = binding protein; K_d = constant of dissociation; RA = retinoic acid.

BP	Ligand	K_d (nM)
CRBP1	Retinol	0.1
	Retinal	10 - 50
CRBP2	Retinol	
	Retinal	
CRABP1	RA	0.4
	RA metabolite	
CRABP2	RA	2.0
	RA metabolite	

2.8 Functions in the adult

Vitamin A is thought to have the greatest effects on the homeostasis of several tissues of all the known vitamins (*89*). And with regard to cancer, retinoids have been shown to have potent effects on several cellular processes, including proliferation, differentiation, immunomodulation and apoptosis, both *in vivo* and *in vitro*, and both in normal cells and in cancer cells. Numerous studies have been conducted on the use of retinoids to block cellular transformation, to inhibit cell growth or differentiation, or to enhance apoptosis.

The following brief overview is mainly limited to those studies focusing on the effects of retinoids on the lung, whether through *in vivo* and *in vitro* lung cancer models, or studies with cohorts composed of patients diagnosed with - or at risk for - lung cancer.

2.9 Retinoids play a role in cancer prevention

As mentioned previously, the evidence suggesting that vitamin A may be linked with cancer was inferred from the results of experimental studies showing that vitamin A-deficient (VAD) rats exhibited squamous hyperplasia and metaplasia of the tracheobronchial, intestinal and genito-urinary mucosae (*49*), and these findings were experimentally confirmed in hamsters (*90*), and in human tracheobronchial cells *in vitro* (*91*). However, these early studies may have overlooked the contribution of certain other factors, since the entire lipid-soluble fraction, which contains other lipid-soluble vitamins such as vitamins D, E and K, and which may contain other lipid-soluble co-factors, all of which may play a role in preventing or reversing cancer, was removed from the food rations.

2.9.1 Experimental evidence

The historical breakthrough linking retinoids with cancer prevention came when RAs and their synthetic analogs were found to reverse the preneoplastic process *in vitro* (in organ culture systems) (*92-97*), as well as *in vivo* (*98,99*). Several subsequent studies found that in certain cancer cell lines, including those derived from the lung, retinoids and/or retinoid receptor agonists (both isoform-specific and pan-agonists) could reduce proliferation (*100-105*), cause apoptosis (*103,105-107*), or inhibit invasion (*108*) *in vitro*, and these findings were again mirrored *in vivo* (*106*). Studies also showed that retinoids can differentially regulate the expression of many differentiation markers, including involucrin, transglutaminase I, cholesterol sulfate, and keratin 13 (*91,109-111*).

Most studies since then have focused on the potential tumor suppressive activities of retinoids.

It is informative to point out that many of the cell lines used in these studies were in fact shown to be unaffected, and unexpectedly, a few cell lines were even shown to be growth-stimulated by ATRA, including 2 NSCLC and 1 colon adenocarcinoma cell lines

(*101,112*). In addition, we have also found that one breast cancer cell line (HS-578T) appears to be growth-stimulated upon treatment with ATRA (results not shown). Interestingly, the mRNA expressions of RARβ2 and Major Histocompatibility class I (MHC class I) are inversely correlated with ATRA concentration (Appendix E), and MHC class I is frequently down-regulated in cancer, and has been shown to be correlated with outcome in NSCLC specifically (*113,114*). These findings suggest that in this cell line, the effects of ATRA are not tumor suppressive as they lead to growth induction and immune evasion.

Differences in proliferative and apoptotic responses to retinoids may arise in various ways. One, the various cancer-derived cell lines may have different cellular origins, including Clara cells, type 2 pneumocytes, or other pulmonary stem cell origins (Appendix A). It is not yet known how these different pulmonary stem cells respond to retinoid treatment, but one study has showed that alveolar epithelial cells require vitamin A (in the form of *all-trans*-retinyl palmitate) for maintenance (*115*). Two, the various cell lines may have acquired different genomic and epigenomic modifications along the pre-carcinogenic and carcinogenic processes, including different levels of expression of retinoid receptor isotypes (RARα, β, γ, RXR α, β, γ), which may have different effects on these processes, for example. Three, polymorphisms affecting the various enzymes involved in retinoid metabolism may affect the resultant levels of bioactive retinoids (*72*). Nonetheless, it is clear from these and other studies that retinoids may have positive as well as negative effects on cellular proliferation depending on cell type and context. On the whole, the mechanisms responsible for these diverging effects remain unclear.

On the other hand, two recent experimental studies reveal possible mechanisms responsible for the negative (even harmful) effects of β-carotene supplementation seen in smokers at risk for lung cancer in the large randomized and placebo-controlled studies [CARET (*77*) and the ATBC (*76,116*)] mentioned in the previous section. It is in the author's opinion that their potential impact on the elucidation of the various factors involved in RA-controlled effects deserves their exegesis.

The first study, by Albright *et al.* in 2004, investigated the effects of depletion of vitamins E and A and consequent abrogation of the anti-oxidant effects thereof on the quenching of reactive oxygen species (ROS) (*117*) in mice. Mouse mammary tumor virus (MMTV)-

driven Polyomavirus middle T antigen (or MMTVPyV) transgenic mice that had developed mammary tumors were fed a diet depleted in vitamins E and A for six weeks and then sacrificed. Results showed that tumor cell mitosis was reduced by 50%, apoptosis was increased by 500% (as reflected by apoptotic cell count), and mice had about three times less metastases than their control counterparts (normal diet). Based on the finding that certain oxidants such as reactive oxygen species (ROS) are essential for tumor cell apoptosis (*118*), Albright *et al.* concluded that the anti-oxidant role of vitamins E and A may be deleterious in the context of cancer. The finding that most lipophilic anti-oxidants, including tocopherols, carotenoids and retinol, are degraded upon exposure to the gaseous phase of cigarette smoke (*119*) is consistent with these findings.

The other study looked at the effects of chronic β-carotene supplementation with or without chronic cigarette smoke inhalation on the lungs of ferrets, and analyzed the products of retinoid degradation (*120*). Ferrets were either supplemented with low or high doses of β-carotene (equivalent to 2.4 mg/70 kg/day or 30 mg/70kg/day in humans, respectively), and exposed or not to cigarette smoke resulting in an accumulation of urinary cotinic acid (equivalent to that observed in smokers of 1.5 packs/day). Results showed that high dose β-carotene was correlated with the focal proliferation of type II pneumocytes and keratinized squamous metaplasia, and these observations were exacerbated in the presence of cigarette smoke. Treatments consisting of smoke, high β-carotene or both resulted in the reduced expression of *RARβ2* protein (by 18, 62 and 73%, respectively), whereas *RARα* and γ were not affected. Also, *c-jun* protein expression was increased (by 28%, 21% and 316%, respectively) and so was that of *c-fos* (19%, 94% and 229%, respectively), thus demonstrating synergistic effects. Moreover, the unexpected presence of normally rare eccentric β-carotene cleavage products (including retinoid structures that may retain bioactivity) and the reduction of ATRA (not detected in either smoke- or β-carotene + smoke-exposed ferrets) in the lungs of the β-carotene + smoke test group suggest that retinoid metabolism is altered in the presence of smoke, probably due to the free radicals. Since β-apo-8'-carotenal, an eccentric cleavage product was shown to up-regulate cytochrome P450 enzymes such as CYP1A1 and CYP1A2 (*121*), was increased (2.5-fold) in smoke-exposed ferrets, then P450 enzymes may play a role in altered retinoid metabolism/signaling. Reduced β-

carotene and increased eccentric β-carotene cleavage products might reduce RARβ2 expression and consequently retinoid signaling, and further increase AP-1 (*c-jun/c-fos*) mitogenic function (described in section 3.10), which is already stimulated in the presence of smoke-derived free radicals.

To date, too few studies have looked at the effects of retinoids on *normal* lung tissue proliferation and pattern formation, from which certain conclusions could be drawn and hypotheses extrapolated to cancer. However, those few studies that have been performed are unexpectedly revealing. Cardoso *et al.,* for example, found that high concentrations of RA proximalized developing mouse airways *in vitro*, inducing proximal while suppressing distal structures (*122*). Nabeyrat *et al.* showed that RA induces the proliferation of type II pneumocytes, which are in fact pulmonary stem cells [(*123*); Appendix A]. Thus, RA has been shown to have tumor-related activity in the sense that it can affect both normal and cancer cell proliferation positively.

Microarray technology might help elucidate the different genetic pathways at play under the control of RA-mediated regulation in different pulmonary cell types. For example, the gene expression profiles of various purified cell types, such as mucus cells versus ciliated epithelial cells, and in different contexts, such as in the presence versus absence of extracellular matrix, could be compared through genechip technology. None are currently available. The author is aware of only one paper in which epithelial-derived cancer cells, not normal cells, were treated with RA and subsequently analyzed via cDNA microarray (*124*). In particular, MCF-7 breast cancer cells were treated with 100 nM RA for 5 days and the resulting RNAs were reverse transcribed, fluorescently labeled and hybridized to a human UniGEM V cDNA microarray containing >7,000 genes. The article focuses on only 4 genes known to have anti-proliferative activity (IGF-BP3, EPLIN, βIG-H3 and FAT10) of the 13 that were strongly induced. It would be of interest to perform data mining on these results and verify whether or not genes positively involved in tumor-related processes were equally induced.

Also, since the stages of carcinogenesis are characterized by increasingly altered conditions, or multi-hits, it is plausible that RARβ2 plays different - even opposing - roles depending on the stage at which carcinogenesis has proceeded. Consequently, cell lines in which carcinogenesis had been reversed by RARβ2, might have evolved to

similar stages of carcinogenesis (i.e. to stages when the genotype resulted in phenotypically-similar cells).

2.9.2 Evidence from epidemiological studies

Several early epidemiological studies based on interview and questionnaire-type surveys revealed that a diet rich in vitamin A was correlated with a decreased risk for several epidermoid cancers, including pulmonary, laryngeal, oral, cervical and urinary bladder cancers, as well as mammary and ovarian cancers albeit to a lesser degree [reviewed in reference (*125*)]. Contrastingly, such a diet was found to be correlated with an increased risk for prostate cancer (*126*) at about the same time (i.e. early 1980s). The main critique concerning interview- and questionnaire-type surveys is the phenomenon of inaccurate recall. In addition, there are numerous confounding factors inherent to epidemiologic studies based on regimen, and these are perhaps of even greater concern. For example, the intake of fruits and vegetables may be correlated with other factors, such as a healthful lifestyle, and these factors may not be taken into account. Also, fruit and vegetable sources are known to contain many other carotenoids and co-factors that may play a beneficial role but are not accounted. For example, of the over 600 naturally occurring carotenoids of which over 40 are known to be consumed in the American diet, only 6 are ever usually studied (i.e. β-carotene, α-carotene, β-cryptoxanthin, lycopene, lutein and zeaxanthin). More recent studies based on serum concentration assays measuring these six carotenoids, as well as α-tocopherol (vitamin E) and selenium, showed that three carotenoids in particular were significantly decreased in patients with lung cancer as compared to healthy controls, including cryptoxanthin (-25.5%), β-carotene (-17.1%), and lutein/zeaxanthin (-10.1%) (*127*). However, as previously stated, retinoid and carotenoid concentrations in the plasma are very difficult to measure and may not reflect the actual level of intake. It was later shown by the First National Health and Nutrition Examination Survey Epidemiologic Follow-up Study that the observed protective effects against lung cancer were correlated with the intake of fresh fruits and vegetables (sources which contain β-carotene), not from the ingestion of pre-formed and purified oral supplements of vitamins A (retinol) or C, and were modified negatively by the intensity of smoking (*128*), in accordance with Albright *et al.* (*117*). Thus, the evidence from epidemiologic data alone does appear to support a trend between a diet rich in fresh produce-derived retinoid equivalents, but is insufficient

or does not support a role for dietary supplements thereof. Most importantly, epidemiologic data has not yet clearly assessed the effects of retinoids, among smokers or anyone else, on lung cancer rates, other than in retrospective studies. Thus, the evidence is insufficient, and it is also inadequate, since the major risk factor is smoking.

2.9.3 Evidence from chemopreventive studies

Chemoprevention is the use of natural and/or synthetic substances to prevent, stop or reverse the multi-step carcinogenic process, known to affect large and diffuse fields of cells within tissues such as the epithelium after long-term exposure to carcinogen sources, such as tobacco smoke. Thus, chemoprevention of carcinogen-induced cancers is based on two theories (*129*): (1) The theory of *field cancerization*, to describe leukoplakia (*130*), a clinical term referring to white patches on the oral mucosa known to be pre-neoplastic forms of oral cancer, and proposed to explain the phenomena of multiple primaries and high recurrence of oral cancer; (2) The theory of *multi-step carcinogenesis*, which explains that sporadic colon cancers evolved through six successive preneoplastic phases in which specific genetic and epigenetic alterations catalyzed the morphological alterations of the next phase (*131*).

The evidence from chemopreventive studies is more abundant and less vague than the evidence from epidemiological studies, but results mainly suggest that supplemental retinoids should not be used in the chemoprevention of certain cancers, including lung.

Retinoids have been tested in the prevention of primary and secondary cancers derived from several types of epithelia. In general, early studies appeared to show that retinoids have a positive effect on reversing metaplasia or preventing recurrence, but only in certain kinds of cancer. For example, the naturally occurring retinoid ATRA was found to have positive results in the primary prevention of cervical cancer, in patients with moderate dysplasia of the uterine cervix (*132*), and in heavy-smoking patients with epidermoid metaplasia (*133*), but was not effective in the treatment of many solid epithelial-derived cancers (*134*). Moreover, prolonged ATRA treatment is associated with some toxicity and it also stimulates its own catabolism, via trans-activation of Cyp26, for example. Pharmacological retinoids have also been widely tested. For example, etretinate was tested in the secondary prevention of urinary cancers, with

positive results (*135*), and in head and neck cancers, but with negative results (*136*). A few other early studies also showed that retinoids reduced the incidence of bronchial metaplasia in chronic smokers (*133,137*), but these studies consisted of very small cohorts and did not contain control (placebo) arms. A series of large and placebo-controlled chemopreventive studies that occurred later showed that there was no reduction in lung cancer incidence, morbidity or mortality. These studies consisted of supplementing the diets of patients at risk for developing a primary lung cancer (*77,138-142*) or a second primary (i.e. recurrence) lung cancer (*143*) with various retinoids and/or carotenoids. β-carotene and vitamin A supplementation were shown *not* to have any benefit and to even have deleterious effects for patients at risk for lung cancer (*144*). In particular, in the large trial consisting of 18,314 American smoking men and women at high risk for developing lung cancer mentioned previously (section 2.9.1), β-carotene and retinol supplementation was correlated with a 28% increase in incidence and a 19% increase in mortality from lung cancer as compared to placebo, and the study was consequently stunted 21 month early [CARET study; (*77*)]. These findings were closely mirrored in the ATBC study (section 2.9.1), in which 29,133 Finnish smoking men supplemented with α-tocopherol and β-carotene, and lung cancer incidence and mortality were increased by 18% and 8%, respectively, as compared to placebo (*141*).

In total, at least six different retinoids have been used in 10 randomized lung cancer/pre-neoplasia trials, either in the prevention of primary cancers in patients at risk, or in the prevention of recurrence in patients who have already been diagnosed and treated for lung cancer. These six retinoids were: (1) β-carotene (in combination with α-tocopherol/vitamin E, or with RA; (2) retinoic acid (in combination with β-carotene); (3) etretinate; (4) isotretinoin; (5) fenretinide; and (6) retinyl palmitate. None of these 10 trials resulted in any of these 6 agents having a positive effect on outcome, and as stated above, 2 trials (using β-carotene/Vitamin E and β-carotene and RA, respectively, in healthy but high-risk smokers) were even shown to be harmful [for a review, please see reference (*129*)].

2.9.4 Aside: Retinoids in the treatment of acute promyelocytic leukemia

Retinoids have been tested and used in the treatment of acute promyelocytic leukemia (APL) and other hematological malignancies including lymphoma. However, their mode

of action is thought to relate to their effect in the context of the oncogenic fusion protein PML-RARα, which is a genetic aberration frequently found in APL patients due to the nearly ubiquitous and leukemia-specific t(15;17)(q22;q21) chromosomal translocation [for a review, see reference (*145*)]. These results may therefore not be used in the study of retinoids in the prevention and treatment of lung cancer.

2.10 Conclusion

Though early studies appeared promising, the majority of the evidence obtained from more current large, randomized and placebo-controlled studies, which provide the highest level of evidence, converges toward the possibility that carotenoids and/or retinoids may not have uniquely protective effects in lung cancer prevention, and may actually be deleterious in certain contexts, such as in smokers and other carcinogen-exposed individuals (e.g. asbestos miners). Certain recent experimental studies, such as those lead by Albright *et al.* (*117*) and Wang *et al.* (*120*) may help shed light on the some of the mechanisms at play, including RA-sequestration of ROS and the effects of eccentric β-carotene cleavage products.

3 Retinoid receptors

The diverse effects of the main biologically active retinoids, ATRA and 9-*cis*-RA (section 2.7.2.1), are mediated through nuclear receptors encoded by two major gene families: (1) the retinoic acid receptors (RARs); and (2) the retinoid X receptors (RXRs).

RARs and RXRs belong to the nuclear hormone receptor superfamily, which includes: (A) the Type I receptors (or steroid receptors): (1) androgen receptors (ARs); (2) estrogen receptors (ERs); (3) progesterone receptors (PRs); (4) glucocorticoid receptors (GR); and (5) mineralocorticoids receptors (MRs); and (B) the Type II receptors (non-steroid receptors): (1) retinoic acid receptors (RARs); (2) retinoid X receptors (RXRs); (3) thyroid hormone receptors (THRs); (4) vitamin D receptors (VDRs); and (5) peroxisome proliferator-activator receptors (PPARs). Orphan receptors represent an additional category for which ligands have not yet been found, for example, SF-1, COUP-TFI and II, and HNF-4.

3.1 Isotypes and isoforms

RAR and RXR isotypes are encoded by six different genes located at six chromosomally-distinct loci and include RARα, β, and γ, and RXRα, β, and γ. Different isotypes have different patterns of tissular expression and also have different functions (Table X). Each RAR gene contains two alternate promoters (P1 and P2), and may be alternatively spliced into 2-4 isoforms, depending on promoter usage and alternative splicing. RXRs form homo- and heterodimers with various other receptors, including RARs, TRs, VDRs, and PPARs. RARs do not appear to form homodimers, but do heterodimerize with RXRs. In fact, RAR function is mainly mediated via RXR-RAR heterodimers.

TABLE X. RAR and RXR isotypes. ATRA = all-trans-retinoic acid; CNS = central nervous system; RAR = retinoic acid receptor; RXR = retinoid x receptor.

Gene	Locus	Isoforms	Expression	Known functions	Endogenous ligands
RARα	17q21.1	α1,α2	Nearly ubiquitous	Differentiation	ATRA 9-cis-RA
RARβ	3p24	β1,β2,β4	CNS, several epithelia, liver, heart, kidney	Proliferation Differentiation Immune function Apoptosis	
RARγ	12q13	γ1,γ2	Skin, cartilage	Chondrogenesis Keratinization	
RXRα	9q34.3	α1,α2	Skin, liver, kidneys, breast	Retinoid metabolism	9-cis-RA
RXRβ	6p21.3	β1,β2	Ubiquitous	Embryonic development	
RXRγ	1q22-23	γ1,γ2	Muscle, CNS	CNS development	

3.2 Ligand selectivity

Ligand binding effectively activates the RXR-RAR receptor. 9-cis-RA is the main bioactive retinoid because it binds both RARs and RXRs with high affinity (though is has a much greater affinity for RXRs); ATRA only binds the RARs (Table XI). Thus, the use of ATRA in experimental procedures is more RAR- than RXR-specific, even though RAR function is mainly mediated via RXR-RAR heterodimers.

TABLE XI. Dissociation constants of mouse RARs and RXRs. Taken from Table II, reference (*146*). ATRA = all-trans-retinoic acid; NB = no binding.

Receptor	Isotype	ATRA	9-cis-RA
RAR	α	0.20	0.24
	β	0.36	0.40
	γ	0.20	0.70
RXR	α	NB	15.70
	β	NB	18.30
	γ	NB	14.10

3.3 Retinoic acid response elements (RAREs)

Ligand-activated homo- and heterodimers gain entry into the nucleus via an unknown mechanism. Once in the nucleus, RXR-RAR heterodimers bind RA response elements (RAREs) located within the promoters of their target genes. RAREs thus ensure hormone-specific multi-gene responses. They consist of two direct repeats (DR) of a core motif of the consensus hexamer sequence "Pu-G-G/T-T-C-A" (or closely related degenerate motifs). DRs are interspaced by 1, 2, 3, 4 or 5 nucleotides, also known as DR1 - DR5, respectively, and these are selectively bound by different RXR heterodimers. RAR-RXRs and PPAR-RXRs bind DR1; VDR-RXRs bind DR3; THR-RXRs bind DR4; and RXR-RARs bind both DR2 and DR5. All combinations are trans-activating, except RAR-RXR/DR1, which is repressive. The most common RAREs are DR5s (bound by RXR-RARs in the 5' to 3' orientation), and these are found in many gene promoters including the RARα2, β2, and γ2 promoters, thereby allowing auto-regulating of their own transcription (Fig. 7).

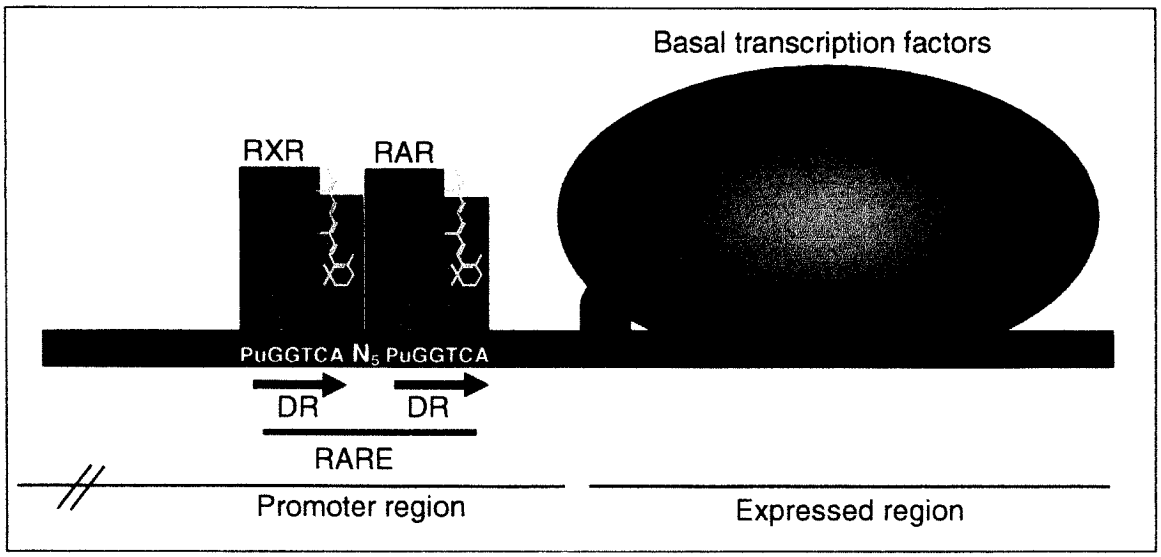

FIGURE 7: Schematic diagram representing an RXR-RAR heterodimer binding a RARE, which consists of two direct repeats (DR) of a core motif of the consensus sequence "Pu-G-G/T-T-C-A" or closely related degenerate motifs, interspaced by 1, 2, or 5 nucleotides (A = adenine; C = cytosine; G = guanine; T = thymine; Pu = purines A or G). DR = Direct repeat; RAR = retinoic acid receptor; RXR = retinoid X receptor; RARE = retinoic acid response element.

Retinoid signaling may result through *direct* ligand-bound RXR-RAR:RARE complexes as described in the preceding paragraph, or via other *indirect* mechanisms not involving ligand-bound RXR-RAR dimers at RAREs, such as through transcription intermediary factors (TIFs).

Balmer and Blomhoff evaluated data from 1,119 papers on RA-target genes and showed that of the 532 genes known to be regulated via RA, 27 are demonstrably regulated directly (Table XII), 105 are candidates for direct regulation, 267 are regulated via RA mechanisms, and 133 are probably regulated indirectly, such as through TIFs (*147*).

TABLE XII. Genes regulated directly via RA-bound RXR-RAR through a RARE. Based on reference (*147*): functions and main cellular processes as per search through OMIM. BP = binding protein; CK = cytokine; TF = Transcription factor; TSG = Tumor suppressor gene.

Gene	Locus	Function	Main processes
ADH3	4q22	Dehydrogenase	Retinoid metabolism
CD38	4p15	Surface protein, ectoenzyme	Metabolism, adhesion, signal transduction, calcium signaling
Cdx-1	5q31-q33	Homeobox TF	Putative TSG (intestinal epithelium)
C/EBPε	14q11.2	TF	Differentiation of myeloid lineages
CRABP2	1q21.3	RA and fatty acid BP and RXR-RAR co-activator	Intracellular retinoid movement and metabolism
CRBPI	3q21-q22	Intracellular lipid binding protein (retinol)	Intracellular retinoid movement and metabolism
CRYA2	11q22.3-23.1	Small heat shock protein	Induced in some neurological autoimmune diseases
DRD2	11q23	Dopamine receptor	Behavior modification (e.g. appetite)
Krox-24	5q31.1	Early growth response gene	TGFβ1 regulation, growth, apoptosis.
Ets-1	11q23.3	TF and oncogene	Proliferation, differentiation, angiogenesis and metastasis
HNF3α	14q13	Forkhead-like TF	Unknown, but expression restricted to tissues of endodermal origin
H1(0)	22q13.1	Histone protein	Chromatin organization
HOXA1, A4,	7p15-p14.2	TF	Morphogenesis and differentiation
B1, B4	17q21-q22		
D4	2q31-q32		

HSD17B1	17q12-q21	Hydroxysteroid dehydrogenase	Estrogen and testosterone metabolism
IL-2Rα	10p14-p15	CK receptor in lymphocytes	CK signaling, regulation of T-cell function
Pck1	20q13.31	Phosphoenolpyruvate carboxykinase-1	Regulation of gluconeogenesis
Pit-1	3p11	Pituitary-specific TF	Pituitary development Hormone expression
RARα2	17q12	Nuclear TFs	Differentiation and neurogenesis
RARβ2/β4	3p24		Proliferation, differentiation, immune function and apoptosis
RARγ2	12q13		Chondogenesis (cartilage) and keratinization (epidermis)
SP-B	2p12-p11.2	Alveolar surfactant protein	Reduction of surface tension at the air-liquid interface
Tgase I	14q11.2	Enzyme (structural protein cross-linking)	Metabolic and energetic pathways; Epidermal differentiation, squamous metaplasia
Ucp1	4q31	Mitochondrial uncoupling protein	Brown adipose tissue respiration

3.4 Modularity

Retinoid receptors share a basic modular structure comprised of five regions known as A/B, C, D, E and F (Fig. 8). However, based on findings which have shown that isoforms are spatiotemporally expressed, it is generally accepted that different isoforms may be responsible for different gene pathways, thereby regulating unique and highly distinct biological functions. The N-terminal A/B region encodes the activator function

(AF-1), though two independent activator functions, AF-1 (ligand-independent) and AF-2 (ligand-dependent), located in the A/B and E regions, respectively, act synergistically. The A region is highly variable both in sequence and length, and is isoform-specific due to the alternate promoter usage and alternative splicing mentioned above, whereas the B region is moderately variable. The C and E regions are the most highly conserved, following a small and centrally located sub-region in region D, which is the most highly conserved region, and is highly RAR-specific. The function of the D region is not yet known, however it is known to act as a steric hinge that allows up to 180 degrees swivel, required in the formation of hormone:receptor:DNA complexes. The C region, which consists of two consecutive zinc-fingers, encodes the DNA binding domain. The zinc-fingers, which are finger-like DNA-binding protein loops in which four cysteines coordinate a zinc ion (Zn^{++}), interact directly with the RARE. Receptor dimerization interfaces are located in the E and C regions. The E region consists of the ligand binding domain (LBD). The F region, for which a function has not yet been found, does not present any degree of homology among RARs and is not existent in RXRs. [For a review, see reference (*148*)].

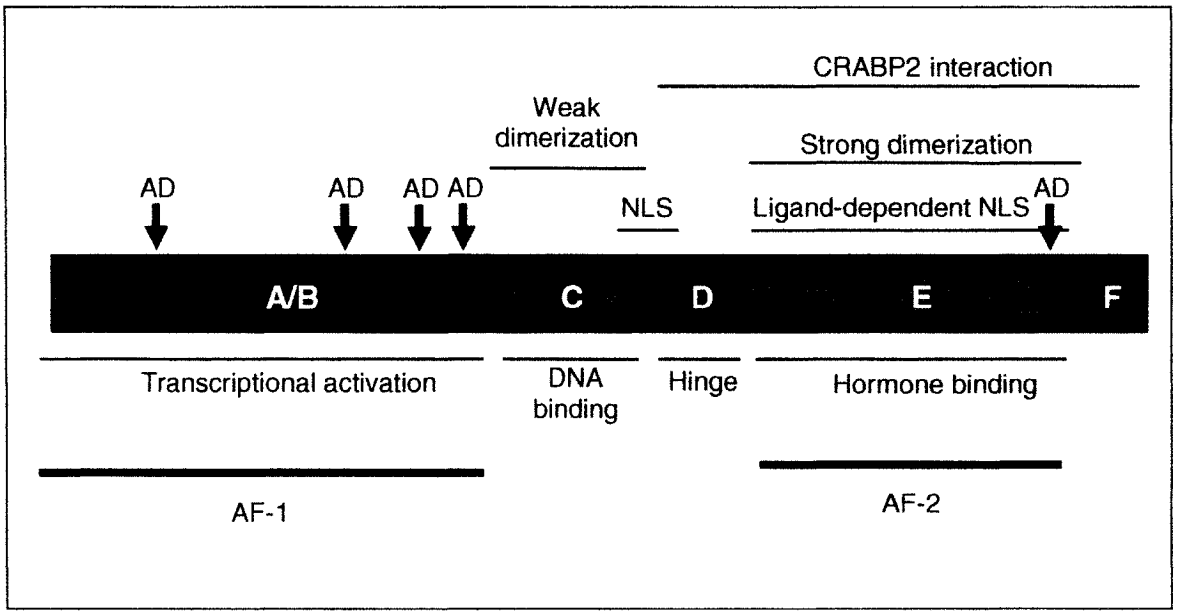

FIGURE 8. Schematic diagram of the modular structure of retinoid receptors. The A region is highly variable and isoform-specific (due to alternate promoter usage and alternative splicing). The B region is moderately variable. Two independent activator functions, AF-1 (ligand-independent) and AF-2 (ligand-dependent), located in the A/B and E regions, respectively, act synergistically. The C and E regions, which encode the DNA binding and the ligand binding domains, respectively, are the most highly conserved after the central region of the D region (function unknown) located in the hinge. Dimerization interfaces are located in the E and C regions. The F region, for which a function has not yet been found, is highly variable among RARs and not existent in RXRs. Pink boxes = modules having high degree of homology; Blue boxes = modules having low degree of homology; Based on Fig. 3 of reference (*149*). AD = autonomous trans-activation domains; AF-1 = Activator function-1; AF-2 = Activator function-2; NLS = nuclear localization signal; CRABP2 = cellular retinoic acid binding protein 2.

3.5 Homology

Regions C and E are highly conserved, sharing 94-97% and 84-90% homology among RARs, respectively (*148*). Each RAR shares a greater degree of homology between species than between isotypes, and this is significant for three reasons. First, the conservation of a DNA sequence through evolution *in general* is thought to reflect the essential nature of the gene product it encodes. Second, the greater inter-species divergence than inter-isotype divergence has been suggested to have occurred prior to invertebrate/vertebrate evolution. Third, the retention of isotypes reflects their likely importance in the fine-tuned regulation of distinct biological processes.

3.6 RAR expression

Most studies on RAR expression have focused on RAR isotypes as opposed to isoforms and have used *in situ* hybridization to assess RAR mRNA expression in the developing mouse embryo. Tissue-specific expression patterns of RAR isoforms have only recently been studied in the developing mouse embryo (*150*).

RARα is nearly ubiquitously expressed, having high expression in nearly all tissues (*151*) except the nervous system (*150*). Its function is generally associated with differentiation. RARα has also been shown to be implicated in murine neurogenesis along with RARβ (*152*).

RARβ expression is restricted mainly to the tracheobronchial, intestinal and reproductive epithelia (*151*), where its function is generally associated with differentiation and homeostasis, and to the central nervous system, where it has been shown to regulate neurogenesis both in the mouse (*152*) and frog (*153*). The RARβ gene encodes four isoforms, β1-β4. RARβ1 is mainly expressed in fetal tissues, especially the fetal nervous system, and it is also expressed in SCLC (*154*). RARβ2 (section 4) is significantly expressed in most adult epithelial tissues, including the pulmonary, mammary and intestinal epithelia (*155*). RARβ3, which was characterized in the mouse, does not have a human homologue (*154,155*). RARβ4 appears to have antagonistic effects against β2 through its truncated N-terminus (*156*), however it is not significantly expressed in non-cancerous tissues.

RARγ is expressed mainly in the epidermis and cartilage (*157*), and its function is generally associated with keratinization and chondrogenesis.

3.7 RAR knock-out mice

Isoform-specific single null mutant mice such as RARα1-/-, RARβ2-/-, and RARγ2-/- appear normal [(*158-160*), respectively], whereas isotype-specific (multi-isoform) single null mutant mice such as RARα-/-, RARγ-/-, RXR+/- and RXRβ-/- exhibit poor viability, shunted growth and male sterility, which are manifestations reminiscent of postnatal

VAD (section 4.2). These limited pathological phenotypes likely reflect the functional redundancy manifested by retinoid receptor isotypes and isoforms, an expected bi-product of gene duplication, proposed to be the mechanism through which the RARs and RXRs evolved. Treatment with RA was shown to prevent and even reverse most of these manifestations, likely because RA induces the expression of certain isoforms, including α2, β2 and γ2 [(*161-163*), respectively]. This finding demonstrates that RAR/RXR heterodimers mediate the physiological effects of vitamin A derivatives.

In contrast to single null mutants, Lohnes *et al.* showed that the majority of compound mutants, especially those involving RARβ and α1 or γ, exhibit extremely poor viability, dying either *in utero* or perinatally (*164*) and many congenital abnormalities [for a review, see (*165*)]. This underlines both the redundancy and the importance of these receptors in basic life processes, previously suggested through the evolutionary conservation of their DNA sequences (section 3.5).

It is important to note that though redundancy of RAR function appears to be common among RAR receptors during ontogeny, multiple factors suggest that isoform specificity is the mechanism through which multiple cellular processes are fine-tuned later on in more advanced stages of development. Moreover, it is essential to note that there are many differences between mouse and human RAR functions, including very basic differences such as the lack of the human mRARβ3 homolog, for instance. Indeed, the multiple known mechanisms through which the diversity of retinoid signaling responses are known to be fine-tuned demonstrate the complex yet elegant mechanisms employed by nature to realize the diversity of retinoid signaling.

3.8 Combinatorial complexity

RAR homologs have been studied in species as diverse as the worm (*Caenorhabditis elegans*), the fruit fly (*Drosophila melanogaster*), and the vertebrates (including *Mus musculus* and *Homo sapiens*). The central theme that has been revealed with regard to the function of retinoid receptors is their contribution to the multiplicity of function of retinoids via their combinatorial effects. Multiplicity of function is mainly derived from seven aspects of retinoid biology: (1) diversity of biologically active retinoids (e.g. retinol, ATRA, 9-cis-RA, and 3-4, didehydroRA to a lesser extent); (2) diversity of cellular retinol

and RA binding proteins (CRBP1 and 2 and CRABP1 and 2); (3) diversity of retinoid receptor function (AF-1, AF-2, trans-repression and trans-activation); (4) diversity of retinoid receptors (RAR α, β, γ, RXR α, β, γ) and related combinatorial effects of their homo- and heterodimerization (resulting in an estimated 84 possible permutations, e.g. RXRα-RARβ); (5) spatiotemporal expression of retinoid receptor isoforms; and (6) numerous and polymorphic RAREs; (7) numerous co-factors, including co-activators (AF-1 and AF-2 agonists), co-repressors (N-CoR, SMRT, NFκB, AF-1 and AF-2 antagonists), and cross-talk with other TFs and TIFs.

3.9 Trans-activational and trans-repressive functions

RARs bind co-activators, such as p300, Creb binding protein (CBP) and p300/CBP-associated factor (P/CAF), and co-repressors, such as silencing mediator for retinoid and thyroid receptors (SMRT) and nuclear repressor corepressor (N-CoR) [for a review, see references (*166,167*)].

In the absence of RAR ligand, co-repressors may associate with the RAR leading to transcription inhibition (RXR subordination). In the presence of RAR ligand but not RXR ligand, the co-repressor is dissociated allowing co-activator recruitment to the LXXLL box (L = leucine; X = any amino acid) and consequently transcriptional activation (Fig. 9). In the presence of both RAR and RXR ligands, the co-activator binds both moieties of the heterodimer cooperatively through both of its LXXLL boxes. Though this mechanism of action was thought to apply to all RAR isotypes, Farboud *et al.* showed that in the absence of hormone, RARα binds SMRT with high affinity thereby repressing target gene transcription, but RARβ and γ bind SMRT with very low affinity and do not repress target gene transcription, but retain their trans-activational function, which is enhanced in the presence of hormone (*168*). In particular, helix 12 occludes the SMRT docking site (present in all RARs) and helix 3-helix 12 interactions help stabilize this occlusion.

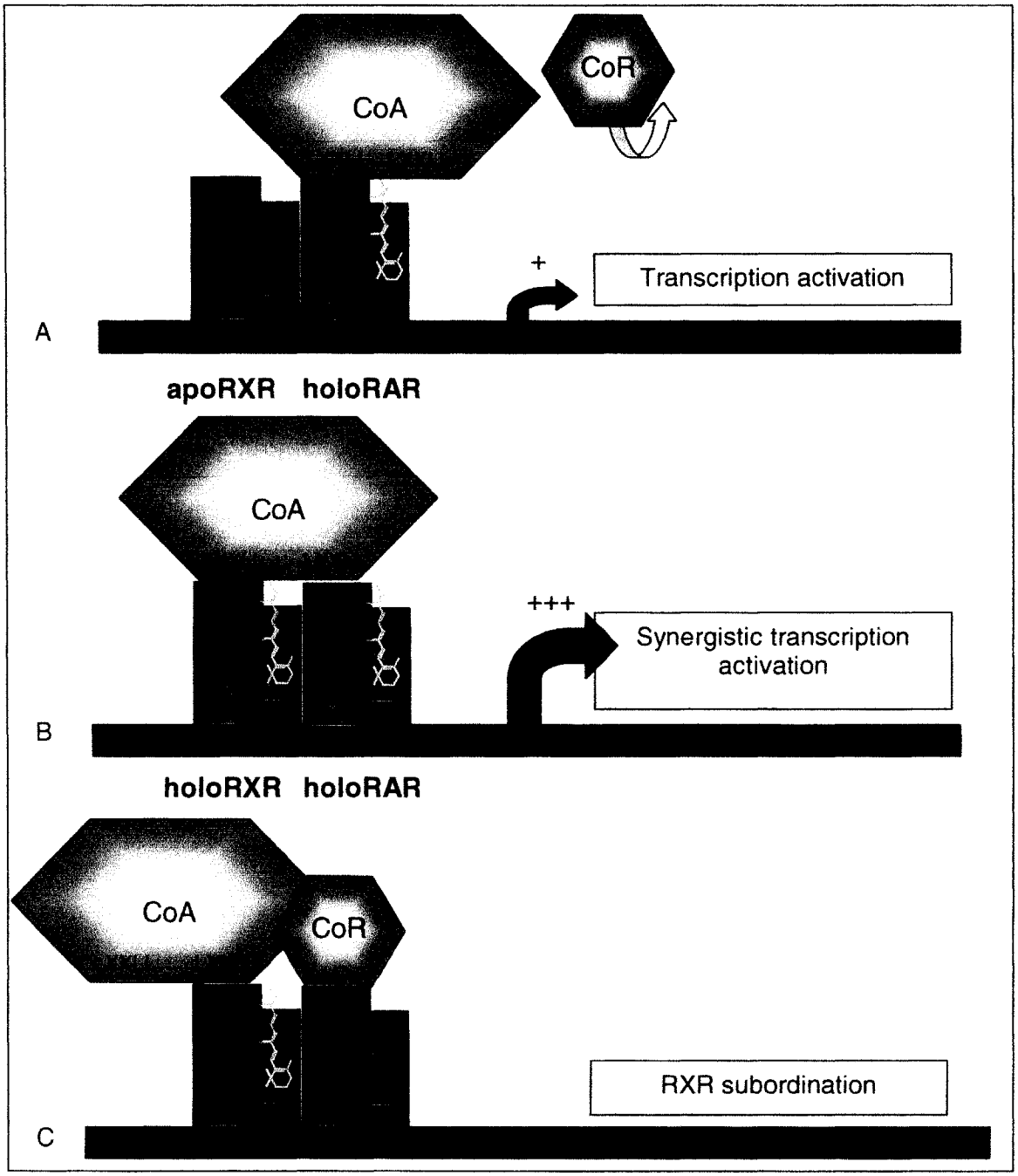

FIGURE 9: Schematic diagram representing co-activator versus co-repressor docking to the RXR-RAR heterodimer and their effects on transcriptional activity. **A**. In the presence of RAR ligand only, the co-repressor is dissociated allowing the co-activator to be recruited, thereby allowing transcriptional activation. **B**. In the presence of both RAR and RXR ligands, the co-activator binds both moieties of the heterodimer cooperatively through both of its LXXLL boxes. **C**. In the absence of RAR ligand, there is association of the co-repressor to the RAR and transcription is inhibited (RXR subordination). Based on Fig. 10 of (*149*). CoA = co-activator; CoR = co-repressor; LXXLL = motif where L is leucine and X is any amino acid.

3.10 RARα, β, and γ-mediated AP-1 sequestration and inactivation

Activator protein-1 (AP-1) complexes are dimeric transcription factors formed by two of the following oncogenes: *c-jun, c-fos, c-maf,* or the transcription factor *atf.* AP-1 binds and transcriptionally activates the expression of genes containing 12-O-tetradecanoylphorbol-13-acetate (TPA) or cAMP response elements (CRE) in their promoter. AP-1 mediates several types of signals, including growth, inflammatory, mitogenic and apoptotic signals, but its activation is normally correlated with increased cellular proliferation.

Schule *et al.* showed that RAR but not RXR receptors bind and sequester AP-1 function, disallowing binding to AP-1 sites in AP-1 target genes (*169*). The binding of AP-1 to certain TSGs, including the putative TSG RARβ2, causes their suppression. [For a review, see reference (*170*)].

3.11 Conclusion

The main theme in retinoid signaling, whether pertaining to retinoids or retinoid receptors, is diversity and complexity. It is important to keep this in mind as the role of RARβ2 in lung cancer is considered, even though it is the main receptor isoform thought to be implicated in lung cancer.

4 RARβ2

4.1 3p alterations in lung cancer

One of the earliest preneoplastic lesions in lung cancer is the loss of the short arm of chromosome 3. Nearly 100% of SCLCs and 90% of NSCLCs show large deletion of the short arm (p) of chromosome 3 (*171,172*). This region potentially harbors one or more TSGs involved in lung carcinogenesis and has been the active interest of many researchers since the early 1980s. The putative TSGs DUTT1/ROBO1 (3p12), FHIT (3p14), RASSF1A (3p21), SEMA 3B (3p21), RARβ2 (3p24), VHL (3p25) and FUS1 (3p31), are among the genes exhibiting tumor suppressor activity located in this genomic region (*173*). SCLCs and squamous cell lung carcinomas exhibit many genetic alterations including the loss of function of the putative TSG RARβ. 100% of SCLCs have deletions spanning 3p13/14-ter, and 85-90% of squamous cell lung carcinomas have deletions spanning 3p24-ter (*174*).

Goeze *et al.* used comparative genomic hybridization (CGH) to assess the relative frequencies of losses and gains acquired by primary and metastatic lung adenocarcinomas as compared to their corresponding normal tissues [(*175*); http://amba.charite.de/cgh]. Analyses showed that chromosome 3p under-representation (Table XIII) is correlated with increased invasive character of lung cancer (*175*). The gene coding for the retinoic acid receptor beta 2 isoform (RARβ2), the focus of this project, is located on chromosome 3p24, and this region is frequently underrepresented in changes prevalent in dissemination and progression. Peterson *et al.* strengthens three major assumptions: (1) chromosomal instability is a frequent event that affects multiple loci in lung adenocarcinoma; (2) the chromosomal alterations observed are clonally related; and (3) the profile of chromosomal alterations is correlated with the metastatic phenotype of the tumor. Of general interest is the fact that CGH analysis of lung adenocarcinoma could potentially distinguish non-metastatic from metastatic tumors. Of specific interest to this project is the fact that the frequent loss of the 3p22-24 region is a change acquired by tumors with a high potential for dissemination and progression. Large deletions in this genomic region were first described by karyotypic analyses by Whang-Peng in 1982 (*171,172*). Indeed, 3p became popular because it was thought to contain candidate TSGs, and RARβ2 was

both found to have TSG activity (*174*) and to play a role in tumor immunomodulation in our laboratory (*176*).

TABLE XIII. Chromosomal imbalances found in pulmonary adenocarcinoma via CGH. Modified from Table 2 of reference (*175*). Note that the region where RARβ2 is localized, 3p24, is frequently underrepresented in changes prevalent in dissemination and progression. *DNA gain with ratio >1.5; DNA loss with ratio <0.5. CGH = Comparative genomic hybridization.

Type of change	DNA Under-representations	DNA Over-representations
High incidence changes (≥ 50%)	3p21-p24, 3p12-p14, 4p13-p14, 4q13-q34, 5q14-q23, 6q 14-q24 8p12-p21, 9p13-p24, 9q12-q22,13q14-q32, 15q21, 18q21-q22	1q21-q43, 5p13-p15, 8q22-qter, 11q13, 17q12-q22, 17q24-25, 19q13.1, 20q11.2-q13.2
Pronounced changes* (≥5)	13q21-q22, 18q22, 8p23	1q21-q25, 1q31-q42, 5p13-pter, 8q22-qter, 14q13-q21, 17q24-q25, 20ql3.2-q13.3
Changes relevant for dissemination/progression	3p12-p14, 3p22-p25, 4p13-p15.1, 4q21-qter, 6q21-qter, 8p,10q, 14q21, 17p12-p13, 20p12, 21q	1q21-q25, 7q11.2, 9q34, 11q12-q13, 14q11-q13, l7q25
Changes prevalent in non-metastatic tumors	19	3p, 4q, 5p, 6q

The recent consensus that RARβ2 may be a true tumor suppressor gene (TSG) lies in the finding that a major mechanism through which it is inactivated in cancer is through DNA methylation, also referred to as methylation-associated gene inactivation (MAGI).

RARβ expression is restricted mainly to the tracheobronchial, intestinal and reproductive epithelia (*151*), where its function is generally associated with differentiation and homeostasis. The RARβ gene encodes three human isoforms (β1, β2 and β4) but only β2 is significantly expressed in these tissues in the adult (*155*). Thus, the RARβ2 isoform is thought to have the most significant implications in lung cancer (*174,177,178*). RARβ2 was independently discovered by two separate groups in 1987 (*179,180*) and was the subject of intense research in cancer in the early 1990s. RARβ2 was thought to

be a TSG because loss of heterozygosity (LOH) was frequently found to affect the chromosomal region in which it is located (3p24) in SCLC and NSCLC, and because expression of exogenous RARβ2 constructs in lung cancer cells having lost endogenous RARβ2 expression resulted in their diminished growth and tumorigenicity. However, RARβ was referred to as a "putative" TSG, reflecting in part the controversial findings surrounding its role in cancer and the fact that no other mutation or deletion was found to affect the remaining allele. Studies on RARβ2 in cancer were significantly decelerated in the late 1990s when this project began, due to the controversial findings mentioned above and the discovery of other genes with TSG activity located near or in the same region, including the fragile histidine triad gene (FHIT), located at 3p14. At the present time, RARβ is considered a true TSG, and this change in vision was due to the findings that it is frequently inactivated via MAGI in cancer.

The following section briefly summarizes the findings that pertain to its tumor immunomodulating and tumor suppressive activities. This project's findings regarding RARβ2 and immunomodulation may be found in Appendices B, C and E, and findings regarding RARβ2 and its characteristics and role as a TSG may be found in Chapters 2 and 3, respectively.

4.2 Retinoid-dependent immunomodulation in cancer and RARβ2

The widespread squamous hyperplasia and metaplasia that are characteristic of vitamin A deficiency (VAD) are accompanied by many immune-related conditions, such as loss of certain immune cell types in the affected epithelia, including pulmonary goblet and mucus cells (*49,181*), and infections of the ear, sinus, respiratory, gastro-intestinal and genital tracts, and concurrent atrophy of the thymus, spleen and lymphoid tissues (*181*). It is plausible that goblet and mucus cells play a role in the control of RA-regulated proliferation and differentiation in the epithelial tissue. The author has not found studies designed to ascertain their role in 3-D culture or organ culture systems where multiple purified lung cell types are grown simultaneously. Monolayer studies consisting of a single cell type may not adequately reflect the situation *in vivo,* and organ culture systems do not yet ascertain the effects on single cell types post-experimentation.

With regard to early experimental evidence, vitamin A and its derivatives were shown to suppress tumorigenicity in four ways: (1) by reducing the formation of tumors in rabbits following inoculation with Papilloma virus (*182*); (2) by decreasing the formation of tumors in rodents following treatment with chemical carcinogens (*183-186*); (3) by increasing the rejection of skin transplants in rabbits (*182*); and (4) by inhibiting the growth and development of transplantable tumors in mice (*187-189*). However, these latter findings were controversial since the majority of studies at the time showed that retinoids did not inhibit transplanted tumor growth (*183,190-194*). Nevertheless, key studies by Dennert, Lotan and colleagues in the '60s and '70s showed that low doses (5-300 μg/day) of RA specifically stimulated the induction of T killer cells up to tenfold while high doses (1,000 μg/day) were inhibitory (*195,196*). These studies helped identify which aspects of the immune system were affected through RA-signaling, namely immature CD8+ T-cells. In addition, since CD8+ T-cells pre-treated with RA were *not* more effective than cells *not pre-treated* with RA in causing the cytolysis of target cells, the hypothesis that RA plays a role in the immunomodulation of T-cells was negated, leading Dennert *et al.* to correctly postulate that RA could "enhance the immunogenicity of antigens (expressed by the target cells) recognized by the T killer cell precursor" (*195*) as early as 1987. Studies from our laboratory by Toulouse *et al.* over 10 years later verified this postulate and identified the cell surface proteins mediating these cytotoxic effects (*176*) [i.e. major histocompatibility complex class I (MHC class I) and intercellular adhesion molecule-1 (ICAM-1), at least in CALU-1 lung cancer cells].

Toulouse *et al.* showed that the MHC class I constant C region and ICAM-1, which are involved in the generation of an effective CD8+ cytotoxic response, were upregulated in lung cancer cells transfected with an RARβ2 construct and further enhanced upon treatment with RA, and were specifically involved in the stimulation of the heterologous CTL response in both the induction and the effector phases by up to threefold (*176*).

The activation of CD8+ T-lymphocytes requires two events (Fig. 10): (1) antigen stimulation, which occurs via MHC class I antigen presentation to the T-cell receptor; and (2) co-stimulation, which occurs through the binding of accessory molecules, such as ICAM-1, B7, or B7-1, to co-stimulatory molecules, such as LFA-1, CD28, or CTLA-4, respectively. Costimulation may occur in three situations, depending on which cell the co-stimulatory molecules are present: (1) *cis*-costimulation occurs at the cancer cell

surface; (2) *trans*-co-stimulation occurs via antigen presenting cells (APCs); (3) transfer co-stimulation occurs through APC-ingestion and presentation of soluble antigen. [For a review see reference (*197*)].

It is important to note that the elimination of newly formed pre-cancerous or cancerous cells is ultimately dependent on CD8+ T-cell removal, since no other immune cell effectively recognizes, targets and cytolyses cancer cells in a *specific* manner. Natural killer (NK) cells recognize cancer cells through non-specific mechanisms, including down-regulated MHC class I expression, and are not clonally selected and amplified to provide a large titer of antigen-specific and MHC-restricted cells, whereas CD8+ precursor T-cells do. Also, MHC class I down-regulation is a nearly ubiquitous phenomenon in cancer, including lung cancer (*198,199*), thereby allowing cancer cells to evade the CD8+ T-cell immune response, provided that the NK response is unsuccessful.

It is also important to note that the MHC class I moiety which was shown to be up-regulated was the constant C region, which is integral to all MHC class I species present on the cancer cell surface (approximately 10^5 molecules/cell), no matter which variable region is expressed. Thus, though multiple neo-antigens, ranging in the hundreds and thousands, are bound and presented by multiple variable regions ($\alpha 1$ and $\alpha 2$ regions, encoded by genes subject to recombination), the constant region is ubiquitous in all of these recombinant forms and upregulation of MHC class I may effectively increase the recognition and targeting of many different neo-antigens.

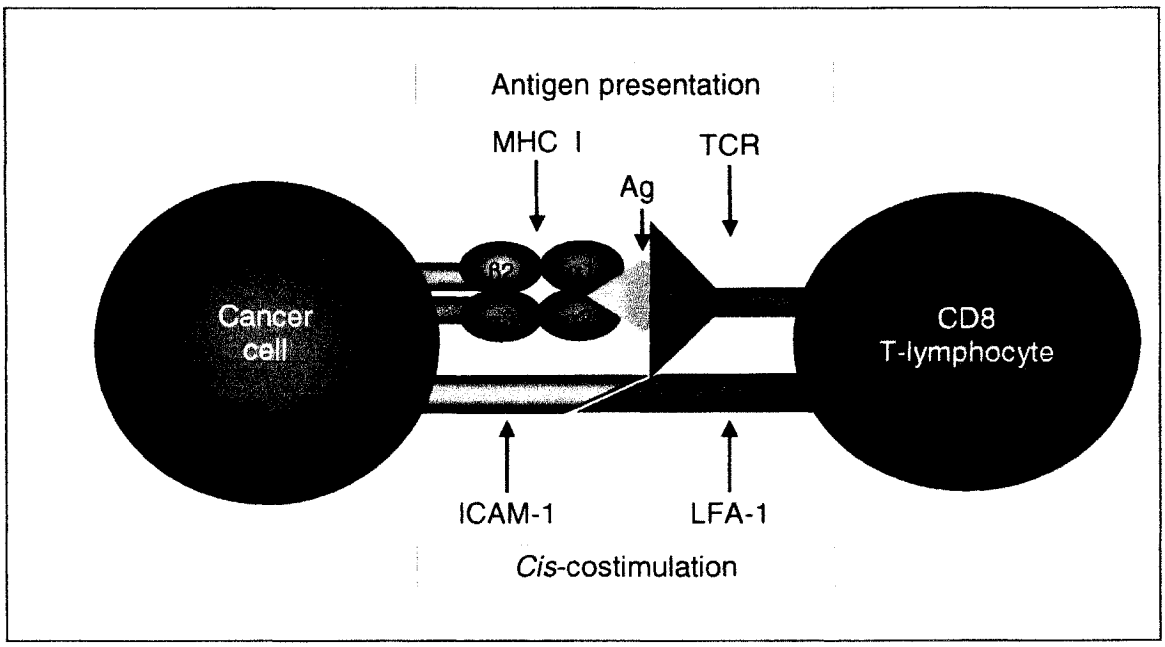

FIGURE 10: Schematic diagram representing the two requirements for CD8+ T-lymphocyte activation: antigen presentation and costimulation. Activation requires both antigen presentation via MHC class I antigen presentation to the T-cell receptor (which is part of the CD3/TCR/CD8 complex) and costimulation, which occurs through the binding of accessory molecules such as ICAM-1 to LFA-1. Absence of co-stimulatory molecules leads to T-cell anergy. Based on (*197*), Fig. 1, p. 194. Ag = Antigen; ICAM-1 = intercellular adhesion molecule-1; LFA-1 = leukocyte function activation-1; MHC I = Major histocompatibility class I.

In contrast, ICAM-1 does not play a uniquely beneficial role; it has been shown to be correlated with poor prognosis in several cancers, including lung (*114,200*), stomach (*201*), colon (*202*), bladder (*203*), liver (*204*), and skin (*205*). The exact mechanism of action remains undetermined, but is likely related to its properties related to cell-cell adhesion.

Other cellular and molecular components of the immune system have been shown to be affected through retinoids, carotenoids, or their analogs. These include CD4+ helper T-cells (*206*), NK cells (*207*), B cells (*208,209*), the interleukin-2 (IL-2) pathway (*210-212*), tumor necrosis factor (TNF) (*213-215*) and interferon gamma (IFNγ) (*216*), for example. However, none of these implicate the cells that are directly involved in the recognition, targeting and cytotoxic response against cancer cells, i.e. CD8+ T cells.

4.3 RARβ2 as tumor suppressor gene

RARβ2 has been studied for its tumor suppressive activities since 1987, when it was initially cloned (*179,180*). Its chromosomal locus, 3p24, was known to be genetically altered at one allelic copy by loss of heterozygosity (LOH) in the great majority of lung cancers (90% and 100% of all NSCLCs and SCLCs, respectively) since 1982 (*172*). But in accordance with the classical "two-hit" hypothesis (*217*), TSGs were required to harbour two genetically-based inactivations, LOH generally affecting one allele and intragenic mutation or deletion affecting the other. Two hits were required because TSGs manifest a recessive behavior, as opposed to oncogenes, which manifest a dominant one, and the types of aberrations described had been observed in many other genes having achieved TSG status.

In 1993, the Bradley laboratory showed that RARβ2 was one of the nuclear receptors responsible for mediating the tumor suppressive effects of retinoic acid (RA) in epithelial cells (*218*). RARβ2 was also shown to be inactivated and to have tumor suppressive properties in numerous tumor-derived cell lines, including those derived from lung, breast and colon cancers, in our laboratory and others' (*101,174,219-224*). In addition, modulation of RARβ2 expression in transgenic mice by dominant-negative or antisense constructs resulted in the appearance of tumors in which endogenous RARβ2 expression is significantly reduced (*225*).

Despite this increasing evidence that RARβ2 had TSG activity, the lack of intragenic mutations or deletions affecting the remaining allele (and thereby resulting in the lack of a "2nd hit") remained a source of doubt.

However, as seen in the previous section, the definition of a TSG is evolving; MAGI is now accepted as one of the two hits required by genes to acquire TSG status (*226,227*). Indeed, it appears that a few other genes lacking intragenic mutations or deletions in the remaining allele are inactivated through MAGI.

4.4 Methylation of the RARβ2 gene

Several groups have shown that methylation of the RARβ2 gene is correlated with its inactivation (*228-235*). Simultaneous treatment with 5-azadeoxycytidine, a methyltransferase inhibitor, and RA caused demethylation of the exonic sequences and reactivation of gene expression (*228,230,233,235,236*). It is of importance to note that all report the existence of cell lines where inactivation is not correlated with methylation, and many show evidence of existence of methylated and non-methylated alleles but do not directly document this phenomenon as it is not central to the context of their study.

These studies have mainly used MSP and have focused on a 146 bp region within the 5' untranslated region (5'UTR) and the first exon coding region A of the RARβ2 protein. As mentioned in section 5.11.2, MSP does not assess the methylation status of individual alleles nor does it provide information about the individual CpG sites in the PCR-amplified region, except for the sites contained in the sequences complementary to the oligos. Moreover, this 146 bp region is downstream of the transcription start site and is not contained within the promoter *per se*. As has been discussed by Jones (*237*), downstream methylation may not necessarily be inhibitory and may even be correlated with transcriptional activity. Moreover, two studies using transfection of *in vitro*-methylated sequences showed that methylated CpG sites need to be proximally located to the promoter in order to be correlated with repression (*238,239*).

4.4.1 Methylation proximal to the RARE

Using bisulfite genomic sequencing (BGS), one report revealed that methylation of 2 of 3 tandem CpG sites in the proximal promoter correlated with gene inactivation in the MCF-7 breast cancer cell line (*240*), and another report described that these 3 sites were the most frequently methylated among 38 sites and may play an important role in the inactivation of RARβ2 in prostate cancer cells (*241*). Figure 11 (top) is a representation of these 3 CpG sites, which are located in and proximal to the RARβ2-RARE within the RARβ2 P2 promoter.

It is not yet clear whether particular patterns of methylation are essential for inactivation, but with regard to MeCP1 and MeCP2, methylation over very short stretches of DNA

may be particularly important in gene inactivation (*240,242*). The location of certain CpG sites relative to key transcription factor binding sites within the gene in question may be of importance as well. For instance, the TATA box is the binding site for TFIID, which consists of the TATA binding protein (TBP) and the TBP-associated factors (TAFs), and promoter sequence and/or architecture in this region has been shown to contribute to the activation of the RARβ2 promoter (*243,244*).

4.4.2 Methylation of RARα2 in breast cancer cells

The expression levels of RARα1 and α2 are reduced in breast cancer cells, and recent evidence from several laboratories shows that these isoforms mediate some of the growth inhibitory signals of RA in breast cancer cells (*245-249*). Farias *et al.* demonstrated that the RARα2 promoter, which is located on a CpG island and contains a RARE that is one nucleotide different than the RARβ2 RARE, is hypermethylated in MCF-7 breast cancer cells, and that treatment with 5-azacytidine followed by RA and trichostatin A (TSA), an HDAC inhibitor, was correlated with the partial demethylation of the promoter and reactivation of RARα2 (*250*). Interestingly, the 3 CpG sites distributed in and around the RARE, and in similar positions as those in/around the RARβ2 RARE, were nearly 100% methylated in all (n=10) bisulfite genomic sequencings (Fig. 11).

Most importantly, Farias *et al.* demonstrated that RARα2 plays an important role in RA-signalled growth suppression, similar if not equal to that of RARβ2, in MCF-7 breast cancer cells, using clonogenic assays, where "matched (isoform) expression levels" were compared in their growth suppression following RA treatment. However, they used retroviral transduction of a bicistronic expression vector containing the RAR isoform of choice and the enhanced green fluorescence protein (EGFP) separated by an internal ribosomal entry site (IRES), and assayed EGFP intensity via fluorescence microscopy to indirectly estimate the level of expression of the RAR isoform. This may not have been the most accurate method, since translation of the first cistron may vary from one experiment to the other (personal experience). Western blot analysis or fluorimetry using antibodies against RARβ2 may have been more precise.

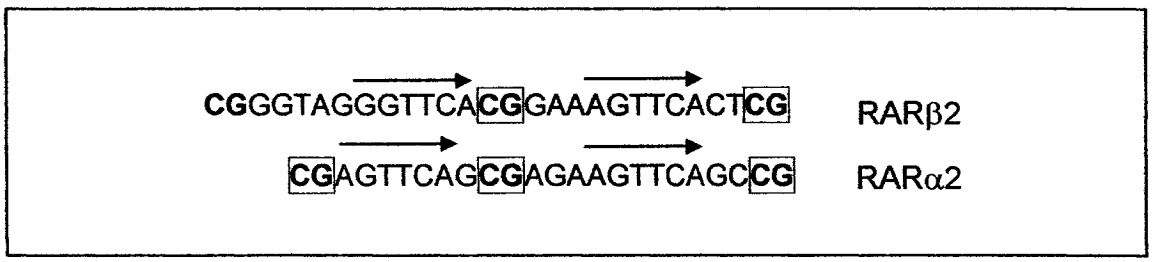

FIGURE 11. Comparison of the DNA sequences and the CpG sites frequently methylated in and around the RAREs of RARβ2 and RARα2 in cancer. **Top**: DNA sequence in and around the RARE located in the RARβ2 P2 promoter. Based on references (*251-253*). **Bottom**: DNA sequences in and around the RARE located in the RARα2 gene promoters, respectively. Based on reference (*250*). Arrows = direct repeats forming the RAREs; Boldface = CpG sites; Boxes = CpG sites found to be frequently methylated in cancer cells. RARE = retinoic acid response element.

4.4.3 Methylation of CRBP1 in cancer cells

CRBP1 (section 2.7.4), which is responsible for the intracellular transport of retinol and is inactivated in several cancer cell lines and tumors (including lung, breast and colorectal), is also inactivated through hypermethylation, and inactivation was found to be correlated with RARβ2 hypermethylation and low dietary retinol intake (*254*). The methylation statuses of the other retinol/RA binding proteins, RBP, CRBP2, CRABP1 and CRABP2, have not yet been reported. In addition, methylation was observed both in human breast cancer cells and in MMTV-Myc induced mouse mammary cells, suggesting that MAGI of CRBP1 is oncogene-induced and may be evolutionarily conserved (*254*).

4.4.4 RARβ2 is one of the genes most frequently methylated in cancer

More recent research investigating the incidence of methylation of various genes in cancer has shown that RARβ2 is the gene (or one of the genes) most frequently methylated in several cancers (Table XIV). All of these studies mainly used MSP as well. The other genes most frequently analyzed included some or all of the following: RASSF1A, CDH1, cyclin D2, p15/Ink4a, APC and DAPK, among others. Consequently, the methylation status of TSGs, including RARβ2, is currently being considered as a potential marker for cancer diagnosis and grading.

TABLE XIV. List of anatomical sites in which cancers are found to frequently have methylated copies of the RARβ2 gene using MSP and corresponding frequency of methylation. MSP = Methylation-specific PCR; NSCLC=non-small cell lung carcinoma.

Site	Frequency of RARβ2 methylation (%)	Ref.
Urinary bladder	88	(*255*)
Nasopharynx	80	(*256*)
Esophagus	73	(*257*)
Stomach	56	(*258*)
Prostate	53	(*259*)
Mammary ducts	46	(*260*)
Mammary lobes	41	
NSCLC	40	(*261*)
Uterine cervix	33	(*262*)
Leukemia	21	(*263*)

4.4.5 Lack of promoter-specific and allele-specific analyses

The majority of studies have used MSP and have focused on the 5'UTR and the first exon. Consequently, promoter-specific analyses, and more importantly, detailed analyses of separate RARβ2 alleles, have not yet been reported.

4.5 MSP in early detection, diagnosis and grading of epithelial cancers and RARβ2

MSP has recently been tested in cancer diagnosis and treatment because it can detect methylated genes in exfoliated cancer cells, such as those present in the sputum (*264*) or bronchoalveolar lavage (*265*) of lung cancer patients, the bronchial brushings of former cigarette smokers (*19*), the mammary ductal lavage of breast cancer patients (*266*), or the plasma, serum, ejaculate and urine of prostate cancer patients (*267*). In fact, occult tumor cells were detected via MSP assayed to detect RARβ2 hypermethylation among peripheral blood mononuclear cells (PBMCs) in SCLC patients

(*268*), but the relation between PBMC methylation and tumor cell methylation has not yet been established.

There is increasing evidence that smoking increases the extent of gene-specific methylation both in preneoplastic and neoplastic lesions. For example, Eguchi *et al.* first demonstrated that the extent of methylation of a TSG, in this case the HIC-1 gene located at the D17S5 locus, was correlated with smoking in patients with NSCLC in 1997 ($p=0.01$) (*269*). Strikingly, the extent of methylation was also found to be correlated with tumor size, lymph node metastasis, and differentiation grade, which are prognostic parameters. Kim *et al.* found that methylation of the p16 gene was correlated with the number of pack-years smoked ($p=0.007$) and the duration of the smoking habit ($p=0.0009$) (*17*), and methylation was again found to be correlated with prognosis in stage I NSCLC. Belinsky *et al.* demonstrated that gene-specific methylation, such as p16 in sputum-derived cell samples for example, could be used to detect and even assess the grade of NSCLC, and they also showed that methylation was correlated with smoking history (*18*). Recent studies, in which MSP has allowed the analysis of multiple genes, confirm these findings. For example, Zochbauer-Müller showed that methylation of at least one of four frequently methylated genes in cancer (RARβ2, CDH13, p16/Ink4a and RASSF1A) is correlated with smoking (in sputum samples) and that RARβ2 was the most frequently methylated gene (27% versus 10%, 5% and 4%, respectively) (*261*).

The results from Zochbauer-Müller *et al.* help shed light on the association between RARβ2 inactivation and epidermoid cancer first described in our laboratory (*174*), since methylation was found to occur more frequently in the central airways as opposed to the peripheral airways. Epidermoid (squamous) cancers usually occur in the central airways, whereas adenocarcinoma and large cell carcinomas are normally peripherally located (Table VII, p. 19). Similar correlations have been found between methylation and aging, both in rodent (*270-272*) and in human cells (*273-280*).

Based on the findings that RARβ2 has beneficial effects both in tumor immunomodulation and suppression, and MAGI appears to be one mechanism through which RARβ2 is inactivated in cancer, there is strong evidence supporting the postulate that RARβ2 has TSG activity.

However, certain questions remain unanswered. *Are there functional reasons why one allele is retained (i.e. not genetically altered) in cancer cells? Is methylation, through its reversibility, an epigenetic aberration reserved for genes whose expression is required during carcinogenesis?*

5 DNA methylation in cancer

In the normal cell, DNA methylation is thought to be directly involved in at least seven processes: (1) differential gene expression during development; (2) protection against invading DNA sequences, such as viral genomes and retrotransposons; (3) masking of non-regulated regions; (4) replication; (5) repair; (6) imprinting; and (7) chromosome X inactivation. The common effect of DNA methylation is thought to be gene-inactivation, but the precise repressive molecular mechanisms remain unclear. However, in the cancer cell, these mechanisms are thought to acquire aberrations, which lead to dysregulated gene expression.

In general, cancer cells share two major characteristics vis-à-vis DNA methylation: (1) under-methylation of bulk DNA (global hypomethylation) (*281,282*); and (2) over-methylation of CpG islands (promoter and promoter-proximal hypermethylation) (*283-285*). In particular, DNA methylation has been shown to be responsible for the inactivation of multiple TSGs. In fact, methylation appears to be the predominant mechanism through which TSGs lacking mutations or deletions in their exonic sequences in cancer cells are inactivated.

Toyota *et al.* postulated the existence of a specific carcinogenesis-related methylation aberration that would cause methylation instability, akin to genetic instability and the mutator phenotype proposed by Loeb (*286*), and result in the quasi-simultaneous MAGI of multiple TSGs (*276*). He coined this aberration the "CpG island methylator phenotype" (CIMP). CIMP is used to describe aberrations not related to the incremental and age-related hypermethylation observed in certain cancers, such as colon cancer [for a review, see reference (*287*)].

5.1 Biochemistry of DNA Methylation

DNA methylation is a post-replicational modification process through which certain bases within specific DNA sequences are reversibly modified by the covalent addition of a methyl group (-CH3; Fig. 12). For example, GATC-adenines, CpN- and CpG-cytosines are DNA bases frequently methylated in bacteria, plants and mice/humans,

respectively. Normally, approximately 3 x 10^7 cytosines (Cs) are methylated per human genome (*288*), which consists of 6 x 10^9 base pairs. Thus 1/200 bases is methylated.

In higher eukaryotes including mouse and man, methylation most frequently involves CpG-cytosines (>90%) (*289*). The methyl group is covalently linked to the carbon in the 5 position of the cytidine ring (Fig. 12), creating 5m-cytosine (5m-C). However, other sequences and other bases are methylated as well, albeit infrequently, such as CpNpG-cytosines, for example (*290*).

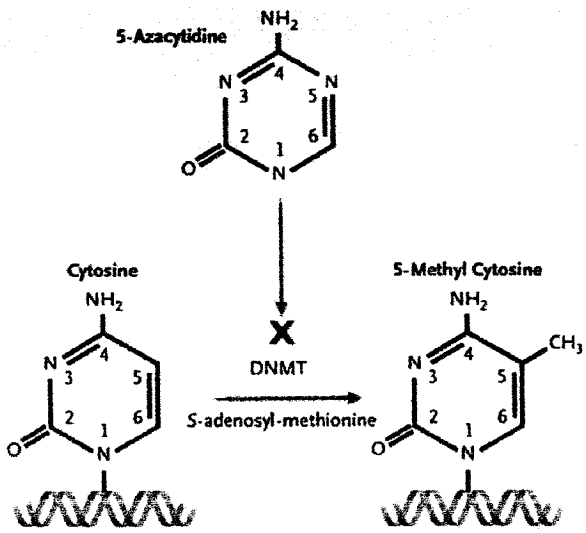

FIGURE 12. Chemical structure representation of the methylation of a CpG-cytosine. Note that a methyl group is covalently linked to the carbon atom in the 5 position (5m-C) of the cytidine ring. Methylation inhibitors, such as 5-azacytidine (shown here) and 5-aza-2'deoxycytidine (not shown) are cytosine analogs that cannot be methylated due to their nitrogen atom in the 5 position. Taken from reference (*291*).

5.2 CpG islands

CpG-cytosines are rare in the normal human genome. For instance, the frequencies of GpC and CpG dinucleotides, which would be expected to occur with about equal frequencies if they were randomly occurring, are in fact approximately 0.043 and 0.010, respectively (*292*). However, certain areas of the genome are particularly CpG-rich and consequently known as CpG islands. While the remaining "bulk" DNA is frequently methylated at CpG sites (70-90%, mouse studies), CpG islands are not normally methylated in healthy normal cells (*293*). The majority of human genes whose

promoters are found to be methylated in cancer cells are located on CpG islands. In fact, the number of CpG islands in the human genome was estimated to be approximately 45,000 (*294*), correlating closely with the estimated number of genes, initially approximated at 50,000 with the completion of the human genome project (*295*), but now closer to 25,000-30,000 (*296*). In addition, endogenous parasitic sequences known as transposons, also have methylated promoters and comprise 35% of the genome, thereby containing the majority of methyl-CpGs (*297*). Repetitive centromeric and peri-centromeric sequences and exogenous parasitic sequences, including dormant viral DNA sequences, are also methylated and inactive.

5.3 *De novo* and maintenance methylation activities

DNA methylation is essential in the differential control of gene expression, both during ontogeny and in later stages of development. Patterns are established for the first time in the genome during gametogenesis (the production of female and male gametes, ova and spermatozoa, respectively), via *de novo* methylation. However, they are re-established in the post-implantation embryo, after a wave of demethylation that takes place in the post-cleavage/pre-implantation embryo (*298*), necessarily via *de novo* methylation. This process of widespread demethylation is thought to be essential in defining which gene copy among meiotic paired alleles of sister chromatids (derived from the maternal and paternal contributions) will be expressed. *De novo* methylation thus refers to the establishment of methylation patterns on non-methylated DNA. The processes and molecular signals responsible for these new patterns are unknown.

Maintenance methylation refers to the methylation of the nascent strand during DNA replication. Since DNA replication is semi-conservative, the DNA in the replication fork is hemi-methylated. The molecular signals responsible for the heritability of methylation patterns during mitosis are also unknown.

5.4 DNA methyltransferases

DNA methylation is catalyzed by four known DNA methyltransferases, DNMT-1, 2, 3A and 3B, and requires the methyl-group donor S-adenosylmethionine (SAM; Fig. 12).

DNMT-1 encodes the major form of methyltransferase activity in human cells and is thought to be responsible for both *de novo* methylation [since its highest expression is found in the testis and ovary (*298*)] and maintenance methylation [since DNMT-1 was likely responsible for the vast extent of the reduction (95%) in genome methylation in somatic cells in DNMT-3B/DNMT-1-disrupted colorectal carcinoma cells (*299*)]. DNMT-1 also encodes a minor splice variant, DNMT-1B, which only accounts for approximately 2-5% of DNMT-1 mRNA (*300*).

With regard to cancer, DNMT-1 was shown to be over-expressed in colon cancer cells (several hundredfold), pre-malignant polyps (60-fold), and normal colon tissue from colon cancer patients (15-fold), as compared to healthy individuals (*301*). DNMT-1 overexpression may thus contribute to the regional hypermethylation affecting TSG promoters observed in cancer. Also, DNMT-1 was found to form a transcription repressive complex consisting of DNMT-1, histone deacetylase 2 (HDAC2) and DNMT-1-associated protein DMAP-1 (*302*). It was also shown to form another repressive complex with *Rb*, *E2F1* and *HDAC1*, capable of repressing E2F1-responsive promoters (*303*). Therefore, there are several lines of evidence that DNMT-1 may be involved in carcinogenesis.

Interestingly, the major polyphenol in green tea, (-)-epigallocatechin-3-gallate (EGCG), was recently shown to inhibit DNMT-1 activity and reactivate methylation-silenced genes including RARβ2 in cancer cells (*304*). This may partially explain the protective effect that green tea has in several forms of cancer.

The minor forms of DNA methyltransferase activity are DNMT-2, 3A and 3B. Very little is known about DNMT-2, and the roles of these methyltransferases in cancer are unclear. DNMT-3A and 3B are thought to have predominantly *de novo* methylation activity. Dnmt3a-/- knockout mice appear normal, but Dnmt3b-/- usually die at 4 weeks of age (*305*). Sporadic or germ-line mutations in Dnmts other than Dnmt 3a have not been reported, but germ-line mutations of Dnmt3b have been linked with the extremely rare and recessive autosomal disorder known as "Immunodeficiency, Centromere instability and Facial abnormalities" (ICF), in which chromosomes are ubiquitously hypomethylated and certain other chromosomes (1, 9 and 16) are abnormally long and fragile (*306*). This may reflect how DNMT-3B aberrations in cancer contribute to both

global hypomethylation and chromosomal instability, however, more research is required.

5.5 Replication and methylation synchronicity

During DNA replication, it is generally thought that the newly synthesized strand is not immediately methylated upon polymerization, as evidenced from the asynchronous replication and methylation observed in *E. coli oriC* (*307*), and that observed in human bladder cancer cells (*308*). (In *E. coli*, GATC sequences remain unmethylated thus allowing mismatch repair enzymes in the methyl-directed pathway to distinguish between the conserved template that is left intact and the newly synthesized and error-prone strand that is corrected). In opposition to these findings, Chuang *et al.* found that DNMT-1 is associated with proliferating cell nuclear antigen (PCNA) located in the replication fork, a structure that forms only at the precise moment of DNA synthesis (*309*). Araujo *et al.* concurs with Chuang *et al.*, since replication and methylation were found to be concurrent in human HeLa and monkey CV-1 cells using the nascent strand extrusion method (*310*). This issue remains controversial.

5.6 Folate metabolism

Folate metabolism, crucial for the syntheses of purines and pyrimidines - the building blocks of DNA - is also the primary source of S-adenosylmethionine (SAM), the methyl group-donor involved in DNA methylation (Fig. 12). Folate metabolism is therefore implicated both in DNA replication and methylation (Fig. 13). Consequently, the genes encoding the enzymes involved in folate metabolism, notably methylene tetrahydrofolate reductase (MTHFR), methionine synthase (MS) and cystathionine β-synthase, may play a role in cancer-related aberrant methylation mechanisms, and may potentially be involved in the root cause of CIMP. However, folate metabolism has only recently acquired scrutiny in methylation research.

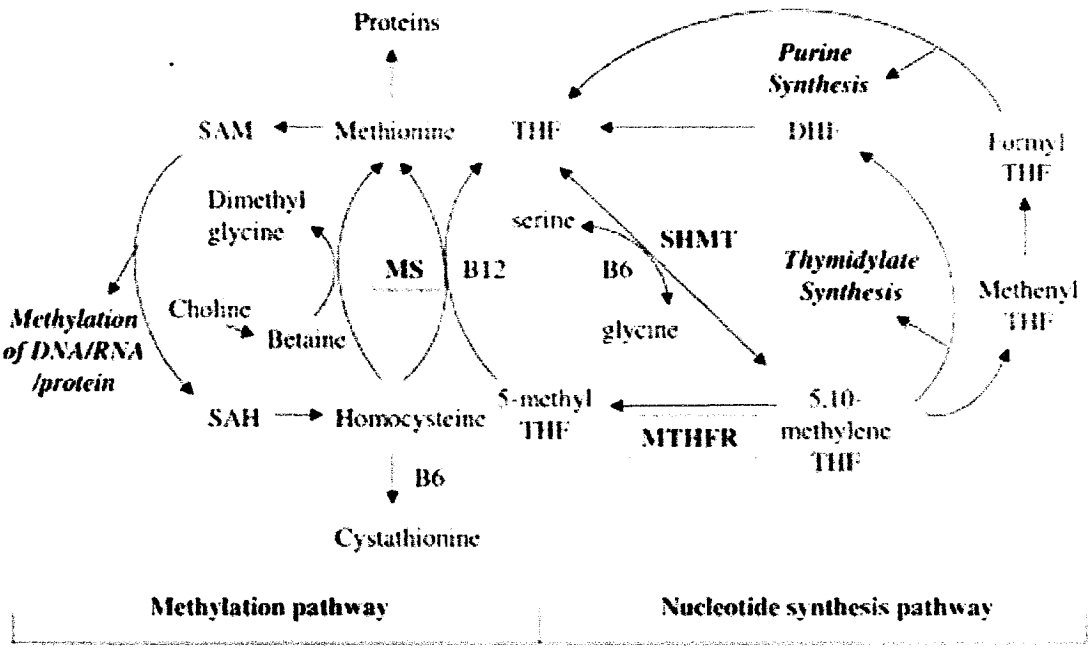

FIGURE 13. Metabolic pathways involved in SAM homeostasis and thus in DNA synthesis and methylation. Taken from reference (*287*).

Studies to date have shown that certain polymorphic variants of genes involved in folate metabolism are correlated with increased incidence of cancer. For example, the low activity haplotype of MTHFR was shown to be correlated with CDKN2 and hMLH1 promoter hypermethylation, and CIMP, in Japanese patients with colorectal cancer (*311*). The C677T MTHFR variant was shown to be correlated with promoter hypermethylation and inversely correlated with the accumulation of certain folate intermediates, including 5,10-methylenetetrahydrofolate and tetrahydrofolate (*312*). This same variant was correlated with constitutively low levels of 5m-C in the genome, but tumors did not reach severe levels of hypomethylation in a large pool of patients (n = 233) with colon, breast or lung cancer (*313*). With regard to studies focusing on lung cancer, three studies did not find a correlation between the C677T variant and the risk for lung cancer (*314-316*), whereas one study found the inverse correlation (*317*).

5.7 DNA demethylation

Methylation is a reversible process. First, newly synthesized DNA is not methylated but requires the activity of DNA methyltransferases to acquire methylation patterns. Thus, for all intents and purposes, the genome can be said to be "demethylated" upon the completion of every mitotic cycle. Second, synthetic inhibitors of methylation, 5-azacytidine and 5-aza-2'deoxycytidine, as well as EGCG (the DNMT-1 inhibitor found in green tea, mentioned above) are known. Third, there is no biochemical reason why 5m-Cs may not undergo natural chemical demethylation in the cell. Fourth, a demethylase activity has been cloned [see below; (*318,319*)].

5.7.1 5-azacytidine and 5-aza-2'deoxycytidine

Synthetic methylation inhibitors, such as 5-azacytidine and 5-aza-2'deoxycytidine, are drugs commonly used to demethylate the genome of cells in methylation research, and 5-aza-2'deoxycytidine is used in the clinical treatment of acute myelocytic leukemia (AML) due to its potent effects on myeloid differentiation (thought to occur mainly through demethylation of target genes). 5-aza-2'deoxycytidine has two known mechanisms of action: (1) it is incorporated into DNA upon DNA synthesis but cannot be methylated due to the nitrogen atom in the 5 position of its cytidine ring; and (2) it selectively inhibits DNMT-1 (*320*). 5-azacytidine acts only through mechanism 1 (above).

Both 5-azacytidine and 5-aza-2'deoxycytidine are known to have a wide range of secondary effects. First, they cause the widespread and non-specific demethylation of the genome, including human genes, dormant retroviral sequences, and transposons, and consequently have the potential to reactivate all three. Second, they bind and inhibit other molecules causing multiple adverse effects, including potent effects on recombinagenicity (*321*).

5.7.2 DNA demethylase

An intracellular DNA demethylase activity (MBD2) was discovered in Szyf's laboratory at McGill University in 1999 (*318,319*), and was shown to be involved in tumorigenesis, and

postulated to "maintain the transformed state" since antisense oligonucleotides against MBD2 inhibit anchorage-independent growth *in vitro* and tumorigenesis *in vivo* (*322*). Also, recent evidence shows that SAM may inhibit MBD2 and is correlated with increased hypermethylation (*323*). Clearly, these findings are somewhat contradictory and evoke many other questions; more research is thus needed.

5.8 Functions of DNA methylation

There are at least four lines of evidence suggesting that DNA methylation is correlated with transcriptional repression. First, methylation of exogenous gene constructs is correlated with decreased or absent expression (*324*). Second, the use of methylation inhibitors such as 5-azacytidine and 5-aza-2'deoxycytidine *in vitro* is correlated with the reactivation of gene expression. Third, transfection of antisense oligonucleotides against MeTase cDNA (DNMT-1) in cancer cells inhibits methylation thereby abrogating transcription block (*325*). Fourth, MeTase (DNMT-1) gene knock-out causes embryonic lethality and developmental stunting in mice, likely through the dysregulation of differential gene expression (*326*). It is curious that the precise molecular mechanism causing death remains unknown. The only documented effects include several abnormalities such as developmental delay and asynchrony, and altered expression of certain imprinted genes and of *Xist* RNA.

In essence, methylation consists of an additional level of information, known as "epigenetic" information. It is not encoded by - or inherited through - the genome *per se*, but rather by the "methylome", a term proposed to define "the complete set of DNA methylation modifications of the genome of a cell" (*327*), and distinguishing, at least in part, the expression pattern of one cell's genome from another.

In lower species, such as the *Drosophila melanogaster*, much less is known about the functions of methylation in the regulation of gene transcription. Current studies are identifying DNMT homologs, such as *Dnmt2-like* in dipteran insects including the fruit fly (*328*), but further studies are required. It is important to clarify that *D. melanogaster* was initially thought to lack methylation (*329*), but in 2000 Lyko *et al.* demonstrated otherwise (*330*), thereby highlighting the fundamental importance of methylation in basic life processes. DNA methylation is catalyzed by the DNA methyltransferases (MeTases)

and homozygous MeTase null mutant mice were shown to die at mid-stage embryogenesis (*326*), further supporting this concept. In bacteria such as *E. coli*, DNA methylation of GATC-adenines mainly acts to protect the genome against the organism's neutralizing mechanisms against retro-transposons and proviruses (*331*), by distinguishing between invading sequences, which are generally not methylated and consequently sensitive to certain endogenous restriction enzymes, versus genomic sequences, which are methylated and are thus resistant to these endogenous restriction enzymes. This prokaryotic function remains one hypothetical function of DNA methylation in higher eukaryotes, including mice and humans.

MAGI is required in the differential expression of parentally-imprinted allele-specific genes, such as *H19*, *IGF2* and *INS2*, of which about 45 are known [for a review, see (*332*)]. DNA methylation is also required in chromosome X-inactivation, which is the silencing of one of the two X chromosomes in females to allow gene dosage to be equivalent between males (XY) and females (XX). [For a review, see reference (*333*)].

5.9 DNA methylation and retinoid signaling share two major features

Interestingly, DNA methylation is essential in the differential control of gene expression both during ontogeny and in later stages of development, reminiscent of retinoid signaling, and it is conserved in a wide range of species from *D. melanogaster* to *H. sapiens*, also reminiscent of retinoid signaling. Is there a link?

To date, the only link between retinoid signaling and methylation stems from the finding that the PML-RARα fusion protein recruits DNA methyltransferases DNMT-1 and 3A and silences genes, including RARβ2, via targeted methylation of their promoters in U937 cells transfected with a PML-RARα cDNA construct (Fig. 14) (*334*). Unfortunately, only one gene, namely RARβ2, was shown to be targeted and silenced via chromatin immunoprecipitation (ChIP), and this gene is known to be frequently methylated in cancer cells including APL cells [21%; (*263*)]. Strikingly, RA treatment was associated with demethylation and derepression of the RARβ2 gene. Also, neither confirmation nor follow-up studies have yet to be performed. Thus, these two alleged associations, one between genetics and epigenetics and the other between the retinoid system and methylation, remain unsubstantiated.

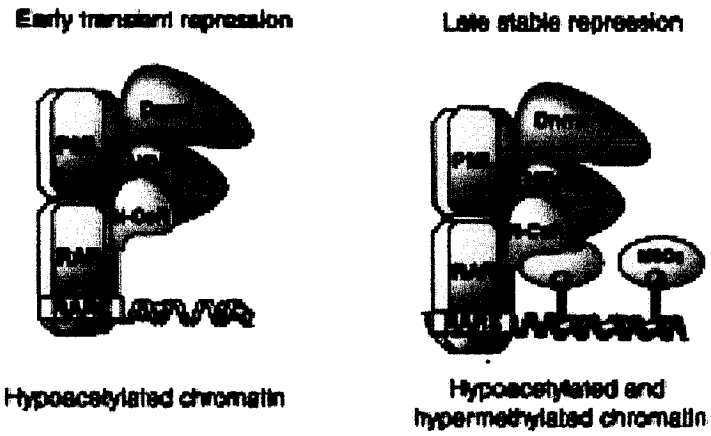

FIGURE 14. Schematic diagram representing PML-RARα recruitment of DNA methyltransferases DNMT-1 and 3A. This results in the silencing of genes through methylation-associated gene inactivation (MAGI). Taken from reference (*335*).

5.10 Potential mechanisms of methylation-associated gene inactivation

With regard to primary structure, methylation increases the risk of mutation considerably because 5m-Cs spontaneously deaminate to thymine (T), hence frequently causing missense mutations. In fact, more than 1/3 of all DNA single base pair mutations implicated in human disease consist of C→T transitions within CpG dinucleotide pairs (*336*), (or the corresponding G→A transition on the opposite strand). This is an unexpectedly high ratio when considering that CpG dinucleotides are rare and methylated CpG-Cs are rarer still, and that there is a DNA repair mechanism, the G/T mismatch-repair system, which is specific for this type of mutation. This hypermutability of 5m-Cs, which are usually found in CpG dinucleotides, has been suggested to be the evolutionary cause of the decreased frequency of CpGs in the genome. The aberrant base O(6)-methylguanine is also responsible for C→T (or G→A) transitions, and Esteller *et al.* found that the enzyme responsible for its repair, O(6)-methylguanine-DNA methyltransferase (MGMT), is frequently hypermethylated and thereby inactivated in colon cancer (*337*).

In addition, the frequency and extent of CpG island *de novo* methylation was found to be correlated with microsatellite instability (and thus DNA repair deficiency) in colorectal carcinomas (*338*), thereby affecting primary structure. Also, mutations in the

methyltransferase DNMT-3B are associated with the hypomethylation of certain chromosomes, and the aberrant structure and fragility of others (1, 9 and 16) (*306*).

In light of the increased potential for mutations in hypermethylated sequences (*336*), Vogelstein's multistage model for colorectal carcinogenesis (*131*), and the 5m-C origin of some of the genetic alterations seen in cancer, then the reversibility of methylation may lead to the presence of mutations at one allele, but the absence of mutation and methylation affecting the other. Data supporting this hypothesis has yet to be documented.

With regard to secondary and tertiary structure, the precise molecular mechanisms through which DNA methylation regulates gene expression remain unclear. In an approach using quantum mechanics, Liu *et al.* determined that the most likely primary function DNA methylation plays is the inhibition of transcription rather than the increased rate of local mutation, since 5m-C has a stronger molecular electrostatic potential (MEP) in the major groove than unmethylated C (*339*).

High levels of *de novo* methylation have been associated with alterations of chromatin structure associated with transcriptional repression (*294*). In addition, nickel, a carcinogen found in the residue phase of tobacco smoke, has been shown to induce methylation and silence genes via specific chromatin condensation (*340,341*), thereby linking the major cause of lung cancer with a mechanism known to be involved in the inactivation of numerous genes in cancer. In addition, hypermethylation of the HIC-1 locus was correlated with poor differentiation grade and smoking history (*269*).

Chromatin, the structural and functional organization of chromosomal material with small basic nuclear protein complexes such as histones, is found in two main forms. It is either (A) loosely organized into open, accessible and non-densely staining structures (euchromatin), which is associated with transcriptional activity; or (B) compactly organized into tightly bound, inaccessible and darkly staining structures (heterochromatin), which is associated with transcriptional repression.

5.10.1 Methyl-CpG density

On the one hand, the level of CpG methylation-associated repression has been shown to be dependent upon both CpG density and promoter strength (*342*), but related experiments were done using episomal systems that do not reflect most situations studied in cancer, since the genes assayed for methylation are intrachromosomal. In 1992, Boyes and Bird found that a weak promoter could overcome a low density of methylation (1 methylated CpG per 100 CpGs; 1/100) but not a high density of methylation (1/10), whereas a strong promoter could (*343*). Since then, no additional information obtained through experimental studies has been published on this topic.

On the other, Koizume *et al.* found that the formation of inactive chromatin involving the CDH1 promoter was independent of density and pattern of methylation, merely requiring a low level of methylation (*344*). Further studies are required to support any such dependence. Pogribny *et al.* assigned a threshold level of 25% to differentiate between unmethylated and methylated (and consequently active versus inactive), but this threshold appears to have been randomly set. Pao *et al.* also categorized their findings with similarly established thresholds (0-10%, 11-25%, 26-50%, and >51%), but these thresholds also appear to be randomly set or based on empiric data.

Nonetheless, it may be assumed that CpG-methylation hinders transcription directly, by altering chromatin structure (*342,345*), or indirectly, by recruiting Methyl CpG-Binding Proteins MeCP1 and 2 (*346,347*), and co-repressors such as Sin3a.

5.10.2 MeCP1 and MeCP2

MeCPs bind methylated DNA and recruit histone deacetylases (HDACs), thereby resulting in the condensation of the local chromatin structure and causing the inhibition of transcription. MeCP1 binds methylated DNA containing at least 12 symmetrically methylated CpGs; MeCP2 binds methylated DNA with substantially more methylated CpGs. MeCP2 has been shown to concentrate in pericentromeric heterochromatin (*348*). Mutations in the MeCP2 gene (Xq28) have been found to be associated with the X-linked neurodevelopmental disorder Rett syndrome, which is nearly always diagnosed

in females (XX), and has thus been proposed to be generally lethal in hemizygous males (XY) (*349*).

5.11 DNA methylation assays

5.11.1 Bisulfite genomic sequencing

Bisulfite was first used to convert 5m-C to uracil (U) in 1970 (*350*), but the bisulfite genomic sequencing (BGS) protocol used today to determine the methylation status of CpG dinucleotides in the genome was first published in 1992 and 1994 (*351,352*). The protocol is based on the differential reactivity of C versus 5m-C to the chemical reaction of sulphonation in the presence of bisulfite ions at high concentration, followed by deamination in a highly alkaline environment (Fig. 15). While Cs are converted to Us, 5m-Cs remain intact by virtue of the methyl-group at position 5 of the cytidine ring (Fig. 12).

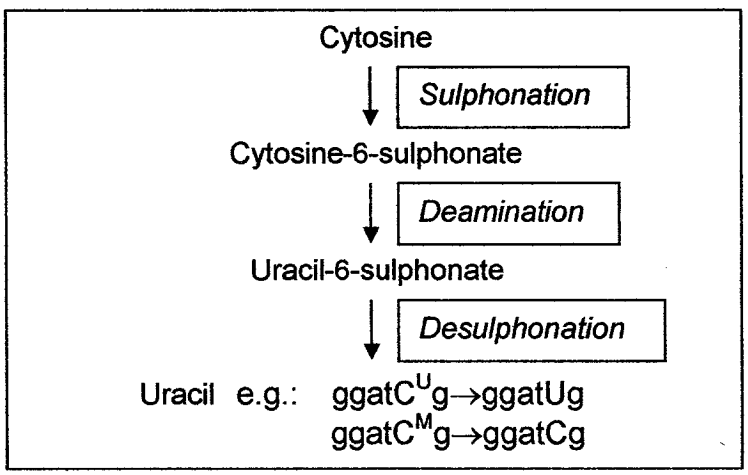

FIGURE 15. Chemical reaction central to the bisulphite genomic sequencing principle. Cytosines (C) are converted to uracil, but 5m-Cs are resistant to conversion and remain intact. The reactions require high concentration bisulfite ions, high heat and alkalinity. U = Unmethylated; M = Methylated.

The protocol has essentially remained unmodified except for several attempts to minimize template degradation, which occurs mainly via HSO_3^--driven oxidative depurination (*352*), or to increase the efficiency of the deamination reaction (*353*). These versions reflect some of the inherent difficulties of the BGS protocol. Indeed,

most studies use the technique known as methylation-specific polymerase chain reaction (MSP).

5.11.2 Methylation-specific PCR

MSP was developed by Herman *et al.* in 1996 (*354*) and is based on the BGS protocol. [For a comparison of the two techniques see reference (*355*); for a review of all current techniques see reference (*356*)]. Though MSP is a high throughput technique capable of identifying the presence of a single methylated allele among 1,000, it does not assess individual alleles, because pooled PCR products of a given population (wild-type, methylated, and unmethylated) are visualized via agarose gel electrophoresis rather than subcloned and sequenced. Therefore the methylation status of all gene copies present in a sample is not available. Additionally, it does not provide information about the individual CpG sites in the PCR-amplified region, except for the sites contained in the sequences complementary to the oligos. Thus, the presence of just a few methylated sites, which may be well below the threshold generally accepted to be correlated with gene inactivation, may wrongly be interpreted as hypermethylation.

In comparison, BGS is neither a high throughput assay nor as sensitive as MSP, but it does provide information about *single* alleles, because subclones of PCR-amplified bisulfite-treated DNA products, necessarily derived from *single* molecules, are by definition, sequenced.

5.11.3 Modern methylation assay techniques

Several recent approaches have been taken to accelerate methylation research in cancer. Methylation-sensitive single nucleotide primer extension (Ms-SNuPE) (*357*) allows the quantitative assessment of the proportion of methylated cytosines at one or more known CpG sites. Restriction landmark genome scanning (RLGS) (*358*) allows the identification of novel methylated sequences, using the restriction enzyme site *Not I* as a landmark. MethyLight (*359*) is a highly sensitive fluorescence-based real-time PCR technique that is capable of quantitating the proportion of 5m-Cs at given CpG sites using PCR primers that are either hybridizable to bisulfite-converted sequences, or sequences resistant to bisulfite conversion. It is capable of identifying a single

methylated sequence among 10,000 unmethylated alleles, representing a sensitivity which is tenfold greater than methylation-specific PCR (MSP) (*354*). Methylation-specific oligonucleotides microarray (*360*) allows the quantitative assessment of 5m-Cs at known CpG sites within multiple genes, using a microarray hybridization approach in which glass slides contain paired oligonucleotides probes capable of distinguishing between methylated and unmethylated CpG dinucleotides. Finally, amplification of inter-methylated sites (AIMS) (*361*) allows methylome profiling using methylation-sensitive restriction enzymes, such as *Sma I*, and adaptor molecules to produce a fingerprint of the epigenome.

5.12 DNA methylation of tumor suppressor genes

Recently, the relative importance of epigenetic modifications in the silencing of TSGs in cancer has become appreciated [(*227*); Table XV). The "MethDB DNA methylation database" (http://www.methdb.de) is an attempt to unify and compare methylation data obtained through these and other studies.

TABLE XV: Tumor suppressor genes found to be inactivated through DNA methylation in cancer since 1990. TSG = Tumor suppressor gene.

TSG	Reference
ER	(*362,363*)
WT	(*364*)
Rb	(*365,366*)
VHL	(*367*)
p16/CDKN2	(*368,369*)
E-cadherin	(*370,371*)
p15/Ink4a	(*372,373*)
Rb	(*374*)
BRCA1	(*375*)
hMLH1	(*376*)
p73	(*377*)
AR	(*378*)
HTR1B	(*379*)
NES1/Kallikrein10	(*380*)
RASSF1A	(*381*)
Connexin 26	(*382*)
p57Kip2	(*383*)
HIC-1	(*269*)

5.13 Conclusion

In conclusion, DNA methylation is now being recognized as a mechanism through which certain TSGs are inactivated in cancer. However, multiple factors play a role in dysregulated DNA methylation, be it at the level of methylation machinery, methyl sources (i.e. folate metabolism), or co-factors that may target the methylation machinery to the genes observed to be frequently methylated in cancer.

6 Evidence contradicting the postulate that RARβ2 acts solely as a tumor suppressor gene

Though RARβ2 has been shown to possess many types of protective, even tumor suppressive, properties and characteristics, many findings in the last decade briefly summarized here and in Table XVI have suggested that RARβ2 may not act solely as a classical TSG.

6.1 Half of all lung cancers express RARβ2

First, as much as half of the normal bronchial epithelium contains cellular *foci* in which RARβ2 is inactivated in patients at risk for - or diagnosed with - lung cancer (*384,385*). This should provide an enormous pool of cells predisposed to the carcinogenic process. However, since half of all tumors express RARβ2 then one may postulate that either the initial turn-off did not provide the advantage to the cells that would be expected from the inactivation of a TSG, or that the initial turn-off is followed by reactivation at some point along the carcinogenic process. Nonetheless, since half of all tumors express RARβ2 at the end of this process then the suppressive effects of RARβ2 may not be insurmountable.

6.2 Carotenoids/retinoids have frequently had negative effects in lung cancer chemoprevention trials

Second, it has been known for almost ten years, since the ATBC and CARET studies (summarized in section 2.9.1), that β-carotene, the metabolic precursor of RAs, the activating ligands of the RARs, is not uniquely protective in lung cancer and may even be harmful (*76,77*). Moreover, eight other randomized placebo-controlled trials having tested the effects of various retinoids against the development of a primary or secondary lung cancer, confirm the lack of positive effects of retinoids in the prevention of lung cancer (*116,139,142,143,386-390*). [For a review, please see reference (*129*)].

6.3 RARβ2 was shown to be a negative prognostic indicator in one study

Third, Khuri *et al.* showed that the prognosis of NSCLC patients whose tumors strongly expressed RARβ2 was significantly worse than those whose tumors weakly expressed RARβ2 (p=0.045) (*391*).

6.4 RARβ2 up-regulates oncogenes directly and indirectly

Fourth, RARβ2 regulates many genes in various cellular pathways, including TSGs as well as oncogenes (e.g. Ets, RET, c-fms, and v-erbA). Some of these oncogenes (e.g. Ets-1) contain a RARE in their promoter region, whereas some are known to be regulated via other RA-dependent/RARE-independent mechanisms. Other RARβ2 target genes are only beginning to be correlated to cancer progression, such as ICAM-1, for example. In particular, ICAM-1 has been shown to be correlated with poor prognosis in several cancers, including lung (*114,200*), stomach (*201*), colon (*202*), bladder (*203*), liver (*204*), and skin (*205*). In addition, we have found that the retroviral transduction of RARβ2 significantly upregulates ICAM-1 expression at the cell surface of 10/13 cancer cell lines, and ATRA treatment exacerbates this effect (Appendix C). Thus, RARβ2-mediated up-regulation of downstream target genes may include the transforming potentials of oncogenes.

6.5 Lack of intragenic mutations

Fifth, while large deletions have been shown to affect one allelic copy of RARβ2, no intragenic mutations or deletions have been documented in the remaining allelic copy to date. Both copies are expected to be altered according to the previous definition of classical TSGs (*392*). However, the definition of a TSG is evolving and has recently recognized the effect of MAGI as a mechanism of TSG inactivation (*226,227*). Nonetheless, genes in which one allelic copy remains genetically intact in cancer are rare.

6.6 Reversibility of DNA methylation

Sixth, even when methylation is found to be correlated with TSG-inactivation, it remains a reversible process and may thus hypothetically be subject to reactivation.

6.7 Lack of hypermethylation

Seventh, numerous groups including our own have analyzed the RARβ2 promoter and all report the existence of cell lines where inactivation is not correlated with methylation (*235,251,393-399*). Additionally, we have found that several cell lines exhibit *divergent methylation*, the coexistence of hypo- and hyper-methylated alleles within the same cell, consequently suggesting the existence of unmethylated alleles (*399*) from which expression should be possible.

TABLE XVI. Summary of lines of evidence converging toward the possibility that RARβ2 may not act solely as a TSG in cancer cells.

RARβ2 may not act solely as a TSG in cancer cells:

1. RARβ2 expression is retained in ~50% of lung tumors;
2. RA has no chemopreventive effect and may even be harmful (ATBC, CARET);
3. RARβ2 expression is correlated with poor prognosis in NSCLC;
4. RARβ2 regulates various TSGs and tumor-related genes;
5. RARβ2 is not mutated or deleted in the remaining allelic copy in cancer cells;
6. RARβ2 is inactivated through methylation, a reversible process;
7. RARβ2 is frequently hypomethylated in ≥1 allele.

7. Rationales and objectives

7.1 Methylation studies

7.1.1 Rationale:

The RARβ2 putative tumor suppressor gene is frequently inactivated in many cancers, and multiple studies have shown that DNA hypermethylation plays a key role. However, the majority of these studies have used methylation-specific polymerase chain reaction (MSP) and have focused on the 5'untranslated region (5'UTR) and the first exon of the coding sequence. Consequently, detailed analyses of separate RARβ2 alleles and promoter region-specific analyses, respectively, have not been reported. Moreover, certain studies including our own suggest that different alleles in a same cell may have different methylation patterns.

7.1.2 Objectives:

1. To determine if the level of methylation, upstream of the transcription start site (within the promoter region), reflects that reported in the 5'UTR and first exon;
2. To elucidate the methylation status of each of the individual CpG sites within or proximal to the hormone response elements or transcription sites;
3. To analyze separate RARβ2 alleles and determine whether the different patterns of methylation within a given cell line reflect intracellular allelic variations and if so to study their heritability (mitotic transmission);
4. To determine whether methylation is subject to allelic bias; and
5. To determine if divergent methylation is correlated with transcription asynchrony.

7.2 Antisense studies

7.2.1 Rationale:

RARβ2 is a putative tumor suppressor gene that undergoes loss of heterozygosity in 90-100% of lung cancers and loss of expression in ~50% of all lung cancers. However, multiple lines of evidence converge toward the possibility that RARβ2 does not act solely

as a tumor suppressor. Three examples in point are that RARβ2 expression is maintained in ~50% of lung cancers; retinoids and carotenoids, the metabolic precursors of certain bioactive retinoids, have been shown to be correlated with negative, even harmful effects in several chemoprevention trials; and RARβ2 expression has been shown to be correlated with poor prognosis in non-small cell lung carcinoma patients. Also, the majority of studies have tested the effects of expressing RARβ2 constructs in cells that have lost RARβ2 expression.

7.2.2 Objectives:

1. To design an efficient antisense oligo against RARβ2 mRNA expression;
2. To determine whether the observed biologic activity is antisense oligo-specific;
3. To test the effects of RARβ2 knockdown on the growth of cancer cells having retained RARβ2 expression versus cancer cells having lost RARβ2 expression;
4. To determine whether programmed cell death is activated in cancer cells treated with antisense oligos;
5. To elucidate and compare the gene expression profiles following retinoid stimulation/ RARβ2 activation versus RARβ2 down-regulation;
6. To validate microarray findings.

References

(*1*) Anonymous. Canadian Cancer Statistics, 2003. Toronto, Canada: National Cancer Institute of Canada; 2003. p. 1-104.

(*2*) American Cancer Society I. Cancer Facts & Figures, 2003. 2003.

(*3*) Alberg AJ, Samet JM. Epidemiology of Lung Cancer. Chest 2003;123:21S-49.

(*4*) Shields PG. Molecular epidemiology of smoking and lung cancer. Oncogene 2002;21:6870-6.

(*5*) Donaldson K, MacNee W. Potential mechanisms of adverse pulmonary and cardiovascular effects of particulate air pollution (PM10). Int J Hyg Environ Health 2001;203:411-5.

(*6*) Anonymous. The World Health Report, 2002. The World Health Organization; 2002.

(*7*) Parkin DM, Pisani P, Lopez AD, Masuyer E. At least one in seven cases of cancer is caused by smoking. Global estimates for 1985. Int J Cancer 1994;59:494-504.

(*8*) Vineis P, Alavanja M, Buffler P, Fontham E, Franceschi S, Gao YT, et al. Tobacco and Cancer: Recent Epidemiological Evidence. J Natl Cancer Inst 2004;96:99-106.

(*9*) Gaudette LA, Illing EM, Hill GB. Canadian Cancer Statistics 1991. Health Rep 1991;3:107-35.

(*10*) Hecht SS. Tobacco smoke carcinogens and lung cancer. J Natl Cancer Inst 1999;91:1194-210.

(*11*) Osann KE. Lung cancer in women: the importance of smoking, family history of cancer, and medical history of respiratory disease. Cancer Res 1991;51:4893-7.

(*12*) Lee JJ, Liu D, Lee JS, Kurie JM, Khuri FR, Ibarguen H, et al. Long-term impact of smoking on lung epithelial proliferation in current and former smokers. J Natl Cancer Inst 2001;93:1081-8.

(*13*) Hittelman WN, Yu R, Kurie J. Evidence for genomic instability and clonal outgrowth in the bronchial epithelium of smokers. Proc. Am. Assoc. Cancer Res. 1997;38:3097.

(*14*) Ahrendt SA, Decker PA, Alawi EA, Zhu Yr YR, Sanchez-Cespedes M, Yang SC, et al. Cigarette smoking is strongly associated with mutation of the K-ras gene in patients with primary adenocarcinoma of the lung. Cancer 2001;92:1525-30.

(*15*) Thun MJ, Lally CA, Flannery JT, Calle EE, Flanders WD, Heath CW, Jr. Cigarette smoking and changes in the histopathology of lung cancer. J Natl Cancer Inst 1997;89:1580-6.

(*16*) Wistuba, II, Lam S, Behrens C, Virmani AK, Fong KM, LeRiche J, et al. Molecular damage in the bronchial epithelium of current and former smokers. J Natl Cancer Inst 1997;89:1366-73.

(17) Kim DH, Nelson HH, Wiencke JK, Zheng S, Christiani DC, Wain JC, et al. p16(INK4a) and histology-specific methylation of CpG islands by exposure to tobacco smoke in non-small cell lung cancer. Cancer Res 2001;61:3419-24.

(18) Belinsky SA, Palmisano WA, Gilliland FD, Crooks LA, Divine KK, Winters SA, et al. Aberrant promoter methylation in bronchial epithelium and sputum from current and former smokers. Cancer Res 2002;62:2370-7.

(19) Soria JC, Rodriguez M, Liu DD, Lee JJ, Hong WK, Mao L. Aberrant promoter methylation of multiple genes in bronchial brush samples from former cigarette smokers. Cancer Res 2002;62:351-5.

(20) Zhang H, Cai B. The impact of tobacco on lung health in China. Respirology 2003;8:17-21.

(21) Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, et al. Initial sequencing and analysis of the human genome. Nature 2001;409:860-921.

(22) Lipscomb MF. The respiratory system. In Kumar V, Cotran RS, Robbins SL, editors. Basic Pathology. 5th ed. ed. Philadelphia: Saunders Co.; 1992. p. 385-436.

(23) Emmendoerffer A, Hecht M, Boeker T, Mueller M, Heinrich U. Role of inflammation in chemical-induced lung cancer. Toxicol Lett 2000;112-113:185-91.

(24) Beckles MA, Spiro SG, Colice GL, Rudd RM. Initial evaluation of the patient with lung cancer: symptoms, signs, laboratory tests, and paraneoplastic syndromes. Chest 2003;123:97S-104S.

(25) Anonymous. Cancer Medicine. Section 28 Neoplasms of the thorax. 88. Cancer of the lung. Natural History. 5th edition, e.5 ed; 2000.

(26) World Health Organization. Histological typing of lung tumours. 1 ed. ed. Geneva: World Health Organization; 1967.

(27) Ordonez NG. The immunohistochemical diagnosis of mesothelioma: a comparative study of epithelioid mesothelioma and lung adenocarcinoma. Am J Surg Pathol 2003;27:1031-51.

(28) Franklin WA. Pathology of lung cancer. J Thorac Imaging 2000;15:3-12.

(29) Travis WD, Colby TV, Sobin LH, Corrin B, Shimosato Y, Brambilla E. Histological Typing of Lung and Pleural Tumours. 3 ed. New York: Springer-Verlag Inc.; 1999.

(30) Organization. IAfRoCWH. General Remarks. In IARC handbook of cancer prevention. Vol 4. Lyon, France: IARC; 1999. p. 20.

(31) Organization WH. Histological typing of lung tumors. In International Histological Classification of Tumours. Geneva, Switzerland: World Health Organization; 1981.

(32) Flint A, Lloyd RV. Pulmonary metastases of colonic carcinoma. Distinction from pulmonary adenocarcinoma. Arch Pathol Lab Med 1992;116:39-42.

(33) Flint A, Lloyd RV. Colon carcinoma metastatic to the lung. Cytologic manifestations and distinction from primary pulmonary adenocarcinoma. Acta Cytol 1992;36:230-5.

(*34*) Garber ME, Troyanskaya OG, Schluens K, Petersen S, Thaesler Z, Pacyna-Gengelbach M, et al. Diversity of gene expression in adenocarcinoma of the lung. Proc Natl Acad Sci U S A 2001;98:13784-9.

(*35*) Bhattacharjee A, Richards WG, Staunton J, Li C, Monti S, Vasa P, et al. Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. Proc Natl Acad Sci U S A 2001;98:13790-5.

(*36*) Beadsmoore CJ, Screaton NJ. Classification, staging and prognosis of lung cancer. Eur J Radiol 2003;45:8-17.

(*37*) Armstrong J, McGibney C. The impact of three-dimensional radiation on the treatment of non-small cell lung cancer. Radiother Oncol 2000;56:157-67.

(*38*) Franklin WA. Diagnosis of Lung Cancer. Pathology of Invasive and Preinvasive Neoplasia. Chest 2000;117:80s-9s.

(*39*) World Health Organization. Histological typing of lung tumors. In International Histological Classification of Tumours. Geneva, Switzerland: World Health Organization; 1981.

(*40*) World Health Organization. Histological typing of lung and pleural tumors. In Organization WH, editor. International Histological Classification of Tumours. Geneva, Switzerland; 1999.

(*41*) Organization WH. Histological typing of lung and pleural tumors. In Organization WH, editor. International Histological Classification of Tumours. Geneva, Switzerland; 1999.

(*42*) Hatcher J, Dover DC. Trends in histopathology of lung cancer in Alberta. Can J Public Health 2003;94:292-6.

(*43*) Franklin WA, Carbone DP. Molecular staging and pharmacogenomics. Clinical implications: from lab to patients and back. Lung Cancer 2003;41 Suppl 1:S147-54.

(*44*) Jemal A, Travis WD, Tarone RE, Travis L, Devesa SS. Lung cancer rates convergence in young men and women in the United States: analysis by birth cohort and histologic type. Int J Cancer 2003;105:101-7.

(*45*) TheEndNoteTeam. EndNote. 6.0.2 ed. Berkeley, CA; Carlsbad, CA; Philadelphia, PA: Thompson ISI ResearchSoft; 2002.

(*46*) McCollum EV, Davis M. The necessity of certain lipids during growth. Journal of Biological Chemistry 1913;15:167-75.

(*47*) Osborne TB, Mendel LB. The relation of growth to the chemical constituents of the diet. Journal of Biological Chemistry 1913;15:311-26.

(*48*) Bloch CE. Klinische Untersuchungen über Dystrophie und Xerophthalmie bei jungen Kindern. Jahrb. Kinderheilkd. 1919;89:409-41.

(*49*) Wolbach S, Howe P. Tissue changes following deprivation of fat-soluble A vitamin. J. Exp. Med. 1925;42:753-77.

(*50*) Schapira M, Raaka BM, Samuels HH, Abagyan R. In silico discovery of novel retinoic acid receptor agonist structures. BMC Struct Biol 2001;1:1.

(51) Anonymous. Molsoft, LLC. In ICM 2.7 Program Manual. MolSoft, San Diego: MolSoft; 1998.

(52) Anonymous. Nutrition Data Inc. Vol 2004: Nutrition Data Inc., Phoenix, Arizona; 2004.

(53) Guo X, Nanus DM, Ruiz A, Rando RR, Bok D, Gudas LJ. Reduced levels of retinyl esters and vitamin A in human renal cancers. Cancer Res 2001;61:2774-81.

(54) Institute of Medicine FaNB. Vitamin A. In Dietary Reference Intakes: Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc. Washington, D.C.: National Academy Press; 2001. p. 82-161.

(55) Heber D, Lu QY. Overview of mechanisms of action of lycopene. Exp Biol Med (Maywood) 2002;227:920-3.

(56) Goodman DS, Huang HS. Biosynthesis of Vitamin a with Rat Intestinal Enzymes. Science 1965;149:879-80.

(57) Tang GW, Wang XD, Russell RM, Krinsky NI. Characterization of beta-apo-13-carotenone and beta-apo-14'-carotenal as enzymatic products of the excentric cleavage of beta-carotene. Biochemistry 1991;30:9829-34.

(58) Anonymous. Position of the American Dietetic Association and Dietitians of Canada: vegetarian diets. Can J Diet Pract Res 2003;64:62-81.

(59) World Health Organization. Micronutrient malnutrition—half of the world's population affected. World Health Organization 1996;78:1–4.

(60) Sanjoaquin MA, Appleby PN, Thorogood M, Mann JI, Key TJ. Nutrition, lifestyle and colorectal cancer incidence: a prospective investigation of 10998 vegetarians and non-vegetarians in the United Kingdom. Br J Cancer 2004;90:118-21.

(61) Key TJ, Fraser GE, Thorogood M, Appleby PN, Beral V, Reeves G, et al. Mortality in vegetarians and nonvegetarians: detailed findings from a collaborative analysis of 5 prospective studies. Am J Clin Nutr 1999;70:516S-24S.

(62) Guo X, Ruiz A, Rando RR, Bok D, Gudas LJ. Esterification of all-trans-retinol in normal human epithelial cell strains and carcinoma lines from oral cavity, skin and breast: reduced expression of lecithin:retinol acyltransferase in carcinoma lines. Carcinogenesis 2000;21:1925-33.

(63) Andreola F, Giandomenico V, Spero R, De Luca LM. Expression of a smaller lecithin:retinol acyl transferase transcript and reduced retinol esterification in MCF-7 cells. Biochem Biophys Res Commun 2000;279:920-4.

(64) Simmons DP, Andreola F, De Luca LM. Human melanomas of fibroblast and epithelial morphology differ widely in their ability to synthesize retinyl esters. Carcinogenesis 2002;23:1821-30.

(65) Yeum KJ, Russell RM. Carotenoid bioavailability and bioconversion. Annu Rev Nutr 2002;22:483-504.

(66) Horton C, Maden M. Endogenous distribution of retinoids during normal development and teratogenesis in the mouse embryo. Dev Dyn 1995;202:312-23.

(67) Jornvall H, Hoog JO, Persson B, Pares X. Pharmacogenetics of the alcohol dehydrogenase system. Pharmacology 2000;61:184-91.

(68) Yang ZN, Davis GJ, Hurley TD, Stone CL, Li TK, Bosron WF. Catalytic efficiency of human alcohol dehydrogenases for retinol oxidation and retinal reduction. Alcohol Clin Exp Res 1994;18:587-91.

(69) Bhat PV, Samaha H. Kinetic properties of the human liver cytosolic aldehyde dehydrogenase for retinal isomers. Biochem Pharmacol 1999;57:195-7.

(70) Taimi M, Helvig C, Wisniewski J, Ramshaw H, White J, Amad M, et al. A novel human cytochrome P450, CYP26C1, involved in metabolism of 9-cis and all-trans isomers of retinoic acid. J Biol Chem 2004;279:77-85.

(71) Niederreither K, Abu-Abed S, Schuhbaur B, Petkovich M, Chambon P, Dolle P. Genetic evidence that oxidative derivatives of retinoic acid are not involved in retinoid signaling during mouse development. Nat Genet 2002;31:84-8.

(72) Muindi JR, Frankel SR, Huselton C, DeGrazia F, Garland WA, Young CW, et al. Clinical pharmacology of oral all-trans retinoic acid in patients with acute promyelocytic leukemia. Cancer Res 1992;52:2138-42.

(73) Fairfield KM, Fletcher RH. Vitamins for chronic disease prevention in adults: scientific review. Jama 2002;287:3116-26.

(74) Willett WC, Stampfer MJ, Underwood BA, Taylor JO, Hennekens CH. Vitamins A, E, and carotene: effects of supplementation on their plasma levels. Am J Clin Nutr 1983;38:559-66.

(75) Johnson LJ, Meacham SL, Kruskall LJ. The antioxidants--vitamin C,vitamin E, selenium, and carotenoids. J Agromedicine 2003;9:65-82.

(76) The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. N Engl J Med 1994;330:1029-35.

(77) Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, et al. Risk factors for lung cancer and for intervention effects in CARET, the Beta-Carotene and Retinol Efficacy Trial. J Natl Cancer Inst 1996;88:1550-9.

(78) Blendon RJ, DesRoches CM, Benson JM, Brodie M, Altman DE. Americans' views on the use and regulation of dietary supplements. Arch Intern Med 2001;161:805-10.

(79) Neuhouser ML, Patterson RE, Levy L. Motivations for using vitamin and mineral supplements. J Am Diet Assoc 1999;99:851-4.

(80) Hind M, Corcoran J, Maden M. Temporal/spatial expression of retinoid binding proteins and RAR isoforms in the postnatal lung. Am J Physiol Lung Cell Mol Physiol 2002;282:L468-76.

(81) Eriksson U, Hansson E, Nordlinder H, Busch C, Sundelin J, Peterson PA. Quantitation and tissue localization of the cellular retinoic acid-binding protein. J Cell Physiol 1987;133:482-90.

(*82*) Astrom A, Tavakkol A, Pettersson U, Cromie M, Elder JT, Voorhees JJ. Molecular cloning of two human cellular retinoic acid-binding proteins (CRABP). Retinoic acid-induced expression of CRABP-II but not CRABP-I in adult human skin in vivo and in skin fibroblasts in vitro. J Biol Chem 1991;266:17662-6.

(*83*) Kreutz M, Fritsche J, Andreesen R, Krause SW. Regulation of cellular retinoic acid binding protein (CRABP II) during human monocyte differentiation in vitro. Biochem Biophys Res Commun 1998;248:830-4.

(*84*) Busch C, Siegenthaler G, Vahlquist A, Nordlinder H, Sundelin J, Saksena P, et al. Expression of cellular retinoid-binding proteins during normal and abnormal epidermal differentiation. J Invest Dermatol 1992;99:795-802.

(*85*) Boylan JF, Gudas LJ. Overexpression of the cellular retinoic acid binding protein-I (CRABP-I) results in a reduction in differentiation-specific gene expression in F9 teratocarcinoma cells. J Cell Biol 1991;112:965-79.

(*86*) Boylan JF, Gudas LJ. The level of CRABP-I expression influences the amounts and types of all-trans-retinoic acid metabolites in F9 teratocarcinoma stem cells. J Biol Chem 1992;267:21486-91.

(*87*) Arapshian A, Bertran S, Kuppumbatti YS, Nakajo S, Mira YLR. Epigenetic CRBP downregulation appears to be an evolutionarily conserved (human and mouse) and oncogene-specific phenomenon in breast cancer. Mol Cancer 2004;3:13.

(*88*) Napoli JL. Retinoid binding-proteins redirect retinoid metabolism: biosynthesis and metabolism of retinoic acid. Semin Cell Dev Biol 1997;8:403-15.

(*89*) Thurnham DI, Northrop-Clewes CA. Optimal nutrition: vitamin A and the carotenoids. Proc Nutr Soc 1999;58:449-57.

(*90*) Harris CC, Silverman T, Smith JM, Jackson F, Boren HG. Proliferation of tracheal epithelial cells in normal and vitamin A-deficient Syrian golden hamsters. J Natl Cancer Inst 1973;51:1059-62.

(*91*) Jetten AM. Multistep process of squamous differentiation in tracheobronchial epithelial cells in vitro: analogy with epidermal differentiation. Environ Health Perspect 1989;80:149-60.

(*92*) Chopra DP, Wilkoff LJ. Reversal by vitamin A analogues (retinoids) of hyperplasia induced by N-methyl-N'-nitro-N-nitrosoguanidine in mouse prostate organ cultures. J Natl Cancer Inst 1977;58:923-30.

(*93*) Chopra DP, Wilkoff LJ. beta-Retinoic acid inhibits and reverses testosterone-induced hyperplasia in mouse prostate organ cultures. Nature 1977;265:339-41.

(*94*) Lasnitzki I, Bollag W. Prevention and reversal by a retinoid of 3,4-benzpyrene- and cigarette smoke condensate-induced hyperplasia and metaplasia of rodent respiratory epithelia in organ culture. Cancer Treat Rep 1982;66:1375-80.

(*95*) Chopra DP. Retinoid reversal of squamous metaplasia in organ cultures of tracheas derived from hamsters fed on vitamin A-deficient diet. Eur J Cancer Clin Oncol 1983;19:847-57.

(*96*) Wille JJ, Chopra DP. Reversal by retinoids of keratinization induced by benzo[alpha]pyrene in normal hamster tracheal explants: comparison with the assay involving organ culture of tracheas from vitamin A-deficient hamsters. Cancer Lett 1988;40:235-46.

(*97*) Shealy YF, Frye JL, Schiff LJ. N-(Retinoyl)amino acids. Synthesis and chemopreventive activity in vitro. J Med Chem 1988;31:190-6.

(*98*) Lasnitzki I. Reversal of methylcholanthrene-induced changes in mouse prostates in vitro by retinoic acid and its analogues. Br J Cancer 1976;34:239-48.

(*99*) Anzano MA, Byers SW, Smith JM, Peer CW, Mullen LT, Brown CC, et al. Prevention of breast cancer in the rat with 9-cis-retinoic acid as a single agent and in combination with tamoxifen. Cancer Res 1994;54:4614-7.

(*100*) Eliason JF, Kaufmann F, Tanaka T, Tsukaguchi T. Anti-proliferative effects of the arotinoid Ro 40-8757 on human cancer cell lines in vitro. Br J Cancer 1993;67:1293-8.

(*101*) Geradts J, Chen JY, Russell EK, Yankaskas JR, Nieves L, Minna JD. Human lung cancer cell lines exhibit resistance to retinoic acid treatment. Cell Growth Differ 1993;4:799-809.

(*102*) Maxwell SA, Mukhopadhyay T. Transient stabilization of p53 in non-small cell lung carcinoma cultures arrested for growth by retinoic acid. Exp Cell Res 1994;214:67-74.

(*103*) Kalemkerian GP, Slusher R, Ramalingam S, Gadgeel S, Mabry M. Growth inhibition and induction of apoptosis by fenretinide in small-cell lung cancer cell lines. J Natl Cancer Inst 1995;87:1674-80.

(*104*) Takatsuka J, Takahashi N, de Luca LM. Retinoic acid metabolism and inhibition of cell proliferation: an unexpected liaison. Cancer Res 1996;56:675-8.

(*105*) Toma S, Isnardi L, Raffo P, Riccardi L, Dastoli G, Apfel C, et al. RARalpha antagonist Ro 41-5253 inhibits proliferation and induces apoptosis in breast-cancer cell lines. Int J Cancer 1998;78:86-94.

(*106*) Lu XP, Fanjul A, Picard N, Pfahl M, Rungta D, Nared-Hood K, et al. Novel retinoid-related molecules as apoptosis inducers and effective inhibitors of human lung cancer cells in vivo. Nat Med 1997;3:686-90.

(*107*) Toma S, Isnardi L, Riccardi L, Bollag W. Induction of apoptosis in MCF-7 breast carcinoma cell line by RAR and RXR selective retinoids. Anticancer Res 1998;18:935-42.

(*108*) Fazely F, Ledinko N, Smith DJ. Inhibition by retinoids of in vitro invasive ability of human lung carcinoma cells. Anticancer Res 1988;8:1387-91.

(*109*) Eckert RL, Rorke EA. Molecular biology of keratinocyte differentiation. Environ Health Perspect 1989;80:109-16.

(*110*) Jetten AM, Shirley JE. Characterization of transglutaminase activity in rabbit tracheal epithelial cells. Regulation by retinoids. J Biol Chem 1986;261:15097-101.

(*111*) Jetten AM, George MA, Smits HL, Vollberg TM. Keratin 13 expression is linked to squamous differentiation in rabbit tracheal epithelial cells and down-regulated by retinoic acid. Exp Cell Res 1989;182:622-34.

(*112*) Stewart LV, Thomas ML. Retinoids differentially regulate the proliferation of colon cancer cell lines. Exp Cell Res 1997;233:321-9.

(*113*) Hirata S, Kubo Y, Kokubo T, Kitada M, Nosaka T. [Expression of major histocompatibility complex of advanced non-small cell lung cancer (NSCLC)]. Nippon Kyobu Geka Gakkai Zasshi 1996;44:138-43.

(*114*) Passlick B, Pantel K, Kubuschok B, Angstwurm M, Neher A, Thetter O, et al. Expression of MHC molecules and ICAM-1 on non-small cell lung carcinomas: association with early lymphatic spread of tumour cells. Eur J Cancer 1996;32A:141-5.

(*115*) Takahashi Y, Miura T, Takahashi K. Vitamin A is involved in maintenance of epithelial cells on the bronchioles and cells in the alveoli of rats. J Nutr 1993;123:634-41.

(*116*) Lippman SM, Lee JJ, Karp DD, Vokes EE, Benner SE, Goodman GE, et al. Randomized phase III intergroup trial of isotretinoin to prevent second primary tumors in stage I non-small-cell lung cancer. J Natl Cancer Inst 2001;93:605-18.

(*117*) Albright CD, Salganik RI, Van Dyke T. Dietary depletion of vitamin e and vitamin a inhibits mammary tumor growth and metastasis in transgenic mice. J Nutr 2004;134:1139-44.

(*118*) Slater AF, Nobel CS, Orrenius S. The role of intracellular oxidants in apoptosis. Biochim Biophys Acta 1995;1271:59-62.

(*119*) Handelman GJ, Packer L, Cross CE. Destruction of tocopherols, carotenoids, and retinol in human plasma by cigarette smoke. Am J Clin Nutr 1996;63:559-65.

(*120*) Wang XD, Liu C, Bronson RT, Smith DE, Krinsky NI, Russell M. Retinoid signaling and activator protein-1 expression in ferrets given beta-carotene supplements and exposed to tobacco smoke. J Natl Cancer Inst 1999;91:60-6.

(*121*) Gradelet S, Leclerc J, Siess MH, Astorg PO. beta-Apo-8'-carotenal, but not beta-carotene, is a strong inducer of liver cytochromes P4501A1 and 1A2 in rat. Xenobiotica 1996;26:909-19.

(*122*) Cardoso WV, Williams MC, Mitsialis SA, Joyce-Brady M, Rishi AK, Brody JS. Retinoic acid induces changes in the pattern of airway branching and alters epithelial cell differentiation in the developing lung in vitro. Am J Respir Cell Mol Biol 1995;12:464-76.

(*123*) Nabeyrat E, Besnard V, Corroyer S, Cazals V, Clement A. Retinoic acid-induced proliferation of lung alveolar epithelial cells: relation with the IGF system. Am J Physiol 1998;275:L71-9.

(*124*) Dokmanovic M, Chang BD, Fang J, Roninson IB. Retinoid-induced growth arrest of breast carcinoma cells involves co-activation of multiple growth-inhibitory genes. Cancer Biol Ther 2002;1:24-7.

(*125*) Graham S. Epidemiology of retinoids and cancer. J Natl Cancer Inst 1984;73:1423-8.

(*126*) Graham S, Haughey B, Marshall J, Priore R, Byers T, Rzepka T, et al. Diet in the epidemiology of carcinoma of the prostate gland. J Natl Cancer Inst 1983;70:687-92.

(*127*) Comstock GW, Alberg AJ, Huang HY, Wu K, Burke AE, Hoffman SC, et al. The risk of developing lung cancer associated with antioxidants in the blood: ascorbic acid,

carotenoids, alpha-tocopherol, selenium, and total peroxyl radical absorbing capacity. Cancer Epidemiol Biomarkers Prev 1997;6:907-16.

(*128*) Yong LC, Brown CC, Schatzkin A, Dresser CM, Slesinski MJ, Cox CS, et al. Intake of vitamins E, C, and A and risk of lung cancer. The NHANES I epidemiologic followup study. First National Health and Nutrition Examination Survey. Am J Epidemiol 1997;146:231-43.

(*129*) Winterhalder RC, Hirsch FR, Kotantoulas GK, Franklin WA, Bunn PA, Jr. Chemoprevention of lung cancer--from biology to clinical reality. Ann Oncol 2004;15:185-96.

(*130*) Slaughter DP, Southwick HW, Smejkal W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. Cancer 1953;6:963-8.

(*131*) Vogelstein B, Kinzler KW. The multistep nature of cancer. Trends Genet 1993;9:138-41.

(*132*) Surwit EA, Graham V, Droegemueller W, Alberts D, Chvapil M, Dorr RT, et al. Evaluation of topically applied trans-retinoic acid in the treatment of cervical intraepithelial lesions. Am J Obstet Gynecol 1982;143:821-3.

(*133*) Misset JL, Mathe G, Santelli G, Gouvela J, Homasson JP, Sudre MC, et al. Regression of bronchial epidermoid metaplasia in heavy smokers with etretinate treatment. Acta Vitaminol Enzymol 1985;7 Suppl:21-5.

(*134*) Smith MA, Parkinson DR, Cheson BD, Friedman MA. Retinoids in cancer therapy. J Clin Oncol 1992;10:839-64.

(*135*) Alfthan O, Tarkkanen J, Grohn P, Heinonen E, Pyrhonen S, Saila K. Tigason (etretinate) in prevention of recurrence of superficial bladder tumors. A double-blind clinical trial. Eur Urol 1983;9:6-9.

(*136*) Bolla M, Lefur R, Ton Van J, Domenge C, Badet JM, Koskas Y, et al. Prevention of second primary tumours with etretinate in squamous cell carcinoma of the oral cavity and oropharynx. Results of a multicentric double-blind randomised study. Eur J Cancer 1994;30A:767-72.

(*137*) Gouveia J, Mathe G, Hercend T, Gros F, Lemaigre G, Santelli G, et al. Degree of bronchial metaplasia in heavy smokers and its regression after treatment with a retinoid. Lancet 1982;1:710-2.

(*138*) Saccomanno G, Moran PG, Schmidt R, Hartshorn DF, Brian DA, Dreher WH, et al. Effects of 13-CIS retinoids on premalignant and malignant cells of lung origin. Acta Cytol 1982;26:78-85.

(*139*) Arnold AM, Browman GP, Levine MN, D'Souza T, Johnstone B, Skingley P, et al. The effect of the synthetic retinoid etretinate on sputum cytology: results from a randomised trial. Br J Cancer 1992;65:737-43.

(*140*) Lee JJ, Ro JY, Lukeman JM, Morice RC, Peters RJ, Pang A, et al. A randomized placebo-controlled chemoprevention trial of 13-cis retinoic acid (cRA) in bronchial squamous metaplasia. Proc. Am. Soc. Clin. Oncol. 1993;12:335.

(*141*) The alpha-tocopherol, beta-carotene lung cancer prevention study: design, methods, participant characteristics, and compliance. The ATBC Cancer Prevention Study Group. Ann Epidemiol 1994;4:1-10.

(*142*) van Zandwijk N, Dalesio O, Pastorino U, de Vries N, van Tinteren H. EUROSCAN, a randomized trial of vitamin A and N-acetylcysteine in patients with head and neck cancer or lung cancer. For the EUropean Organization for Research and Treatment of Cancer Head and Neck and Lung Cancer Cooperative Groups. J Natl Cancer Inst 2000;92:977-86.

(*143*) Pastorino U, Infante M, Maioli M, Chiesa G, Buyse M, Firket P, et al. Adjuvant treatment of stage I lung cancer with high-dose vitamin A. J Clin Oncol 1993;11:1216-22.

(*144*) Marwick C. Trials reveal no benefit, possible harm of beta carotene and vitamin A for lung cancer prevention. Jama 1996;275:422-3.

(*145*) Altucci L, Wilhelm E, Gronemeyer H. Leukemia: beneficial actions of retinoids and rexinoids. Int J Biochem Cell Biol 2004;36:178-82.

(*146*) Allenby G, Bocquel MT, Saunders M, Kazmer S, Speck J, Rosenberger M, et al. Retinoic acid receptors and retinoid X receptors: interactions with endogenous retinoic acids. Proc Natl Acad Sci U S A 1993;90:30-4.

(*147*) Balmer JE, Blomhoff R. Gene expression regulation by retinoic acid. J Lipid Res 2002;43:1773-808.

(*148*) Chambon P. A decade of molecular biology of retinoic acid receptors. Faseb J 1996;10:940-54.

(*149*) Anonymous. General Remarks. In Group TIW, editor. Retinoids. Vol 4. Lyon, France: International Agency for Research on Cancer; 1999. p. 15-93.

(*150*) Mollard R, Viville S, Ward SJ, Decimo D, Chambon P, Dolle P. Tissue-specific expression of retinoic acid receptor isoform transcripts in the mouse embryo. Mech Dev 2000;94:223-32.

(*151*) Dolle P, Ruberte E, Leroy P, Morriss-Kay G, Chambon P. Retinoic acid receptors and cellular retinoid binding proteins. I. A systematic study of their differential pattern of transcription during mouse organogenesis. Development 1990;110:1133-51.

(*152*) Yamagata T, Momoi MY, Yanagisawa M, Kumagai H, Yamakado M, Momoi T. Changes of the expression and distribution of retinoic acid receptors during neurogenesis in mouse embryos. Brain Res Dev Brain Res 1994;77:163-76.

(*153*) Sharpe CR, Goldstone K. Retinoid receptors promote primary neurogenesis in Xenopus. Development 1997;124:515-23.

(*154*) Houle B, Pelletier M, Wu J, Goodyer C, Bradley WE. Fetal isoform of human retinoic acid receptor beta expressed in small cell lung cancer lines. Cancer Res 1994;54:365-9.

(*155*) Toulouse A, Morin J, Pelletier M, Bradley WE. Structure of the human retinoic acid receptor beta 1 gene. Biochim Biophys Acta 1996;1309:1-4.

(*156*) Berard J, Gaboury L, Landers M, De Repentigny Y, Houle B, Kothary R, et al. Hyperplasia and tumours in lung, breast and other tissues in mice carrying a RAR beta 4-like transgene. Embo J 1994;13:5570-80.

(*157*) Kastner P, Krust A, Mendelsohn C, Garnier JM, Zelent A, Leroy P, et al. Murine isoforms of retinoic acid receptor gamma with specific patterns of expression. Proc Natl Acad Sci U S A 1990;87:2700-4.

(*158*) Li E, Sucov HM, Lee KF, Evans RM, Jaenisch R. Normal development and growth of mice carrying a targeted disruption of the alpha 1 retinoic acid receptor gene. Proc Natl Acad Sci U S A 1993;90:1590-4.

(*159*) Lohnes D, Kastner P, Dierich A, Mark M, LeMeur M, Chambon P. Function of retinoic acid receptor gamma in the mouse. Cell 1993;73:643-58.

(*160*) Luo J, Pasceri P, Conlon RA, Rossant J, Giguere V. Mice lacking all isoforms of retinoic acid receptor beta develop normally and are susceptible to the teratogenic effects of retinoic acid. Mech Dev 1995;53:61-71.

(*161*) Leroy P, Nakshatri H, Chambon P. Mouse retinoic acid receptor alpha 2 isoform is transcribed from a promoter that contains a retinoic acid response element. Proc Natl Acad Sci U S A 1991;88:10138-42.

(*162*) Zelent A, Mendelsohn C, Kastner P, Krust A, Garnier JM, Ruffenach F, et al. Differentially expressed isoforms of the mouse retinoic acid receptor beta generated by usage of two promoters and alternative splicing. Embo J 1991;10:71-81.

(*163*) Lehmann JM, Zhang XK, Pfahl M. RAR gamma 2 expression is regulated through a retinoic acid response element embedded in Sp1 sites. Mol Cell Biol 1992;12:2976-85.

(*164*) Lohnes D, Mark M, Mendelsohn C, Dolle P, Dierich A, Gorry P, et al. Function of the retinoic acid receptors (RARs) during development (I). Craniofacial and skeletal abnormalities in RAR double mutants. Development 1994;120:2723-48.

(*165*) Kastner P, Mark M, Chambon P. Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? Cell 1995;83:859-69.

(*166*) Sterner DE, Berger SL. Acetylation of histones and transcription-related factors. Microbiol Mol Biol Rev 2000;64:435-59.

(*167*) Wei LN. Retinoid receptors and their coregulators. Annu Rev Pharmacol Toxicol 2003;43:47-72.

(*168*) Farboud B, Hauksdottir H, Wu Y, Privalsky ML. Isotype-restricted corepressor recruitment: a constitutively closed helix 12 conformation in retinoic acid receptors beta and gamma interferes with corepressor recruitment and prevents transcriptional repression. Mol Cell Biol 2003;23:2844-58.

(*169*) Schule R, Rangarajan P, Yang N, Kliewer S, Ransone LJ, Bolado J, et al. Retinoic acid is a negative regulator of AP-1-responsive genes. Proc Natl Acad Sci U S A 1991;88:6092-6.

(*170*) Shaulian E, Karin M. AP-1 as a regulator of cell life and death. Nat Cell Biol 2002;4:E131-6.

(*171*) Whang-Peng J, Bunn PA, Jr., Kao-Shan CS, Lee EC, Carney DN, Gazdar A, et al. A nonrandom chromosomal abnormality, del 3p(14-23), in human small cell lung cancer (SCLC). Cancer Genet Cytogenet 1982;6:119-34.

(*172*) Whang-Peng J, Kao-Shan CS, Lee EC, Bunn PA, Carney DN, Gazdar AF, et al. Specific chromosome defect associated with human small-cell lung cancer; deletion 3p(14-23). Science 1982;215:181-2.

(*173*) Zabarovsky ER, Lerman MI, Minna JD. Tumor suppressor genes on chromosome 3p involved in the pathogenesis of lung and other cancers. Oncogene 2002;21:6915-35.

(*174*) Houle B, Leduc F, Bradley WE. Implication of RARB in epidermoid (Squamous) lung cancer. Genes Chromosomes Cancer 1991;3:358-66.

(*175*) Goeze A, Schluns K, Wolf G, Thasler Z, Petersen S, Petersen I. Chromosomal imbalances of primary and metastatic lung adenocarcinomas. J Pathol 2002;196:8-16.

(*176*) Toulouse A, Loubeau M, Morin J, Pappas JJ, Wu J, Bradley WE. RARbeta involvement in enhancement of lung tumor cell immunogenicity revealed by array analysis. Faseb J 2000;14:1224-32.

(*177*) Lotan R. Roles of retinoids and their nuclear receptors in the development and prevention of upper aerodigestive tract cancers. Environ Health Perspect 1997;105 Suppl 4:985-8.

(*178*) Toulouse A, Morin J, Dion PA, Houle B, Bradley WE. RARbeta2 specificity in mediating RA inhibition of growth of lung cancer-derived cells. Lung Cancer 2000;28:127-37.

(*179*) Benbrook D, Lernhardt E, Pfahl M. A new retinoic acid receptor identified from a hepatocellular carcinoma. Nature 1988;333:669-72.

(*180*) Brand N, Petkovich M, Krust A, Chambon P, de The H, Marchio A, et al. Identification of a second human retinoic acid receptor. Nature 1988;332:850-3.

(*181*) Blackfan K, Wolbach S. Vitamin A deficiency in infants: a clinical and pathological study. J. Pediatrics 1933;3:679-706.

(*182*) McMichael H. Inhibition of growth of Shope rabbit papilloma by hypervitaminosis A. Cancer Res 1965;25:947-55.

(*183*) Bollag W. Prophylaxis of chemically induced benign and malignant epithelial tumors by vitamin A acid (retinoic acid). Eur J Cancer 1972;8:689-93.

(*184*) Davies RE. Effect of vitamin A on 7,12-Dimethylbenz(alpha)anthracene-induced papillomas in rhino mouse skin. Cancer Res 1967;27:237-41.

(*185*) Chu EW, Malmgren RA. An inhibitory effect of vitamin A on the induction of tumors of forestomach and cervix in the Syrian hamster by carcinogenic polycyclic hydrocarbons. Cancer Res 1965;25:884-95.

(*186*) Sporn MB, Squire RA, Brown CC, Smith JM, Wenk ML, Springer S. 13-cis-retinoic acid: inhibition of bladder carcinogenesis in the rat. Science 1977;195:487-9.

(*187*) Tannock IF, Suit HD, Marshall N. Vitamin A and the radiation response of experimental tumors: an immune-mediated effect. J Natl Cancer Inst 1972;48:731-41.

(*188*) Felix EL, Loyd B, Cohen MH. Inhibition of the growth and development of a transplantable murine melanoma by vitamin A. Science 1975;189:886-8.

(*189*) Trown PW, Buck MJ, Hansen R. Inhibition of growth and regression of a transplantable rat chondrosarcoma by three retinoids. Cancer Treat Rep 1976;60:1647-53.

(*190*) Meltzer MS, Cohen BE. Tumor suppression by Mycobacterium bovis (strain BCG) enhanced by vitamin A. J Natl Cancer Inst 1974;53:585-7.

(*191*) Felix EL, Cohen MH, Loyd BC. Immune and toxic anti-tumor effects of systemic and intralesional vitamin A. J Surg Res 1976;21:307-12.

(*192*) Kurata T, Micksche M. Suppressed tumor growth and metastasis by vitamin A + BCG in Lewis lung tumor bearing mice. Oncology 1977;34:212-5.

(*193*) Patek PQ, Collins JL, Yogeeswaran G, Dennert G. Anti-tumor potential of retinoic acid: stimulation of immune mediated effectors. Int J Cancer 1979;24:624-8.

(*194*) Baron S, Kleyn KM, Russell JK, Blalock JE. Retinoic acid: enhancement of a tumor and inhibition of interferon's antitumor action. J Natl Cancer Inst 1981;67:95-7.

(*195*) Dennert G, Lotan R. Effects of retinoic acid on the immune system: stimulation of T killer cell induction. Eur J Immunol 1978;8:23-9.

(*196*) Dennert G, Crowley C, Kouba J, Lotan R. Retinoic acid stimulation of the induction of mouse killer T-cells in allogeneic and syngeneic systems. J Natl Cancer Inst 1979;62:89-94.

(*197*) Yang GC, VChen LP. Costimulation in immune responses against tumors. In Liu Y, editor. Molecular Approaches to Tumor Immunotherapy. River Edge, New Jersey: World Scientific Publishing; 1998. p. 191-211.

(*198*) Doyle A, Martin WJ, Funa K, Gazdar A, Carney D, Martin SE, et al. Markedly decreased expression of class I histocompatibility antigens, protein, and mRNA in human small-cell lung cancer. J Exp Med 1985;161:1135-51.

(*199*) Redondo M, Ruiz-Cabello F, Concha A, Cabrera T, Perez-Ayala M, Oliva MR, et al. Altered HLA class I expression in non-small cell lung cancer is independent of c-myc activation. Cancer Res 1991;51:2463-8.

(*200*) Grothey A, Heistermann P, Philippou S, Voigtmann R. Serum levels of soluble intercellular adhesion molecule-1 (ICAM-1, CD54) in patients with non-small-cell lung cancer: correlation with histological expression of ICAM-1 and tumour stage. Br J Cancer 1998;77:801-7.

(*201*) Maruo Y, Gochi A, Kaihara A, Shimamura H, Yamada T, Tanaka N, et al. ICAM-1 expression and the soluble ICAM-1 level for evaluating the metastatic potential of gastric cancer. Int J Cancer 2002;100:486-90.

(*202*) Alexiou D, Karayiannakis AJ, Syrigos KN, Zbar A, Kremmyda A, Bramis I, et al. Serum levels of E-selectin, ICAM-1 and VCAM-1 in colorectal cancer patients: correlations with clinicopathological features, patient survival and tumour surgery. Eur J Cancer 2001;37:2392-7.

(*203*) Roche Y, Pasquier D, Rambeaud JJ, Seigneurin D, Duperray A. Fibrinogen mediates bladder cancer cell migration in an ICAM-1-dependent pathway. Thromb Haemost 2003;89:1089-97.

(*204*) Meng C, Chen X. [Association of VEGF, uPA, ICAM-1 and PCNA expression with metastasis and recurrence in hepato cellular carcinoma]. Zhonghua Wai Ke Za Zhi 2002;40:673-5.

(*205*) Haritopoulos KN, Lazaris AC, Kavantzas N, Tseleni-Balafouta S, Thomopoulou G, Aroni K. ICAM-1 and beta(3) integrin immunoexpression in malignant melanoma cells: can they be used as additional predictors? Apmis 2003;111:421-9.

(*206*) Garbe A, Buck J, Hammerling U. Retinoids are important cofactors in T cell activation. J Exp Med 1992;176:109-17.

(*207*) Prabhala RH, Maxey V, Hicks MJ, Watson RR. Enhancement of the expression of activation markers on human peripheral blood mononuclear cells by in vitro culture with retinoids and carotenoids. J Leukoc Biol 1989;45:249-54.

(*208*) Buck J, Ritter G, Dannecker L, Katta V, Cohen SL, Chait BT, et al. Retinol is essential for growth of activated human B cells. J Exp Med 1990;171:1613-24.

(*209*) Blomhoff HK, Smeland EB, Erikstein B, Rasmussen AM, Skrede B, Skjonsberg C, et al. Vitamin A is a key regulator for cell growth, cytokine production, and differentiation in normal B cells. J Biol Chem 1992;267:23988-92.

(*210*) Sidell N, Rieber P, Golub SH. Immunological aspects of retinoids in humans. I. Analysis of retinoic acid enhancement of thymocyte responses to PHA. Cell Immunol 1984;87:118-25.

(*211*) Sidell N, Ramsdell F. Retinoic acid upregulates interleukin-2 receptors on activated human thymocytes. Cell Immunol 1988;115:299-309.

(*212*) Sidell N, Chang B, Bhatti L. Upregulation by retinoic acid of interleukin-2-receptor mRNA in human T lymphocytes. Cell Immunol 1993;146:28-37.

(*213*) Shklar G, Schwartz J. Tumor necrosis factor in experimental cancer regression with alphatocopherol, beta-carotene, canthaxanthin and algae extract. Eur J Cancer Clin Oncol 1988;24:839-50.

(*214*) Manna SK, Aggarwal BB. All-trans-retinoic acid upregulates TNF receptors and potentiates TNF-induced activation of nuclear factors-kappaB, activated protein-1 and apoptosis in human lung cancer cells. Oncogene 2000;19:2110-9.

(*215*) Witcher M, Ross DT, Rousseau C, Deluca L, Miller WH, Jr. Synergy between all-trans retinoic acid and tumor necrosis factor pathways in acute leukemia cells. Blood 2003;102:237-45.

(*216*) Marth C, Daxenbichler G, Dapunt O. Synergistic antiproliferative effect of human recombinant interferons and retinoic acid in cultured breast cancer cells. J Natl Cancer Inst 1986;77:1197-202.

(*217*) Knudson AG. Mutation and Cancer: Statistical study of retinoblastoma. Proc. Nat'l Acad. Sci. USA. 1971;68:820-3.

(*218*) Houle B, Rochette-Egly C, Bradley WE. Tumor-suppressive effect of the retinoic acid receptor beta in human epidermoid lung cancer cells. Proc Natl Acad Sci U S A 1993;90:985-9.

(*219*) Gebert JF, Moghal N, Frangioni JV, Sugarbaker DJ, Neel BG. High frequency of retinoic acid receptor beta abnormalities in human lung cancer. Oncogene 1991;6:1859-68.

(*220*) Nervi C, Vollberg TM, George MD, Zelent A, Chambon P, Jetten AM. Expression of nuclear retinoic acid receptors in normal tracheobronchial cells and in lung carcinoma cells. Exp Cell Res 1991;195:163-70.

(*221*) Roman SD, Clarke CL, Hall RE, Alexander IE, Sutherland RL. Expression and regulation of retinoic acid receptors in human breast cancer cells. Cancer Res 1992;52:2236-42.

(*222*) Swisshelm K, Ryan K, Lee X, Tsou HC, Peacocke M, Sager R. Down-regulation of retinoic acid receptor beta in mammary carcinoma cell lines and its up-regulation in senescing normal mammary epithelial cells. Cell Growth Differ 1994;5:133-41.

(*223*) Moghal N, Neel BG. Evidence for impaired retinoic acid receptor-thyroid hormone receptor AF-2 cofactor activity in human lung cancer. Mol Cell Biol 1995;15:3945-59.

(*224*) Seewaldt VL, Caldwell LE, Johnson BS, Swisshelm K, Collins SJ, Tsai S. Inhibition of retinoic acid receptor function in normal human mammary epithelial cells results in increased cellular proliferation and inhibits the formation of a polarized epithelium in vitro. Exp Cell Res 1997;236:16-28.

(*225*) Berard J, Laboune F, Mukuna M, Masse S, Kothary R, Bradley WE. Lung tumors in mice expressing an antisense RARbeta2 transgene. Faseb J 1996;10:1091-7.

(*226*) Esteller M. Cancer epigenetics: DNA methylation and chromatin alterations in human cancer. Adv Exp Med Biol 2003;532:39-49.

(*227*) Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 2003;33 Suppl:245-54.

(*228*) Cote S, Sinnett, D., Momparler, R. L. Demethylation by 5-aza-2'-deoxycytidine of specific 5-methylcytosine sites in the promoter region of the retinoic acid receptor beta gene in human colon carcinoma cells. Anticancer Drugs 1998;9:743-50.

(*229*) Bovenzi V, Le, N. L., Cote, S., Sinnett, D., Momparler, L. F., Momparler, R. L. DNA methylation of retinoic acid receptor beta in breast cancer and possible therapeutic role of 5-aza-2'-deoxycytidine. Anticancer Drugs 1999;10:471-6.

(*230*) Sirchia SM, Ferguson, A. T., Sironi, E., Subramanyan, S., Orlandi, R., Sukumar, S., Sacchi, N. Evidence of epigenetic changes affecting the chromatin state of the retinoic acid receptor beta2 promoter in breast cancer cells. Oncogene 2000;19:1556-63.

(*231*) Virmani AK, Rathi, A., Zochbauer-Muller, S.. Sacchi, N., Fukuyama, Y., Bryant, D., Maitra, A., Heda, S., Fong, K. M., Thunniss N, F., Minna, J. D., Gazdar, A. F. Promoter methylation and silencing of the retinoic acid receptor-beta gene in lung carcinomas. J Natl Cancer Inst 2000;92:1303-7.

(*232*) Bovenzi V, Momparler, R. L. Antineoplastic action of 5-aza-2'-deoxycytidine and histone deacetylase inhibitor and their effect on the expression of retinoic acid receptor beta and

estrogen receptor alpha genes in breast carcinoma cells. Cancer Chemother Pharmacol 2001;48:71-6.

(*233*) Sirchia SM, Ren, M., Pili, R., Sironi, E., Somenzi,G., Ghidoni, R., Toma, S., Nicolo, G., Sacchi, N. Endogenous reactivation of the RARbeta2 tumor suppressor gene epigenetically silenced in breast cancer. Cancer Res 2002;62:2455-61.

(*234*) Yang Q, Mori I, Shan L, Nakamura M, Nakamura Y, Utsunomiya H, et al. Biallelic inactivation of retinoic acid receptor beta2 gene by epigenetic change in breast cancer. Am J Pathol 2001;158:299-303.

(*235*) Widschwendter M, Berger J, Hermann M, Muller HM, Amberger A, Zeschnigk M, et al. Methylation and silencing of the retinoic acid receptor-beta2 gene in breast cancer. J Natl Cancer Inst 2000;92:826-32.

(*236*) Cote S, Momparler, R. L. Activation of the retinoic acid receptor beta gene by 5-aza-2'-deoxycytidine in human DLD-1 colon carcinoma cells. Anticancer Drugs 1997;8:56-61.

(*237*) Jones PA. The DNA methylation paradox. Trends Genet 1999;15:34-7.

(*238*) Keshet I, Yisraeli J, Cedar H. Effect of regional DNA methylation on gene expression. Proc Natl Acad Sci U S A 1985;82:2560-4.

(*239*) Murray EJ, Grosveld F. Site specific demethylation in the promoter of human gamma-globin gene does not alleviate methylation mediated suppression. Embo J 1987;6:2329-35.

(*240*) Arapshian A, Kuppumbatti, Y. S., Mira-y-Lopez, R. Methylation of conserved CpG sites neighboring the beta retinoic acid response element may mediate retinoic acid receptor beta gene silencing in MCF-7 breast cancer cells. Oncogene 2000;19:4066-70.

(*241*) Nakayama T, Watanabe, M., Yamanaka, M., Hirokawa, Y., Suzuki, H., Ito, H., Yatani, R., Shiraishi, T. The role of epigenetic modifications in retinoic acid receptor beta2 gene expression in human prostate cancers. Lab Invest 2001;81:1049-57.

(*242*) Deng G, Peng, E., Gum J, Terdiman, J., Sleisenger, M. and Kim, Y.S. Methylation of hMLH1 promoter correlates with the gene silencing with a region-specific manner in colorectal cancer. Br. J Cancer 2002;86:574-9.

(*243*) Folkers GE, van der Burg B, van der Saag PT. Promoter architecture, cofactors, and orphan receptors contribute to cell-specific activation of the retinoic acid receptor beta2 promoter. J Biol Chem 1998;273:32200-12.

(*244*) Bhattacharyya N, Dey A, Minucci S, Zimmer A, John S, Hager G, et al. Retinoid-induced chromatin structure alterations in the retinoic acid receptor beta2 promoter. Mol Cell Biol 1997;17:6481-90.

(*245*) Sheikh MS, Shao ZM, Li XS, Dawson M, Jetten AM, Wu S, et al. Retinoid-resistant estrogen receptor-negative human breast carcinoma cells transfected with retinoic acid receptor-alpha acquire sensitivity to growth inhibition by retinoids. J Biol Chem 1994;269:21440-7.

(*246*) van der Leede BJ, Folkers GE, van den Brink CE, van der Saag PT, van der Burg B. Retinoic acid receptor alpha 1 isoform is induced by estradiol and confers retinoic acid sensitivity in human breast cancer cells. Mol Cell Endocrinol 1995;109:77-86.

(*247*) Dawson MI, Chao WR, Pine P, Jong L, Hobbs PD, Rudd CK, et al. Correlation of retinoid binding affinity to retinoic acid receptor alpha with retinoid inhibition of growth of estrogen receptor-positive MCF-7 mammary carcinoma cells. Cancer Res 1995;55:4446-51.

(*248*) Fitzgerald P, Teng M, Chandraratna RA, Heyman RA, Allegretto EA. Retinoic acid receptor alpha expression correlates with retinoid-induced growth inhibition of human breast cancer cells regardless of estrogen receptor status. Cancer Res 1997;57:2642-50.

(*249*) Schneider SM, Offterdinger M, Huber H, Grunt TW. Activation of retinoic acid receptor alpha is sufficient for full induction of retinoid responses in SK-BR-3 and T47D human breast cancer cells. Cancer Res 2000;60:5479-87.

(*250*) Farias EF, Arapshian A, Bleiweiss IJ, Waxman S, Zelent A, Mira YLR. Retinoic acid receptor alpha2 is a growth suppressor epigenetically silenced in MCF-7 human breast cancer cells. Cell Growth Differ 2002;13:335-41.

(*251*) Arapshian A, Kuppumbatti YS, Mira-y-Lopez R. Methylation of conserved CpG sites neighboring the beta retinoic acid response element may mediate retinoic acid receptor beta gene silencing in MCF-7 breast cancer cells. Oncogene 2000;19:4066-70.

(*252*) Deng G, Peng E, Gum J, Terdiman J, Sleisenger M, Kim YS. Methylation of hMLH1 promoter correlates with the gene silencing with a region-specific manner in colorectal cancer. Br J Cancer 2002;86:574-9.

(*253*) Nakayama T, Watanabe M, Yamanaka M, Hirokawa Y, Suzuki H, Ito H, et al. The role of epigenetic modifications in retinoic acid receptor beta2 gene expression in human prostate cancers. Lab Invest 2001;81:1049-57.

(*254*) Esteller M, Guo M, Moreno V, Peinado MA, Capella G, Galm O, et al. Hypermethylation-associated Inactivation of the Cellular Retinol-Binding-Protein 1 Gene in Human Cancer. Cancer Res 2002;62:5902-5.

(*255*) Chan MW, Chan LW, Tang NL, Tong JH, Lo KW, Lee TL, et al. Hypermethylation of multiple genes in tumor tissues and voided urine in urinary bladder cancer patients. Clin Cancer Res 2002;8:464-70.

(*256*) Kwong J, Lo KW, To KF, Teo PM, Johnson PJ, Huang DP. Promoter hypermethylation of multiple genes in nasopharyngeal carcinoma. Clin Cancer Res 2002;8:131-7.

(*257*) Kuroki T, Trapasso F, Yendamuri S, Matsuyama A, Alder H, Mori M, et al. Allele loss and promoter hypermethylation of VHL, RAR-beta, RASSF1A, and FHIT tumor suppressor genes on chromosome 3p in esophageal squamous cell carcinoma. Cancer Res 2003;63:3724-8.

(*258*) Oue N, Motoshita J, Yokozaki H, Hayashi K, Tahara E, Taniyama K, et al. Distinct promoter hypermethylation of p16INK4a, CDH1, and RAR-beta in intestinal, diffuse-adherent, and diffuse-scattered type gastric carcinomas. J Pathol 2002;198:55-9.

(*259*) Maruyama R, Toyooka S, Toyooka KO, Virmani AK, Zochbauer-Muller S, Farinas AJ, et al. Aberrant promoter methylation profile of prostate cancers and its relationship to clinicopathological features. Clin Cancer Res 2002;8:514-9.

(*260*) Fackler MJ, McVeigh M, Evron E, Garrett E, Mehrotra J, Polyak K, et al. DNA methylation of RASSF1A, HIN-1, RAR-beta, Cyclin D2 and Twist in in situ and invasive lobular breast carcinoma. Int J Cancer 2003;107:970-5.

(*261*) Zochbauer-Muller S, Fong KM, Virmani AK, Geradts J, Gazdar AF, Minna JD. Aberrant promoter methylation of multiple genes in non-small cell lung cancers. Cancer Res 2001;61:249-55.

(*262*) Narayan G, Arias-Pulido H, Koul S, Vargas H, Zhang FF, Villella J, et al. Frequent Promoter Methylation of CDH1, DAPK, RARB, and HIC1 Genes in Carcinoma of Cervix Uteri: Its Relationship to Clinical Outcome. Mol Cancer 2003;2:24.

(*263*) Chim CS, Wong SY, Kwong YL. Aberrant gene promoter methylation in acute promyelocytic leukaemia: profile and prognostic significance. Br J Haematol 2003;122:571-8.

(*264*) Palmisano WA, Divine KK, Saccomanno G, Gilliland FD, Baylin SB, Herman JG, et al. Predicting lung cancer by detecting aberrant promoter methylation in sputum. Cancer Res 2000;60:5954-8.

(*265*) Ahrendt SA, Chow JT, Xu LH, Yang SC, Eisenberger CF, Esteller M, et al. Molecular detection of tumor cells in bronchoalveolar lavage fluid from patients with early stage lung cancer. J Natl Cancer Inst 1999;91:332-9.

(*266*) Evron E, Dooley WC, Umbricht CB, Rosenthal D, Sacchi N, Gabrielson E, et al. Detection of breast cancer cells in ductal lavage fluid by methylation-specific PCR. Lancet 2001;357:1335-6.

(*267*) Goessl C, Krause H, Muller M, Heicappell R, Schrader M, Sachsinger J, et al. Fluorescent methylation-specific polymerase chain reaction for DNA-based detection of prostate cancer in bodily fluids. Cancer Res 2000;60:5941-5.

(*268*) Oshita F, Sekiyama A, Suzuki R, Ikehara M, Yamada K, Saito H, et al. Detection of occult tumor cells in peripheral blood from patients with small cell lung cancer by promoter methylation and silencing of the retinoic acid receptor-beta. Oncol Rep 2003;10:105-8.

(*269*) Eguchi K, Kanai Y, Kobayashi K, Hirohashi S. DNA hypermethylation at the D17S5 locus in non-small cell lung cancers: its association with smoking history. Cancer Res 1997;57:4913-5.

(*270*) Ono T, Tawa R, Shinya K, Hirose S, Okada S. Methylation of the c-myc gene changes during aging process of mice. Biochem Biophys Res Commun 1986;139:1299-304.

(*271*) Slagboom PE, Uitterlinden AG, Vijg J. Methylation status of cKi-ras and MHC genes in rat pituitary glands during aging and tumorigenesis. Aging (Milano) 1991;3:141-6.

(*272*) Yenbutr P, Hilakivi-Clarke L, Passaniti A. Hypomethylation of an exon I estrogen receptor CpG island in spontaneous and carcinogen-induced mammary tumorigenesis in the rat. Mech Ageing Dev 1998;106:93-102.

(*273*) Li Q, Jedlicka A, Ahuja N, Gibbons MC, Baylin SB, Burger PC, et al. Concordant methylation of the ER and N33 genes in glioblastoma multiforme. Oncogene 1998;16:3197-202.

(*274*) Issa JP, Ottaviano YL, Celano P, Hamilton SR, Davidson NE, Baylin SB. Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. Nat Genet 1994;7:536-40.

(*275*) Ahuja N, Li Q, Mohan AL, Baylin SB, Issa JP. Aging and DNA methylation in colorectal mucosa and cancer. Cancer Res 1998;58:5489-94.

(*276*) Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci U S A 1999;96:8681-6.

(*277*) Habuchi T, Takahashi T, Kakinuma H, Wang L, Tsuchiya N, Satoh S, et al. Hypermethylation at 9q32-33 tumour suppressor region is age-related in normal urothelium and an early and frequent alteration in bladder cancer. Oncogene 2001;20:531-7.

(*278*) Issa JP, Ahuja N, Toyota M, Bronner MP, Brentnall TA. Accelerated age-related CpG island methylation in ulcerative colitis. Cancer Res 2001;61:3573-7.

(*279*) Kang GH, Lee HJ, Hwang KS, Lee S, Kim JH, Kim JS. Aberrant CpG island hypermethylation of chronic gastritis, in relation to aging, gender, intestinal metaplasia, and chronic inflammation. Am J Pathol 2003;163:1551-6.

(*280*) Tamura G. Promoter methylation status of tumor suppressor and tumor-related genes in neoplastic and non-neoplastic gastric epithelia. Histol Histopathol 2004;19:221-8.

(*281*) Gama-Sosa MA, Slagel VA, Trewyn RW, Oxenhandler R, Kuo KC, Gehrke CW, et al. The 5-methylcytosine content of DNA from human tumors. Nucleic Acids Res 1983;11:6883-94.

(*282*) Feinberg AP, Gehrke CW, Kuo KC, Ehrlich M. Reduced genomic 5-methylcytosine content in human colonic neoplasia. Cancer Res 1988;48:1159-61.

(*283*) de Bustros A, Nelkin BD, Silverman A, Ehrlich G, Poiesz B, Baylin SB. The short arm of chromosome 11 is a "hot spot" for hypermethylation in human neoplasia. Proc Natl Acad Sci U S A 1988;85:5693-7.

(*284*) Nelkin BD, Przepiorka D, Burke PJ, Thomas ED, Baylin SB. Abnormal methylation of the calcitonin gene marks progression of chronic myelogenous leukemia. Blood 1991;77:2431-4.

(*285*) Makos M, Nelkin BD, Lerman MI, Latif F, Zbar B, Baylin SB. Distinct hypermethylation patterns occur at altered chromosome loci in human lung and colon cancer. Proc Natl Acad Sci U S A 1992;89:1929-33.

(*286*) Loeb LA. A mutator phenotype in cancer. Cancer Res 2001;61:3230-9.

(*287*) Choi SW, Mason JB. Folate status: effects on pathways of colorectal carcinogenesis. J Nutr 2002;132:2413S-8S.

(*288*) Bestor TH, Tycko B. Creation of genomic methylation patterns. Nat Genet 1996;12:363-7.

(*289*) Gruenbaum Y, Stein R, Cedar H, Razin A. Methylation of CpG sequences in eukaryotic DNA. FEBS Lett 1981;124:67-71.

(*290*) Clark SJ, Harrison J, Frommer M. CpNpG methylation in mammalian cells. Nat Genet 1995;10:20-7.

(*291*) Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003;349:2042-54.

(*292*) Subak-Sharpe H, Burk RR, Crawford LV, Morrison JM, Hay J, Keir HM. An approach to evolutionary relationships of mammalian DNA viruses through the analysis of the pattern of nearest neighbor base sequences. Cold Spring Harbor Symposium 1966;31:737-48.

(*293*) Bird A, Taggart M, Frommer M, Miller OJ, Macleod D. A fraction of the mouse genome that is derived from islands of nonmethylated, CpG-rich DNA. Cell 1985;40:91-9.

(*294*) Antequera F, Bird A. Number of CpG islands and genes in human and mouse. Proc Natl Acad Sci U S A 1993;90:11995-9.

(*295*) Schuler GD, Boguski MS, Stewart EA, Stein LD, Gyapay G, Rice K, et al. A Gene Map of the Human Genome. Science 1996;274:540-6.

(*296*) Southan C. Has the yo-yo stopped? An assessment of human protein-coding gene number. Proteomics 2004;4:1712-26.

(*297*) Yoder JA, Walsh CP, Bestor TH. Cytosine methylation and the ecology of intragenomic parasites. Trends Genet 1997;13:335-40.

(*298*) Trasler JM. Origin and roles of genomic methylation patterns in male germ cells. Semin Cell Dev Biol 1998;9:467-74.

(*299*) Rhee I, Bachman KE, Park BH, Jair KW, Yen RW, Schuebel KE, et al. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature 2002;416:552-6.

(*300*) Bonfils C, Beaulieu N, Chan E, Cotton-Montpetit J, MacLeod AR. Characterization of the human DNA methyltransferase splice variant Dnmt1b. J Biol Chem 2000;275:10754-60.

(*301*) el-Deiry WS, Nelkin BD, Celano P, Yen RW, Falco JP, Hamilton SR, et al. High expression of the DNA methyltransferase gene characterizes human neoplastic cells and progression stages of colon cancer. Proc Natl Acad Sci U S A 1991;88:3470-4.

(*302*) Rountree MR, Bachman KE, Baylin SB. DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. Nat Genet 2000;25:269-77.

(*303*) Robertson KD, Ait-Si-Ali S, Yokochi T, Wade PA, Jones PL, Wolffe AP. DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. Nat Genet 2000;25:338-42.

(*304*) Fang MZ, Wang Y, Ai N, Hou Z, Sun Y, Lu H, et al. Tea polyphenol (-)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. Cancer Res 2003;63:7563-70.

(*305*) Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 1999;99:247-57.

(*306*) Xu GL, Bestor TH, Bourc'his D, Hsieh CL, Tommerup N, Bugge M, et al. Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature 1999;402:187-91.

(*307*) Kornberg A, Baker T. DNA Replication. In Kornberg A, editor. DNA Replication: W.H. Freeman & Company; 1992. p. 831-3.

(*308*) Bender CM, Gonzalgo ML, Gonzales FA, Nguyen CT, Robertson KD, Jones PA. Roles of cell division and gene transcription in the methylation of CpG islands. Mol Cell Biol 1999;19:6690-8.

(*309*) Chuang LS, Ian HI, Koh TW, Ng HH, Xu G, Li BF. Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21WAF1. Science 1997;277:1996-2000.

(*310*) Araujo FD, Knox JD, Szyf M, Price GB, Zannis-Hadjopoulos M. Concurrent replication and methylation at mammalian origins of replication. Mol Cell Biol 1998;18:3475-82.

(*311*) Oyama K, Kawakami K, Maeda K, Ishiguro K, Watanabe G. The association between methylenetetrahydrofolate reductase polymorphism and promoter methylation in proximal colon cancer. Anticancer Res 2004;24:649-54.

(*312*) Kawakami K, Ruszkiewicz A, Bennett G, Moore J, Watanabe G, Iacopetta B. The folate pool in colorectal cancers is associated with DNA hypermethylation and with a polymorphism in methylenetetrahydrofolate reductase. Clin Cancer Res 2003;9:5860-5.

(*313*) Paz MF, Avila S, Fraga MF, Pollan M, Capella G, Peinado MA, et al. Germ-line variants in methyl-group metabolism genes and susceptibility to DNA methylation in normal tissues and human primary tumors. Cancer Res 2002;62:4519-24.

(*314*) Siemianowicz K, Gminski J, Garczorz W, Slabiak N, Goss M, Machalski M, et al. Methylenetetrahydrofolate reductase gene C677T and A1298C polymorphisms in patients with small cell and non-small cell lung cancer. Oncol Rep 2003;10:1341-4.

(*315*) Jatoi A, Daly BD, Kramer G, Mason JB. Folate status among patients with non-small cell lung cancer: a case-control study. J Surg Oncol 2001;77:247-52.

(*316*) Shen H, Spitz MR, Wang LE, Hong WK, Wei Q. Polymorphisms of methylene-tetrahydrofolate reductase and risk of lung cancer: a case-control study. Cancer Epidemiol Biomarkers Prev 2001;10:397-401.

(*317*) Jeng YL, Wu MH, Huang HB, Lin WY, You SL, Chu TY, et al. The methylenetetrahydrofolate reductase 677C-->T polymorphism and lung cancer risk in a Chinese population. Anticancer Res 2003;23:5149-52.

(*318*) Bhattacharya SK, Ramchandani S, Cervoni N, Szyf M. A mammalian protein with specific demethylase activity for mCpG DNA. Nature 1999;397:579-83.

(*319*) Ramchandani S, Bhattacharya SK, Cervoni N, Szyf M. DNA methylation is a reversible biological signal. Proc Natl Acad Sci U S A 1999;96:6107-12.

(*320*) Robert MF, Morin S, Beaulieu N, Gauthier F, Chute IC, Barsalou A, et al. DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells. Nat Genet 2003;33:61-5.

(*321*) Cunha KS, Reguly ML, Graf U, de Andrade HH. Somatic recombination: a major genotoxic effect of two pyrimidine antimetabolitic chemotherapeutic drugs in Drosophila melanogaster. Mutat Res 2002;514:95-103.

(*322*) Slack A, Bovenzi V, Bigey P, Ivanov MA, Ramchandani S, Bhattacharya S, et al. Antisense MBD2 gene therapy inhibits tumorigenesis. J Gene Med 2002;4:381-9.

(*323*) Detich N, Hamm S, Just G, Knox JD, Szyf M. The methyl donor S-Adenosylmethionine inhibits active demethylation of DNA: a candidate novel mechanism for the pharmacological effects of S-Adenosylmethionine. J Biol Chem 2003;278:20812-20.

(*324*) Rhodes K, Rippe RA, Umezawa A, Nehls M, Brenner DA, Breindl M. DNA methylation represses the murine alpha 1(I) collagen promoter by an indirect mechanism. Mol Cell Biol 1994;14:5950-60.

(*325*) Ramchandani S, MacLeod AR, Pinard M, von Hofe E, Szyf M. Inhibition of tumorigenesis by a cytosine-DNA, methyltransferase, antisense oligodeoxynucleotide. Proc Natl Acad Sci U S A 1997;94:684-9.

(*326*) Li E, Bestor TH, Jaenisch R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 1992;69:915-26.

(*327*) Feinberg AP. Methylation meets genomics. Nat Genet 2001;27:9-10.

(*328*) Marhold J, Rothe N, Pauli A, Mund C, Kuehle K, Brueckner B, et al. Conservation of DNA methylation in dipteran insects. Insect Mol Biol 2004;13:117-23.

(*329*) Urieli-Shoval S, Gruenbaum Y, Sedat J, Razin A. The absence of detectable methylated bases in Drosophila melanogaster DNA. FEBS Lett 1982;146:148-52.

(*330*) Lyko F, Ramsahoye BH, Jaenisch R. DNA methylation in Drosophila melanogaster. Nature 2000;408:538-40.

(*331*) Bestor TH. DNA methylation: evolution of a bacterial immune function into a regulator of gene expression and genome structure in higher eukaryotes. Philos Trans R Soc Lond B Biol Sci 1990;326:179-87.

(*332*) Reik W, Walter J. Genomic imprinting: parental influence on the genome. Nat Rev Genet 2001;2:21-32.

(*333*) Vasques LR, Klockner MN, Pereira LV. X chromosome inactivation: how human are mice? Cytogenet Genome Res 2002;99:30-5.

(*334*) Di Croce L, Raker VA, Corsaro M, Fazi F, Fanelli M, Faretta M, et al. Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. Science 2002;295:1079-82.

(*335*) Marx J. CANCER RESEARCH: Leukemia Protein Spurs Gene Silencing. Science 2002;295:943b-5.

(*336*) Cooper DN, Krawczak M. Cytosine methylation and the fate of CpG dinucleotides in vertebrate genomes. Hum Genet 1989;83:181-8.

(*337*) Esteller M, Risques RA, Toyota M, Capella G, Moreno V, Peinado MA, et al. Promoter hypermethylation of the DNA repair gene O(6)-methylguanine-DNA methyltransferase is associated with the presence of G:C to A:T transition mutations in p53 in human colorectal tumorigenesis. Cancer Res 2001;61:4689-92.

(*338*) Ahuja N, Mohan AL, Li Q, Stolker JM, Herman JG, Hamilton SR, et al. Association between CpG island methylation and microsatellite instability in colorectal cancer. Cancer Res 1997;57:3370-4.

(*339*) Liu CQ, Wang Y, Huang JF, Zhang H. A quantum biological approach to the relations of DNA methylation with gene transcription and mutation. J Theor Biol 1991;148:145-55.

(*340*) Lee YW, Broday L, Costa M. Effects of nickel on DNA methyltransferase activity and genomic DNA methylation levels. Mutat Res 1998;415:213-8.

(*341*) Lee YW, Klein CB, Kargacin B, Salnikow K, Kitahara J, Dowjat K, et al. Carcinogenic nickel silences gene expression by chromatin condensation and DNA methylation: a new model for epigenetic carcinogens. Mol Cell Biol 1995;15:2547-57.

(*342*) Hsieh CL. Dependence of transcriptional repression on CpG methylation density. Mol Cell Biol 1994;14:5487-94.

(*343*) Boyes J, Bird A. Repression of genes by DNA methylation depends on CpG density and promoter strength: evidence for involvement of a methyl-CpG binding protein. Embo J 1992;11:327-33.

(*344*) Koizume S, Sekiya T, Shiraishi M. Specific methylation status of the entire CpG island is not a prerequisite for the formation of an inactive chromatin at the promoter region in cancer cells. Biol Pharm Bull 2003;26:127-8.

(*345*) Antequera F, Boyes J, Bird A. High levels of de novo methylation and altered chromatin structure at CpG islands in cell lines. Cell 1990;62:503-14.

(*346*) Boyes J, Bird A. DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. Cell 1991;64:1123-34.

(*347*) Lewis JD, Meehan RR, Henzel WJ, Maurer-Fogy I, Jeppesen P, Klein F, et al. Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA. Cell 1992;69:905-14.

(*348*) Nan X, Meehan RR, Bird A. Dissection of the methyl-CpG binding domain from the chromosomal protein MeCP2. Nucleic Acids Res 1993;21:4886-92.

(*349*) Amir RE, Van den Veyver IB, Wan M, Tran CQ, Francke U, Zoghbi HY. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. Nat Genet 1999;23:185-8.

(*350*) Hayatsu H, Wataya Y, Kai K, Iida S. Reaction of sodium bisulfite with uracil, cytosine, and their derivatives. Biochemistry 1970;9:2858-65.

(*351*) Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, et al. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci U S A 1992;89:1827-31.

(*352*) Clark SJ, Harrison J, Paul CL, Frommer M. High sensitivity mapping of methylated cytosines. Nucleic Acids Res 1994;22:2990-7.

(*353*) Feil R, Charlton J, Bird AP, Walter J, Reik W. Methylation analysis on individual chromosomes: improved protocol for bisulphite genomic sequencing. Nucleic Acids Res 1994;22:695-6.

(*354*) Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 1996;93:9821-6.

(*355*) Wang RY, Gehrke CW, Ehrlich M. Comparison of bisulfite modification of 5-methyldeoxycytidine and deoxycytidine residues. Nucleic Acids Res 1980;8:4777-90.

(*356*) Oakeley EJ. DNA methylation analysis: a review of current methodologies. Pharmacol Ther 1999;84:389-400.

(*357*) Gonzalgo ML, Jones PA. Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE). Nucleic Acids Res 1997;25:2529-31.

(*358*) Smiraglia DJ, Fruhwald MC, Costello JF, McCormick SP, Dai Z, Peltomaki P, et al. A new tool for the rapid cloning of amplified and hypermethylated human DNA sequences from restriction landmark genome scanning gels. Genomics 1999;58:254-62.

(*359*) Eads CA, Danenberg KD, Kawakami K, Saltz LB, Blake C, Shibata D, et al. MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res 2000;28:E32.

(*360*) Gitan RS, Shi H, Chen CM, Yan PS, Huang TH. Methylation-specific oligonucleotide microarray: a new potential for high-throughput methylation analysis. Genome Res 2002;12:158-64.

(*361*) Frigola J, Ribas M, Risques RA, Peinado MA. Methylome profiling of cancer cells by amplification of inter-methylated sites (AIMS). Nucleic Acids Res 2002;30:e28.

(*362*) Falette NS, Fuqua SA, Chamness GC, Cheah MS, Greene GL, McGuire WL. Estrogen receptor gene methylation in human breast tumors. Cancer Res 1990;50:3974-8.

(*363*) Ferguson AT, Lapidus RG, Baylin SB, Davidson NE. Demethylation of the estrogen receptor gene in estrogen receptor-negative breast cancer cells can reactivate estrogen receptor gene expression. Cancer Res 1995;55:2279-83.

(*364*) Royer-Pokora B, Schneider S. Wilms' tumor-specific methylation pattern in 11p13 detected by PFGE. Genes Chromosomes Cancer 1992;5:132-40.

(*365*) Ohtani-Fujita N, Fujita T, Aoike A, Osifchin NE, Robbins PD, Sakai T. CpG methylation inactivates the promoter activity of the human retinoblastoma tumor-suppressor gene. Oncogene 1993;8:1063-7.

(*366*) Stirzaker C, Millar DS, Paul CL, Warnecke PM, Harrison J, Vincent PC, et al. Extensive DNA methylation spanning the Rb promoter in retinoblastoma tumors. Cancer Res 1997;57:2229-37.

(*367*) Herman JG, Latif F, Weng Y, Lerman MI, Zbar B, Liu S, et al. Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. Proc Natl Acad Sci U S A 1994;91:9700-4.

(*368*) Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, et al. 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nat Med 1995;1:686-92.

(*369*) Otterson GA, Khleif SN, Chen W, Coxon AB, Kaye FJ. CDKN2 gene silencing in lung cancer by DNA hypermethylation and kinetics of p16INK4 protein induction by 5-aza 2'deoxycytidine. Oncogene 1995;11:1211-6.

(*370*) Graff JR, Herman JG, Lapidus RG, Chopra H, Xu R, Jarrard DF, et al. E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. Cancer Res 1995;55:5195-9.

(*371*) Ribeiro-Filho LA, Franks J, Sasaki M, Shiina H, Li LC, Nojima D, et al. CpG hypermethylation of promoter region and inactivation of E-cadherin gene in human bladder cancer. Mol Carcinog 2002;34:187-98.

(*372*) Herman JG, Jen J, Merlo A, Baylin SB. Hypermethylation-associated inactivation indicates a tumor suppressor role for p15INK4B. Cancer Res 1996;56:722-7.

(*373*) Cameron EE, Baylin SB, Herman JG. p15(INK4B) CpG island methylation in primary acute leukemia is heterogeneous and suggests density as a critical factor for transcriptional silencing. Blood 1999;94:2445-51.

(*374*) Sakai T, Toguchida J, Ohtani N, Yandell DW, Rapaport JM, Dryja TP. Allele-specific hypermethylation of the retinoblastoma tumor-suppressor gene. Am J Hum Genet 1991;48:880-8.

(*375*) Rice JC, Massey-Brown KS, Futscher BW. Aberrant methylation of the BRCA1 CpG island promoter is associated with decreased BRCA1 mRNA in sporadic breast cancer cells. Oncogene 1998;17:1807-12.

(*376*) Fleisher AS, Esteller M, Wang S, Tamura G, Suzuki H, Yin J, et al. Hypermethylation of the hMLH1 gene promoter in human gastric cancers with microsatellite instability. Cancer Res 1999;59:1090-5.

(*377*) Banelli B, Casciano I, Romani M. Methylation-independent silencing of the p73 gene in neuroblastoma. Oncogene 2000;19:4553-6.

(*378*) Jarrard DF, Kinoshita H, Shi Y, Sandefur C, Hoff D, Meisner LF, et al. Methylation of the androgen receptor promoter CpG island is associated with loss of androgen receptor expression in prostate cancer cells. Cancer Res 1998;58:5310-4.

(*379*) Takai D, Yagi Y, Wakazono K, Ohishi N, Morita Y, Sugimura T, et al. Silencing of HTR1B and reduced expression of EDN1 in human lung cancers, revealed by methylation-sensitive representational difference analysis. Oncogene 2001;20:7505-13.

(*380*) Li B, Goyal J, Dhar S, Dimri G, Evron E, Sukumar S, et al. CpG methylation as a basis for breast tumor-specific loss of NES1/kallikrein 10 expression. Cancer Res 2001;61:8014-21.

(*381*) Dammann R, Yang G, Pfeifer GP. Hypermethylation of the cpG island of Ras association domain family 1A (RASSF1A), a putative tumor suppressor gene from the 3p21.3 locus, occurs in a large percentage of human breast cancers. Cancer Res 2001;61:3105-9.

(*382*) Tan LW, Bianco T, Dobrovic A. Variable promoter region CpG island methylation of the putative tumor suppressor gene Connexin 26 in breast cancer. Carcinogenesis 2002;23:231-6.

(*383*) Kikuchi T, Toyota M, Itoh F, Suzuki H, Obata T, Yamamoto H, et al. Inactivation of p57KIP2 by regional promoter hypermethylation and histone deacetylation in human tumors. Oncogene 2002;21:2741-9.

(*384*) Xu XC, Sozzi G, Lee JS, Lee JJ, Pastorino U, Pilotti S, et al. Suppression of retinoic acid receptor beta in non-small-cell lung cancer in vivo: implications for lung cancer development. J Natl Cancer Inst 1997;89:624-9.

(*385*) Ayoub J, Jean-Francois R, Cormier Y, Meyer D, Ying Y, Major P, et al. Placebo-controlled trial of 13-cis-retinoic acid activity on retinoic acid receptor-beta expression in a population at high risk: implications for chemoprevention of lung cancer. J Clin Oncol 1999;17:3546-52.

(*386*) Lee JS, Lippman SM, Benner SE, Lee JJ, Ro JY, Lukeman JM, et al. Randomized placebo-controlled trial of isotretinoin in chemoprevention of bronchial squamous metaplasia. J Clin Oncol 1994;12:937-45.

(*387*) McLarty JW, Holiday DB, Girard WM, Yanagihara RH, Kummet TD, Greenberg SD. Beta-Carotene, vitamin A, and lung cancer chemoprevention: results of an intermediate endpoint study. Am J Clin Nutr 1995;62:1431S-8S.

(*388*) Hennekens CH, Buring JE, Manson JE, Stampfer M, Rosner B, Cook NR, et al. Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease. N Engl J Med 1996;334:1145-9.

(*389*) Kurie JM, Lee JS, Khuri FR, Mao L, Morice RC, Lee JJ, et al. N-(4-hydroxyphenyl)retinamide in the chemoprevention of squamous metaplasia and dysplasia of the bronchial epithelium. Clin Cancer Res 2000;6:2973-9.

(*390*) Hirsch FR, Franklin WA, Gazdar AF, Bunn PA, Jr. Early detection of lung cancer: clinical perspectives of recent advances in biology and radiology. Clin Cancer Res 2001;7:5-22.

(*391*) Khuri FR, Lotan R, Kemp BL, Lippman SM, Wu H, Feng L, et al. Retinoic acid receptor-beta as a prognostic indicator in stage I non-small-cell lung cancer. J Clin Oncol 2000;18:2798-804.

(*392*) Haber D, Harlow E. Tumour-suppressor genes: evolving definitions in the genomic age. Nat Genet 1997;16:320-2.

(*393*) Cote S, Sinnett D, Momparler RL. Demethylation by 5-aza-2'-deoxycytidine of specific 5-methylcytosine sites in the promoter region of the retinoic acid receptor beta gene in human colon carcinoma cells. Anticancer Drugs 1998;9:743-50.

(*394*) Bovenzi V, Le NL, Cote S, Sinnett D, Momparler LF, Momparler RL. DNA methylation of retinoic acid receptor beta in breast cancer and possible therapeutic role of 5-aza-2'-deoxycytidine. Anticancer Drugs 1999;10:471-6.

(*395*) Bovenzi V, Momparler RL. Quantitation of inhibition of DNA methylation of the retinoic acid receptor beta gene by 5-Aza-2'-deoxycytidine in tumor cells using a single-nucleotide primer extension assay. Anal Biochem 2000;281:55-61.

(*396*) Virmani AK, Rathi A, Zochbauer-Muller S, Sacchi N, Fukuyama Y,. Bryant D, et al. Promoter methylation and silencing of the retinoic acid receptor-beta gene in lung carcinomas. J Natl Cancer Inst 2000;92:1303-7.

(*397*) Gazdar AF, Zochbauer-Moller S, Virmani A, Kurie J, Minna JD, Lam S. RESPONSE: Re: Promoter Methylation and Silencing of the Retinoic Acid Receptor-beta Gene in Lung Carcinomas. J Natl Cancer Inst 2001;93:67-8.

(*398*) Lamy A, Metayer J, Thiberville L, Frebourg T, Sesboue R. Re: Promoter methylation and silencing of the retinoic acid receptor-beta gene in lung carcinomas. J Natl Cancer Inst 2001;93:66-8.

(*399*) Pappas JJ, Hebert J, Fetni R, Bradley WEC. Divergent methylation of the RARbeta P2 promoter and methylation allelic bias in cancer. (To be submitted).

CHAPTER 2

MANUSCRIPT 1

Divergent methylation of the RARβ2 promoter and methylation allelic bias in cancer

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Abbreviations: ATRA = *all trans* retinoic acid; **BGS** = bisulfite genomic sequencing; **CpG** = cytosine-guanine dinucleotide; **FISH** = fluorescence *in situ* hybridization; **MSP** = Methylation-specific PCR; **LOH** = loss of heterozygosity; **NSCLC** = non-small cell lung carcinoma; **RA** = retinoic acid; **RAR** = retinoic acid receptor; **RARβ2** = RAR isoform β2; **RARE** = retinoic acid-responsive element; **ROH** = retention of heterozygosity; **RXR** = retinoid X receptor; **SCLC** = small cell lung carcinoma; **SKY** = spectral karyotyping; **THRβ2** = thyroid hormone receptor isoform β2; TSG = Tumor suppressor gene.

Abstract

INTRODUCTION: The putative tumor suppressor gene RARβ2 is frequently inactivated in cancer, and methylation, which has been reported in the 5' untranslated region (5'UTR) and the first exon, is known to play a key role. Our objectives were: (1) to determine if the level of methylation upstream of the transcription start site (within the promoter region) reflects that reported in the 5'UTR and first exon; (2) to elucidate the methylation status of each of the individual CpG sites within or proximal to the hormone response elements or transcription sites; (3) to determine whether the different patterns of methylation within a given cell line reflect intracellular allelic variations and if so to study their heritability (mitotic transmission); (4) to determine whether methylation is subject to an allelic bias; and (5) to determine if such a bias is correlated with transcription asynchrony. **METHODS**: We used bisulfite genomic sequencing (BGS), a protocol allowing high resolution analysis, to analyse the methylation status of each of the CpG sites (n=22) within a 0.5 kb region upstream of the start site in 20 cancer cell lines (9 lung, 6 breast, and 5 colon) and 1 normal lung cell line. We also analyzed these sites in 5 subcloned cancer cell lines to determine whether these methylation patterns are heritable and hence stem from intracellular/allelic variations as opposed to population dynamics. We assessed retention of heterozygosity (ROH) at four proximal loci [the thyroid hormone receptor β2 (THRβ2) locus, the polymorphic marker sites D3S1583 and D3S2335, and the rs755661 single nucleotide polymorphism (SNP)], to determine whether the co-existence of hypo- and hypermethylation statuses correlates with heterozygosity at 3p24, hence reflecting methylation allelic bias. We then used the rs755661 polymorphism as a marker of allelic identity following methylation-sensitive restriction enzyme digestion to determine whether methylation is subject to an allelic preference. Finally, we used fluorescence *in situ* hybridization (FISH) to label the allelic copies of chromosome 3 in replication in order to assay the proportion of nuclei that replicate synchronously versus asynchronously. **RESULTS**: We found that the level of promoter methylation is correlated with RARβ2 inactivation in most cell lines (14/16, 88%) reflecting the correlation previously reported regarding the 5'UTR and first exon, and that methylation in and around the RARE and TATA box may be of importance, especially in lung cancer, confirming previous results. However, we also found that hypo- and hypermethylated alleles frequently coexist in lines in which RARβ2 is inactivated (5/11 or 45%), and such divergent patterns are conserved in the majority of subclones analyzed (6/8 or 75%), supporting mitotic transmission. A correlation between divergence of methylation and ROH at THRβ2 and D3S1583 was found for 2/5 cell lines (CALU-1 and LS-180), supporting the hypothesis of methylation allelic bias in these lines. In addition, we found that alleles are indeed subject to an allelic bias in one cancer cell line (LS-180) and two breast cancer biopsy samples: the T allele was more frequently methylated than the C allele by a ratio of at least 2:1. Methylation divergence is not correlated with replication

asynchrony in the LS-180 cell line, but, interestingly, both alleles of RARβ2 are replicated late in the great majority of nuclei analysed, as opposed to the RARα and ETO control genes, which are replicated in early to middle S phase. **CONCLUSIONS**: This work is the first to report the co-existence and heritability of hypo- and hypermethylated allelic copies of a gene completely inactivated in cancer; that hypermethylation in cancer is subject to allelic bias; and that a promoter methylation-independent *cis*-acting silencing mechanism is involved in RARβ2 inactivation. Methylation divergence does not appear to be correlated with replication asynchrony. Methylation divergence represents a novel finding which may have significant impact in the field of cancer research and therapy.

1. Introduction

The process of carcinogenesis involves the inactivation of a number of tumor suppressor genes (TSGs). Due to their recessive nature, TSGs are generally thought to require inactivation of both alleles, in accordance with the classical "two-hit" hypothesis (*1*). Alterations in DNA sequence, including mutations and long deletions that cause loss of heterozygosity (LOH), have frequently been documented as events responsible for inactivation (*2*), and the relative importance of epigenetic modifications in silencing these genes has become appreciated (*3*). These changes involve the methylation of C residues in CpG dinucleotide pairs within CG-rich promoter sequences (within CpG islands), followed by the binding of methyl CpG binding proteins (MeCPs) and the recruitment of histone deacetylases (HDACs). This results in the condensing of the local chromatin structure such that transcription does not occur.

RARβ2 is one of the nuclear receptors responsible for mediating the tumor suppressive effects of retinoic acid (RA) in epithelial cells (*4*). RARβ2 has been shown to be inactivated and to have tumor suppressive properties in numerous tumor-derived cell lines, including those derived from lung, breast and colon cancers (*5-12*). The gene coding RARβ2 is located at 3p24 and is one of two isoforms (β2 and β4) generated from one (P2) of two promoters, P1 and P2. Though large deletions of the region of chromosome 3p carrying RARβ are known to occur in cancer, no mutations affecting the remaining allele and thereby resulting in a "2nd hit," have been found within the coding sequence of the gene. However, using methylation-specific PCR (MSP), several groups have shown that methylation in the 5' untranslated region (5'UTR) and first exon coding region A of the RARβ2 protein is correlated with the gene's inactivation (*13-18*). Treatment with 5-azadeoxycytidine, a methyltransferase inhibitor, simultaneously caused demethylation of the exonic sequences and reactivation of gene expression (*13,15,18,19*). In addition, one report describes that upon direct sequencing of cloned amplified fragments after bisulfite conversion, methylation of two of three tandem CpGs in the proximal promoter correlated with gene inactivation in the MCF-7 breast cancer cell line (*20*), and another describes that all three sites may play an important role in inactivating RARβ2 in prostate cancer cells (*21*). More recent research investigating the incidence of methylation of various genes in cancer has shown that RARβ2 is one of the genes most frequently methylated in several cancers, including lung (*22*),

esophageal (*23*), breast (*24*), cervical (*25*), ovarian (*26*) and urinary cancer (*27*), mainly using MSP as well. It is not yet clear whether particular patterns as opposed to general levels of methylation are essential for inactivation, but methylation over very short stretches of DNA may be particularly important in gene inactivation (*20,28*).

The majority of studies have used MSP and have focused on the 5'UTR and the first exon, downstream of the start site and hence external to the promoter.

We were interested in determining if the region upstream of the start site (i.e. within the promoter region), as opposed to downstream of the start site, is affected by DNA methylation. We studied a 0.5 kb region upstream of the transcription start site in 20 cancer-derived cell lines (10 lung, 4 breast and 7 colon) and 1 normal cell line (lung) as part of a comparative study. However, since we wanted to analyze the methylation status of each of the 22 CpG sites within this target promoter region (in order to elucidate the pattern of methylation), we used the bisulfite genomic sequencing (BGS) protocol, a method that allows high resolution analysis, rather than MSP. Importantly, we analyzed multiple separately BGS-treated samples in order to decrease the probability that the same sequence was analyzed twice due to PCR artefacts, and to increase the probability that all alleles present were reflected.

We found that hypo- and hypermethylated alleles coexist in nearly half (5/11 or 45%) of the lines in which RARβ2 is inactivated. We then analyzed 8 subclones of 3 of these lines to determine whether the results found in parental cell lines were similar in subcloned cell lines and therefore supported the hypothesis that these divergent patterns are intracellular and heritable. We found that divergence is present in the majority of subclones analyzed (6/8 or 75%). We therefore use the term "methylation divergence" to represent the co-existence or simultaneous occurrence of hypo- and hypermethylated allelic gene copies within a same cell. We then proceeded to determine whether these divergent methylation patterns are attributable to an allelic bias for methylation. A database search showed that one high frequency C/T single nucleotide polymorphism (SNP), rs755661, exists near the RARβ promoter. We designed an experiment based on the ability of this SNP to be used as a diagnostic marker of allelic identity following methylation-sensitive restriction enzyme digestion. LS-180, a colorectal adenocarcinoma cell line, was both divergently methylated and heterozygous at this SNP site, and hence amenable to this study. No other more

proximal SNPs known at the time of this study were amenable to such an analysis. We found that the T allele is more frequently methylated than the C allele at nearly all CpGs analysed by a ratio of at least 2:1. Finally, we asked whether there is a correlation between divergent methylation and asynchronous replication of the RARβ locus in the LS180 line, as opposed to two other loci (RARα and ETO) using fluorescence *in situ* hybridization (FISH). Methylation divergence was not correlated with replication asynchrony in this cell line, but, interestingly, both alleles of RARβ2 are replicated late in the great majority of nuclei, whereas RARα and ETO are replicated in early to middle S phase.

2. Materials and Methods

2.1 Cell culture

CALU-1, CACO-2, SW-1222 and LS-180 were grown in α-MEM medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS, Wisent Inc.). SK-MES, NCI-H23, NCI-H125, NCI-H520, Qu-DB and HS-578T were grown in RPMI-1640 medium (Invitrogen) supplemented with 5% heat-inactivated FCS. NCI-H82, NCI-H157, MM-1, T47D, MDA-MB-231 (MB-231), ZR-75B, COLO-201, COLO-205 and HCT-15 were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FCS. NBE-E_6E_7 was grown in Keratinocyte-Serum Free medium (Invitrogen), supplemented with 50 ug/ml bovine pituitary extract and 5 ng/ml recombinant human epidermal growth factor (Invitrogen). Where indicated, cells were treated with freshly-prepared all-*trans* retinoic acid (ATRA; Sigma) dissolved in dimethyl sulfoxide (DMSO).

2.2 Subcloning

Subclones of the CALU-1 cell line, C19, C30, C59 and C64, were isolated after transfection of the neomycin-resistance gene and selection in G418 (*4*). The lines LS-180 and MB-231 were subcloned by limiting dilution of single-cell suspensions into several 96-well cloning trays such that the majority of wells yielded zero colonies. To assure that colonies arose from single cells rather than the fortuitous proximity of two cells, wells were microscopically inspected every 2 days starting at 9 days after seeding, at which point most single colonies had 150-250 cells. Colonies of more

than 500 cells (representing about 10% of those in single-colony wells) were considered as possibly arising from 2 cells (not clonal), and hence were rejected.

2.3 RARβ2 mRNA analyses

Reverse transcription was performed on total RNA using the Moloney Murine Leukemia Virus (MMLV) reverse transcriptase as per protocol (Invitrogen, Carlsbad, California).

A 514 bp exon-specific region of RARβ2 was amplified using standard PCR reaction conditions and the upper oligo 5'-GGGTAGGATCCGGAACGCATT-3' and the lower oligo 5'-GACGAGTTCCTCAGAGCTGG-3'. Cycling conditions were: 94°C/4 min x 1 cycle; 94°C/1 min, 50°C/1 min, 70°C/1 min x 30 cycles; 72°C/5 min x 1 cycle. RARα2, which is constitutively expressed, was amplified using standard PCR reaction conditions and the exon-specific upper oligo 5'-GAGGACTTGTCCTGACAGA-3' and lower oligo 5'-CTTCTGACTGTGGCCGCT-3'. Cycling conditions were: 94°C/4 min x 1 cycle; 94°C/1 min, 45°C/1 min, 70°C/1 min x 5 cycles; 94°C/1 min, 48°C/1 min, 70°C/1 min x 30 cycles; 72°C/5 min x 1 cycle. The optimal number of cycles for RARβ and α was determined after standard curves were established.

2.4 Bisulphite genomic sequencing (BGS)

The target sequence within the promoter is 541 bp and contains 22 CpG dinucleotides as published by Shen *et al.* (*29*). Multiple DNA samples were treated independently with bisulfite as per the BGS protocol (*30*) with some minor modifications, and desalinated using Wizard Magic Miniprep DNA Purification Resins (Promega) or sephaglas (Invitrogen). The PCR amplification process consisted of two rounds of amplification (the second round being a semi-nested reaction). Positions (below) refer to the promoter sequence as published by Shen and colleagues (*29*). Round 1 oligos consisted of the upper oligo 5'-GGAGTGGAAAAATATATAAGTTATAAGGAA-3', RARβ position 381, and the lower oligo 5'-AAAAAAATCCACCCAACTCCATCAAACTCT-3', RARβ position 1250. Round 2 oligos consisted of the same upper oligo as round 1 and the lower oligo 5'-AAAATTCTAATCCCCCCT-TTAACAAAAAAT-3', RARβ position 920. Cycling conditions were: 94°C/4 min x 1 cycle; 94°C/1min, 61°C/2 min, 72°C/2 min x 5

cycles; 94°C/1 min, 61°C/1.5 min, 72°C/1.5-2 min x 25 cycles; 72°C/5 min x 1 cycle. PCR products were gel extracted, ligated to the pBS SK+ or the pCR2.1 vector, transformed into competent DH5α *E. coli* cells (Invitrogen, Carlsbad, California), and plasmid DNA was then sequenced using universal T3 and/or T7 primers.

2.5 ROH at THRβ2 analyses

The upper oligo 5'-TCATTCGAGTTAGTGCAAAG-3' and the lower oligo 5'-ACGTTAGTGGCTCATATGAG-3' were used to amplify a 432 bp region in the thyroid hormone receptor β2 (THRβ2) locus at 3p24, using standard PCR reaction conditions. Cycling conditions were: 94°C/4 min x 1 cycle; 94°C/1 min, 46°C/1 min, 70°C/1 min x 30 cycles; 72°C/5 min x 1 cycle. The product was subsequently digested with an excess of Hind III (New England Biolabs, Ontario, Canada) and analysed on a 1.5% agarose gel for the BH302 restriction fragment length polymorphism (*31*).

2.6 ROH at D3S1583 and D3S2335 analyses

The upper oligo 5'-AGCTTGTAAATAGGTCCTAACAGAG-3' and the lower oligo 5'-TGGTTTAATAGGCACCGTTT-3' were used to amplify a 149-173 bp region in 3p24 for D3S1583 (GenBank Accession no. Z23961). The upper oligo 5'-GCTGAATGCTTCTGAATGTAT-3' and the lower oligo 5'-AAGAGATGGGGTGCTTT-3' were used to amplify a 146-168 bp region in at 3p24 for D3S2335 (GenBank Accession no. Z51343). Standard reaction conditions were used except that the dCTP solutions consisted of 1:100 dilutions of radiolabeled dCTP:cold dCTP. Cycling conditions were: 94°C/4 min x 1 cycle; 94°C/1min, 55°C/1 min, 70°C/1 min x 35 cycles; 72°C/5 min x 1 cycle. Products were visually analysed on 15% polyacrilamide gels.

2.7 Western blot analyses of DNMT protein levels

DNMT 1, 2, 3A and 3B protein expression levels were analysed in the 5 divergently methylated cell lines (CALU-1, LS-180, MDA-MB-231, Hs-578T and A-549). Cells were plated at 1 x 10^6 cells per 10 cm dish and treated the following day with or without 1 uM ATRA for 24 hrs. Cells were then rinsed twice with ice-cold PBS and subsequently lysed using ice-cold lysis buffer (20mM Tris-HCl pH 7.5, 150 mM NaCl,

1 mM EGTA, 1 mM EDTA, 1% Triton-X-100 and 0.1% SDS) to which the following agents were freshly added: 1 ug/ml leupeptin; 1 ug/ml pepstatin; 1 mM PMSF; 1.5 ug/ml aprotinin; 1 mM NaF; 1 mM NaV. Protein concentrations were measured using the Bradford reagent method. Approximately 40 ug of protein were loaded on 8% acrylamide SDS-PAGE gels and transferred to nitrocellulose membranes (Hybond, Amersham Biosciences, Baie d'Urfe, QC, Canada). Uniform loading was confirmed using the Ponceau Red staining method. Membranes were then incubated with antibodies against DNMT-1, 2, 3A or 3B proteins (Santa Cruz Biotechnologies, CA, USA), using a 1/500 dilutions in hybridization buffer, overnight. The membranes were washed and subsequently incubated using a bovine anti-goat IgG-HRP conjugate antibody (Santa Cruz Biotechnologies, CA, USA) for 1.5 hrs. Proteins were detected using the ECL chemiluminescence method (Amersham Biosciences, Baie d'Urfe, QC, Canada).

2.8 Methylation allelic bias assays

2.8.1 ROH at rs755661 analyses

First, 200 ng of genomic DNA was used to amplify a 408 bp product with Taq polymerase (Amersham, Baie d'Urfe, QC, Canada), using the upper oligo 5'-TGTTGGAATCCCAGGGAACT-3' and the lower oligo 5'-TTTCAGAAATTCCAGTGTC-3' (oligos N and O, respectively; Fig. 22), and the supplier's recommended reaction conditions. Cycling conditions were: 94°C/4 min x 1 cycle; 94°C/1 min, 50°C/1 min, 70°C/1 min x 30 cycles; 72°C/5 min x 1 cycle.

Subsequently, a volume of 10 ul (1/5 vol.) of the PCR product was digested with 5 units of Mnl I at 37°C using the supplier's suggested conditions till digestion was complete (a minimum of 2 hours), and digestion products were analyzed visually using agarose gel electrophoresis. Mnl I differential digestion yields either two fragments, 113 and 295 bp, for the allele carrying the T nucleotide within the polymorphism, or three fragments, 67, 113 and 228 bp, for the allele carrying the C polymorphism (Fig. 22).

In order to determine whether the tested phenomenon was also present in breast tumor samples, DNA samples from 18 available breast cancer biopsies (T1-T18) kindly provided to us by D^r. Mark Basik (*32*) were tested in the same manner (Table XIX).

2.8.2 Methylation-sensitive restriction enzyme digestion

The DNA samples that tested positive for ROH at the rs755661 SNP, corresponding to LS-180 and T1-T10, were digested with one of three methylation-sensitive restriction enzymes: (1) Nae I, whose recognition site is 5'-GCCGGC; (2) Hha, whose recognition site is 5'-GCGC-3'; or (3) Hpa II, whose recognition site is 5'-CCGG (all restriction enzymes were purchased at New England Biolabs, Ontario, Canada). Nae I tested CpG site #17, Hha I tested site #18, and Hpa II tested CpG site #20, which are all located within the promoter (see Fig.'s 17 and 22, top). In addition, Hha I tested one other site downstream from the transcription start site, and Hpa II tested four other sites downstream from the transcription start site (Hp1 to Hp4, and Hh, respectively; Fig. 22).

Briefly, 1 ug of DNA was digested with 20 units of methylation-sensitive restriction enzyme at 37°C using the supplier's recommended reaction conditions overnight. To assure that digestion was complete, only new and tested enzyme activities were used, the digestions were allowed to proceed overnight, and a 4-fold excess of activity (10-20 U/ug DNA) was used. Test digestions conducted previously showed that at 1 U/ug DNA, >60 minutes is a sufficient period of time, but <5 minutes is not (data not shown).

2.8.3 CpG sites downstream from the promoter; Hpa II and Hha I sites

The rs755661 SNP (and Mnl I site therein) is more than 2.2 kb downstream from the promoter-associated CpG island. The target region analyzed, which is is 2,896 bp long, contains: (1) three CpG methylation site(s) within the promoter region under analysis (CpG #18, 19 or 20); (2) five non-island CpGs (four Hpa II and one Hha I site) ; and (3) the SNP/Mnl I site (Fig. 22). These four Hpa II sites and one Hha I site (Hp1, Hp2, Hp3, Hp4, Hh; Fig. 16), are bulk/non-CpG island CpGs, hence no methylation bias should exist at these sites (*33,34*) (also described in Results, section 3.8).

2.8.3.1 PCR round 1 amplifications

2.8.3.1.1 Four Hpa II sites downstream from the promoter

We began by amplifying DNA digested or not with Hpa II at 37°C overnight (see section 2.8.2, above), using the appropriate primer pairs (D/P, G/P, I/P, K/P, for Hp1-Hp4, respectively, and L/O for Hh; Fig. 22 and Table XX). This allowed us to determine whether or not methylation allelic bias was indeed absent at these non-promoter sites. The four sets of oligos were: 5'-AAGTAGTAGGAAGTGAGCTGTTCAG-3' (D) and 5'-ACTCTGCAAGGGCACAAGCAATATT-3' (P) for Hp1-Hp4 (2,637 bp); 5'-GGCTTTTTGCAAGCATTTACTTGGA-3' (G) and (P) for Hp2-Hp4 (2,468 bp), 5'-TGCTAGAAAACTACTGGCAATGAAC-3' (I) and (P) for Hp3-Hp4 (1,368 bp), OR 5'-CAGGATTTAAATTGCCCTCTCTGAA-3' (K) and (P) for Hp4 alone (873 bp). The reaction conditions (not standard) were the following: 100 ng Hpa II-digested or control non-digested DNA, 300 nM each oligo, 0.4 mM dNTP mix, 1x Buffer 2, and 1.75 U enzyme activity/100 ng DNA, Expand Long Template PCR System (Roche, Ontario, Canada), in a total reaction volume of 50 ul. Cycling conditions were: 94°C/2 min x 1 cycle; 94°C/10 sec, 60°C/30 sec, 70°C/4 min x 10 cycles; 94°C/15 sec, 60°C/30 sec, 70°C/4 min, 5 sec + 5 sec/cycle x 15 cycles; 70°C/7 min x 1 cycle.

These products were then used as templates for fully-nested PCR amplifications (see section 2.8.3.2).

2.8.3.1.2 One Hha I site downstream from the promoter

For Hha I-digested DNA (see section 2.8.2, above), we used the upper and lower oligos 5'-TAAGAATAAAGACTTGCTTCTTG-3' (L) and 5'-AGACACTGGAATTTCTGAAA-3' (O), respectively, for Hh (719 bp) (Fig. 22 and Table XX). These products were immediately digested with Mnl I (see section 2.8.4) without the need for nested (round 2) PCR (section 2.8.3.2), as in all other cases (Hp1-Hp4). Products were loaded on 3% agarose gels and analysed visually and using spot densitometry.

2.8.3.2 PCR round 2 (fully-nested) amplifications

An internal region of 408 bp containing the Mnl I site, was amplified using 5 ul (1/10 vol.) of the initial PCR reaction, Taq polymerase (Amersham, Baie d'Urfe, QC, Canada) and the upper and lower oligos 5'-TGTTGGAATCCCAGGGAACT-3' (N) and 5'-TTTCAGAAATTCCAGTGTC3' (O), respectively, using standard PCR reaction conditions (Fig. 22 and Table XX). Cycling conditions were: 94°C/4 min x 1 cycle; 94°C/1 min, 50°C/1 min, 70°C/1 min x 30 cycles; 72°C/5 min x 1 cycle.

2.8.4 Mnl I differential digestion

5 ul (1/10) of the initial PCR product was digested with 10 U Mnl I using the supplier's recommended reaction conditions for a minimum of 2 hours or till digestion was complete, at 37°C, and digestion products were loaded on 3% agarose gels and analysed visually and using spot densitometry.

2.8.5 Three CpG sites within the promoter; Nae I, Hha I and Hpa II

The entire 2,896 bp test target region (see section 2.8.3, above) was amplified using PCR. The reaction conditions (not standard) were: 100 – 200 ng (1/10 vol.) of Nae I-, Hha I-, or Hpa II-digested (section 2.8.2), or control non-digested DNA, using the Expand Long Template PCR System (Roche, Ontario, Canada), 300 nM, each oligo, 0.4 mM dNTP mix, 1x Buffer 2, 1.75 U enzyme activity/100 ng DNA. The upper and lower oligos used following Nae I (CpG #17), Hha I (CpG #18) or Hpa II (CpG #20) digestion were the same for all three sites: 5'-CCTGCTCATTTTAAAAGCACTTCTTG-3' (A) and 5'-ACTCTGCAAGGGCACAAGCAATATT-3' (P) (Fig. 22 and Table XX). Cycling conditions were: 94°C/2 min x 1 cycle; 94°C/10 sec, 62°C/30 sec, 70°C/4 min x 10 cycles; 94°C/15 sec, 62°C/30 sec, 70°C/4 min, 5 sec + 5 sec/cycle x 15 cycles; 70°C/7 min x 1 cycle.

PCR round 2 amplifications followed by Mnl I digestion, as described in sections 2.8.3.2 and 2.8.4 above, were then carried out.

2.9 Methylation ratio of six CpG sites

2.9.1 Four Hpa II sites downstream from the promoter

0.5 ug DNA, from LS-180, T2, T8, and two control cell lines, NCI-H596 and A-549, was digested with Hpa II as descibed in section 2.8.2, above. Then, four sets of oligos were used to assay the methylated:non-methylated ratio at the four downstream/non-CpG island CpG/Hpa II sites (Hp1 to Hp4; Fig. 22 and Table XX).

The upper and lower oligos, which were designed to flank the CpG sites, were: 5'-AAGTAGTAGGAAGTGAGCTGTTCAG-3' (D) and 5'-TCCAAGTAAATGCTTGCAAAAAGCC-3' (F) (Site Hp1, 194 bp); 5'-GGCTTTTTGCAAGCATTTACTTGGA-3' (G) and 5'-AAGCCAGCTAAAAGCCTGTAATTGA-3' (H) (Site Hp2, 135 bp), 5'-TGCTAGAAAACTACTGGCAATGAAC-3' (I) and 5'-TTCAGAGAGGGCAATTTAAATCCTG-3' (J) (Site Hp3, 520 bp), and 5'-CAGGATTTAAATTGCCCTCTCTGAA-3' (K) and 5'-GAGCAGCTGAAGATTAAGGCGCC-3' (M) (Site Hp4, 202 bp), respectively. In every case, the cycling conditions were: 94°C/4 min x 1 cycle; 94°C/1 min, 61°C/1 min, 70°C/1 min x 28 cycles; 72°C/5 min x 1 cycle. Note that the DNA was predigested with Pst I (20 U/ug DNA) at 37°C overnight, before Hpa II digestion, in all cases except amplification using the I/J oligo set (for site Hp3). Also note that the optimal number of cycles, 28, was determined following the establishment of a standard curve. Products were loaded on 2% agarose gels and analysed visually.

2.9.2 Two Hpa II sites within the promoter: CpG sites #17 and #20

0.5 ug DNA, from LS-180, T2, T8, and two control cell lines, NCI-H596 and A-549, was digested with Hpa II, or not, as descibed in section 2.8.2, above. Then, the upper oligo 5'-CCTGCTCATTTTAAAAGCACTTCTTG-3' (A) and lower oligo 5'-GATTCTCACAGAGCTTAAAAACTCC-3' (C) (for CpG #17), or 5'-GGAGTTTTTAAGCTCTGTGAGAATC-3' (B) and 5'-CTGAACAGCTCACTTCCTACTACTT-3' (E) (for CpG #20) were used to assay the methylated:non-methylated ratio at the mentioned CpG island/promoter CpG/Hpa II sites (Fig. 22 and Table XX). The cycling conditions were: 94°C/4 min x 1 cycle; 94°C/1 min, 61°C/1 min, 70°C/1 min x 28 cycles; 72°C/5 min x 1 cycle. Note that the

DNA was predigested with Pst I (20 U/ug DNA) at 37°C overnight, prior to Hpa II digestion. Products were loaded on 2% agarose gels and analysed visually.

2.10 G-banding and spectral karyotyping (SKY)

Metaphase spreads of LS-180 cells were prepared according to standard cytogenetic procedures. G-banding chromosome analyses were performed on 20 metaphases according to standard protocol. Image acquisition and analyses were performed using CytoVision software version 3.6 (Applied Imaging Corp., San Josee, California, U.S.A.) coupled with a Zeiss Axioscop II Plus microscope (Carl Zeiss, Jena, Germany).

Spectral karyotyping was conducted on 10 metaphases. Briefly, fresh slides were prepared from fixed chromosome suspensions and treated with pepsin at a final concentration of 12 ng/ml for 2 minutes. Hybridization and detection were then carried out as described by the Applied Spectral Imaging protocol (SkyPaint kit). Metaphases were captured using a SpectraCube coupled to an Axioplan 2 microscope (Carl Zeiss, Jena, Germany) and analysed by the SKYView software (Applied Imaging Corp., San Jose, California, U.S.A.).

2.11 Fluorescence *in situ* hybridization (FISH)/Replication timing

FISH experiments on LS-180 cells were conducted using three probes, separately: BAC clone RP11-421F9, GenBank accession no. AC133141.2 (Research Genetics/Invitrogen, Burlington, Ontario, Canada), which includes the whole RARβ gene and which we localized to chromosome band 3p24 on normal metaphases (data not shown); and the controls, LSI AML1/ETO on chromosome 8q22; or LSI RARα on chromosome 17q21.1 (Vysis Inc., Downers Grove, Illinois, U.S.A.). Prior to harvesting, LS-180 cells were exposed to bromodeoxyuridine (BrdU) at a final concentration of 30 ug/ml for 60 minutes, allowing selective analysis of cells in S phase. Probe labeling and FISH were performed as previously described (*35*) with slight modifications. BrdU-positive nuclei (n=200) were scored for replication status by two different blinded observers, each, using a fluorescence Axioscop II Plus microscope.

3. Results

3.1 RARβ2 mRNA analyses

All 21 cell lines were assayed by RNase protection analysis (*4,5,36*) and/or RT-PCR (Table XVII). A few lines, such as CALU-1, T47-D, ZR-75B, NCI-H125 and MB-231, have been described as negative for RARβ2 mRNA expression by some groups, or as positive by others, but generally only at trace levels of expression (*4-12,37*). We found that all of these lines were negative, except NCI-H125. The RT-PCR analyses of three breast cancer cell lines in particular, T47-D, MB-231, and ZR-75B, were chosen to illustrate that they do not express RARβ2 in our conditions, even upon stimulation with various concentrations of *all trans* retinoic acid (ATRA) as high as 1uM, and even after prolonged treatment (5 days; Fig. 16). HS-578T was used as a positive control.

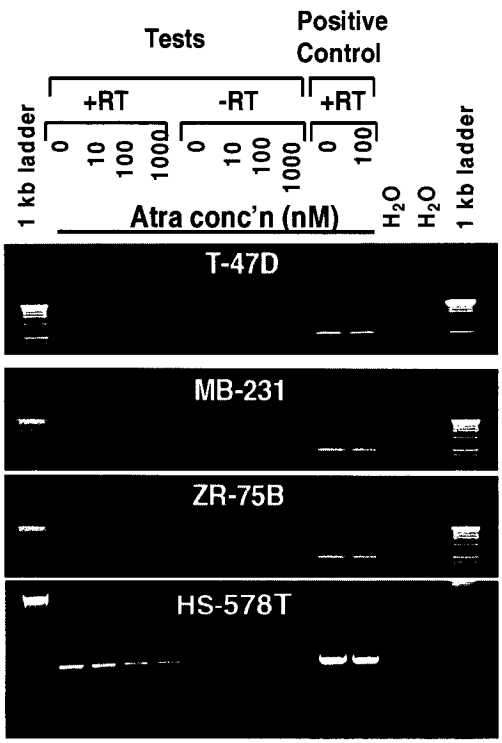


FIGURE 16: Representative 1.5% agarose gels of RARβ2 mRNA expression analyses (RT-PCR) in three RARβ2-negative breast cancer cell lines, T-47D, MB-231 and ZR-75B, and one RARβ2-positive breast cancer cell line, HS-578T, treated with *all-trans* retinoic acid (ATRA) at various concentrations ranging from 0 to 1000 nM, for 5 days. The RT-PCR positive controls are CALU-1 RARβ2-transfectant subclone C64, a strong RARβ2-expressor, treated or not with ATRA (4). Reverse transcriptase negative (-RT) controls and PCR water (H_2O) controls are also shown.

TABLE XVII: Molecular characteristics of the 21 cell lines analysed in this study, including: histological classification, RARβ2 mRNA expression status, ROH at the RARβ2-linked THRβ2 locus status (RFLP marker BH302), and ROH at the rs755661 SNP status. NEG = Negative; N/I = Non-informative; N/T = Not tested; and ROH = Retention of heterozygosity; SNP = single nucleotide polymorphism.

Molecular characteristics of the 21 cell lines analysed in this study							
Type	Tissue	Histological classification	No.	Cell line	RARβ2 expression	ROH at THRβ2	ROH at rs755661
Cancer	Lung	Epidermoid	1	CALU-1	NEG	ROH	N/I
			2	SK-MES	NEG	N/I	N/I
			3	NCI-H157	POS	N/I	N/T
			4	NCI-H520	POS	N/I	N/T
		Adenocarcinoma	5	NCI-H23	POS	N/I	N/T
		Adenosquamous	6	NCI-H596	NEG	N/I	N/I
			7	NCI-H125	POS	N/I	N/T
		SCLC	8	NCI-H82	POS	N/I	N/T
			9	MM-1	NEG	N/I	N/I
		Large Cell	10	QU-DB	POS	N/I	N/T
	Breast	Adenocarcinoma	11	T47D	NEG	N/I	N/T
			12	MB-231	NEG	N/I	N/I
			13	ZR-75B	NEG	N/I	N/I
			14	HS-578T	POS	ROH	N/T
	Colon	Adenocarcinoma	15	CACO-2	POS	ROH	N/T
			16	SW-1222	POS	ROH	N/T
			17	LS-180	NEG	ROH	ROH
			18	HCT-15	NEG	N/I	N/T
			19	COLO-201	NEG	N/I	N/T
			20	COLO-205	NEG	N/I	N/T
Normal	Lung	Epithelial	21	NBE-E6E7	POS	ROH	N/T

3.2 RARβ P2 promoter methylation analyses of parental cell lines

The 541 bp region we chose within the P2 promoter contains 22 CpG sites and several transcription factor binding elements, including a cAMP response element binding site (CREB), an activator protein-1-like site (AP-1-like), and a retinoic acid responsive element (RARE), as well as a TATA box (Fig. 17).

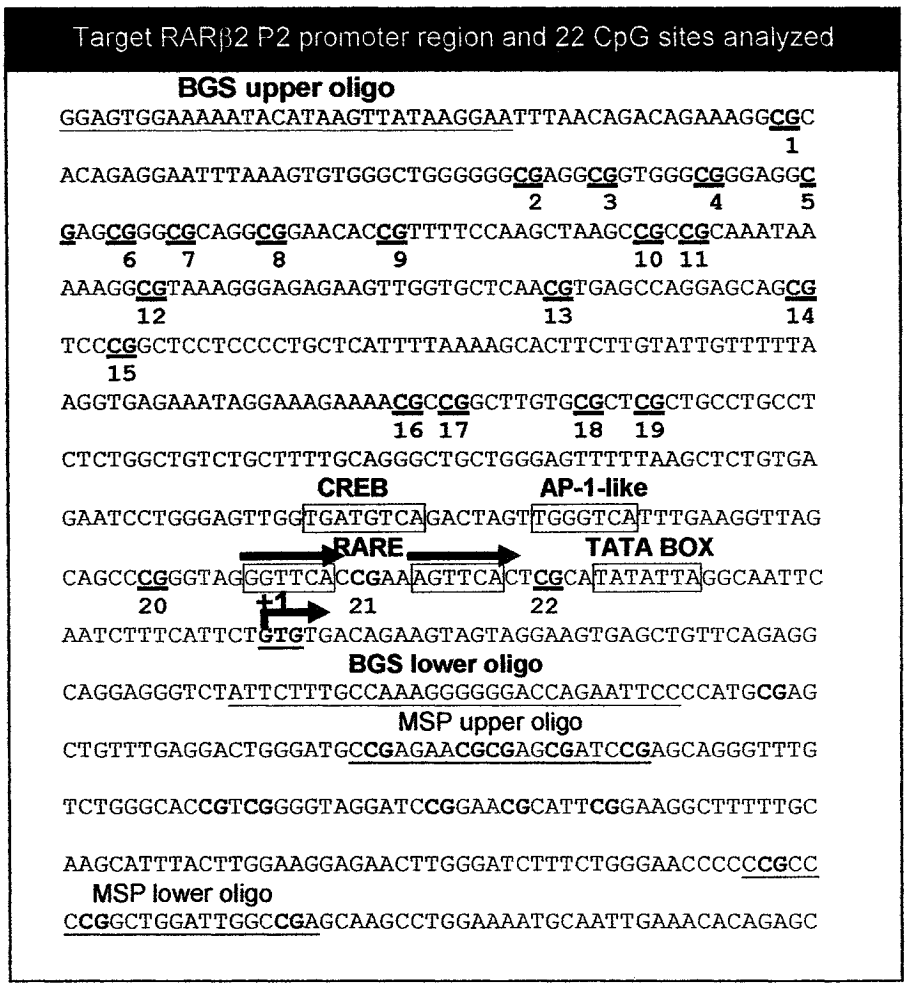


FIGURE 17: Target RARβ2 P2 promoter region (541 bp) containing the 22 CpG sites analyzed in this study. Shown are the main regulatory elements, including the CRE, the AP-1-like site, and the two direct repeats of the RARE (between which is CpG site #21), as well as the TATA box (upstream of which is CpG site #22). Also shown is the sequence which has been frequently analyzed using MSP, and which is located downstream of the transcription start site. Boxes = promoter elements; Straight arrows = direct repeats of the RARE; Bent arrow = Transcription start site; Underlined sequences = regions complementary to oligos used in our study (BGS), or oligos generally used in other studies (MSP). BGS = bisulfite genomic sequencing; MSP = methylation specific PCR.

All 10 cancer-derived cell lines which express RARβ2 (6 lung: NCI-H157, NCI-H520, NCI-H23, NCI-H125, NCI-H82, QU-DB; 1 breast: HS-578T; and 2 colon: CACO-2 and SW-1222) showed virtually no methylation at any of the CpG sites in the RARβ2 P2 promoter, and the normal bronchial epithelial cell line (NBE-E6E7) showed a low density (14 to 36%) of methylation (Fig. 18a).

Among the 11 cell lines which do not express RARβ2, three different groups could be discerned based on their methylation patterns. First, 4 cell lines were hypermethylated (NCI-H596, COLO-201, COLO-205 and HCT-15), with approximately 70% to 90% or more of the 22 CpGs methylated per cell line (Fig. 18, top). Second, 2 cell lines (MM-1 and T47-D) were not methylated (Fig. 18, bottom), indicating that promoter methylation was not responsible for inactivation. The third group, consisting of 5 lines (SK-MES, ZR-75B, CALU-1, MB-231 and LS-180) showed a novel pattern of methylation, consisting of the combination of both hypermethylated and hypomethylated alleles (Fig. 19). For example, several sequencings of SK-MES (Fig. 19a) each had zero (0%) CpG sites methylated, whereas one sequencing revealed 21/22 (95%), and another had an intermediate level of 7/22 (32%). Similar extremes of heterogeneity were observed in ZR-75B (Fig. 19b), with 1/22 (5%) versus 20/22 (91%), and in CALU-1 (Fig. 19c, top), with 2/22 (9%) versus 17/22 (77%). In MB-231 (Fig. 19d, top) the divergence was not as great but there were again two clearly distinct levels of methylation, approximately 25% versus 80-90%. LS-180 (Fig. 19e, top) appeared to present three levels of methylation, with the third being intermediate (45%) between hypermethylated and hypomethylated. In all these lines, the level of methylation in the hypomethylated allele was unlikely sufficient to be responsible for inactivation (*38*).

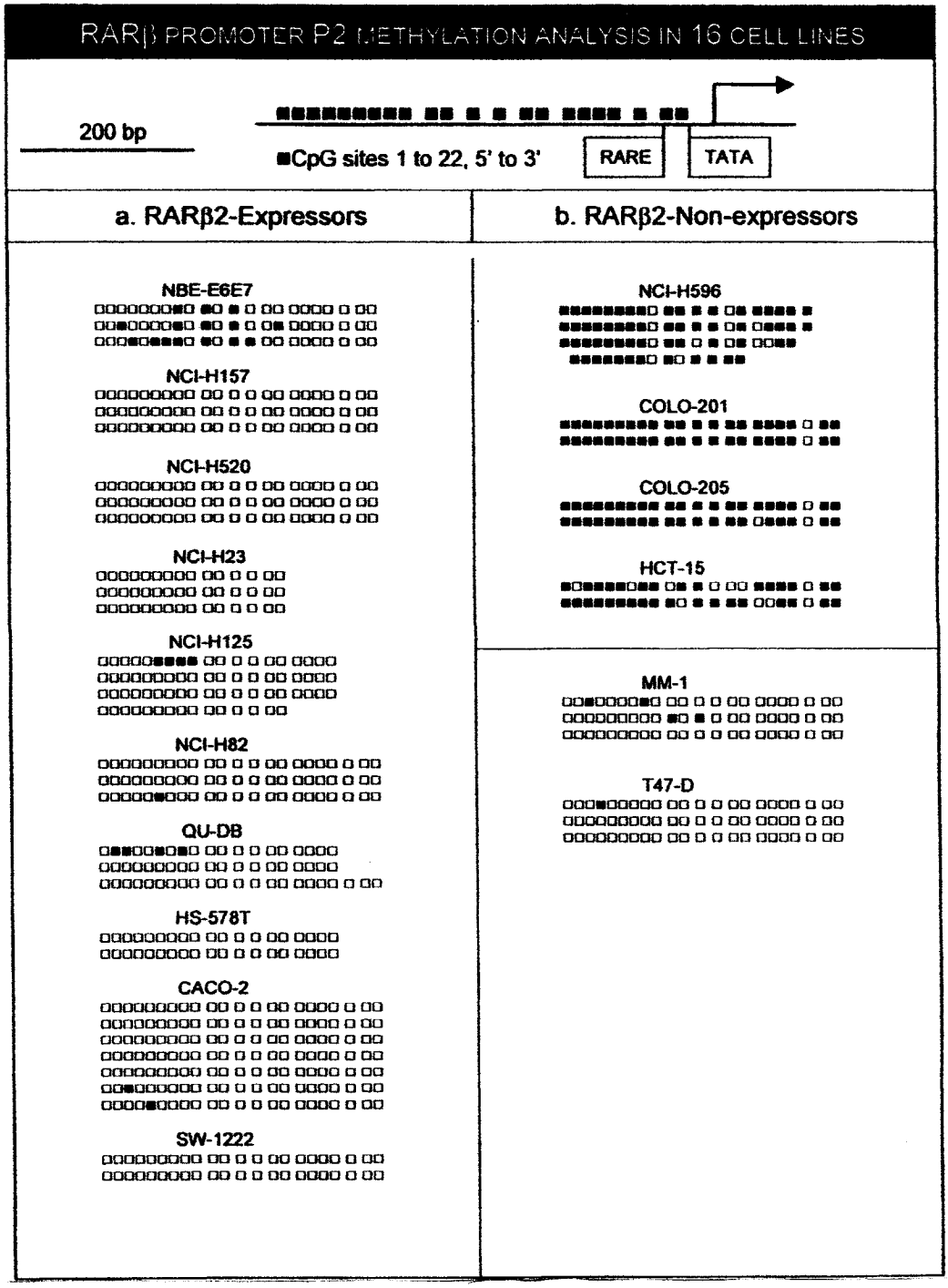


FIGURE 18: Schematic representation of RARβ P2 promoter methylation analyses in 16 cell lines with homogeneous methylation patterns. Schematic representation of the 541 bp promoter region analyzed, showing approximate distribution of CpG sites. CpG site #21 is located between the two direct repeats of the retinoic acid response element (RARE); CpG site #22 is located 4 bp upstream of the TATA box. **a**. 10 RARβ2-expressing cell lines; **b**. 6 RARβ2-non-expressing cell lines. Note that each cell line was subjected to several independent bisulfite treatments in order to decrease the probability that the same sequence was analyzed twice due to PCR artefacts, and to increase the probability that all alleles present were reflected. Boxes represent CpG sites. Solid box = Methylated; Empty box = Not methylated; Absent box = Methylation status was not available.

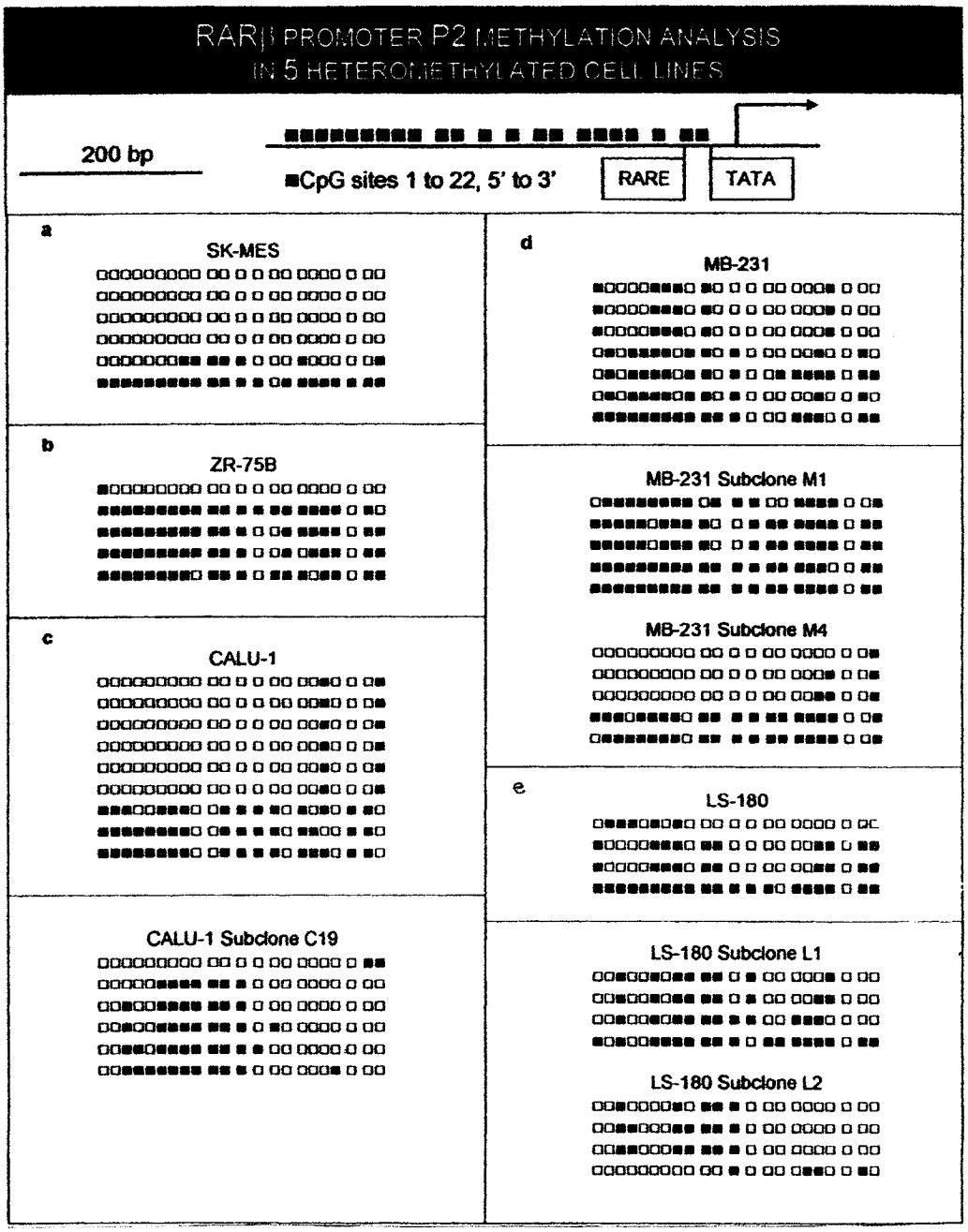


FIGURE 19: Schematic representation of RARβ P2 promoter methylation analyses in 5 cell lines and 5 subclones with divergent methylation patterns. Note that each cell line was subjected to several independent bisulfite treatments in order to decrease the probability that the same sequence was analyzed twice due to PCR artefacts, and to increase the probability that all alleles present were reflected. Boxes represent CpG sites. **a**. SK-MES; **b**. ZR-75B; **c**. CALU-1 and subclone C19; **d**. MB-231 and subclones M1 and M4; **e**. LS-180 and subclones L1 and L2. Boxes represent CpG sites. Solid box = methylated; Empty box = not methylated.

3.3 RARβ P2 promoter methylation analyses of subclones

Briefly, the divergent methylation patterns seen in 5/11 cell lines analyzed (above) could arise from mixed cell populations within the same culture (extracellular divergence), or, they could arise from differential methylation within the same cell or cell clone, hence representing different allelic methylation patterns (intracellular divergence). For a more elaborate discussion regarding this issue, please refer to Discussion, section 4.1. To distinguish between these possibilities, we isolated and analyzed subclones (Materials and Methods, section 2.2), since these new cell lines are likely derived from single cells. All the subclones analyzed revealed the co-existence of hypo- and hypermethylated alleles, akin to their parental cell lines. CALU-1 subclones C19 and C59, which were generated in the course of a previous study (*4*) and had partially reactivated the endogenous RARβ gene, expressed very low levels of endogenous RARβ2, whereas C64 was a strong expressor of the exogenous RARβ gene, and C30, the mock control, did not express RARβ2. We found that C19 had alleles with two clearly divergent methylation patterns bearing similarity to the hypomethylated and hypermethylated alleles of the parental cell line, respectively (Fig. 19c, bottom). The three other subclones, C30, C59 and C64, showed a lower level of methylation than C19 or the parental line but divergent patterns were still present (results not shown). In the newly generated subclones of MB-231, M1 and M4, we found that one subclone, M4, exhibited divergent methylation patterns strikingly similar to the respective parental cell line, strongly supporting the hypothesis that divergent methylation is indeed intracellular and heritable, while the other subclone, M1, yielded a more homogeneous pattern that also resembled one of the parental alleles. LS-180 revealed one subclone to be relatively hypomethylated (about 18-32%), and the other up to about 80% methylated, with extremes of divergence about as great as those seen in the original culture (Fig. 19e, bottom).

3.4 Methylation frequency at 22 individual CpG sites within the RARβ P2 promoter

We divided the BGS sequencings into 5 categories: (1) hypomethylated/expressed; (2) hypermethylated/not expressed; (3) hypomethylated/not expressed; (4) divergent methylation with general level below threshold of expression (<38%); or (5) divergent methylation with general level above threshold of expression (≥38%). We chose

38% as a cutting off rate distinguishing permissible expression from inactivation; this approximation of a threshold level has been made in the past (*39*).

We then plotted the frequency of methylation for each individual CpG site. We found that though CpG sites #21 and 22 were always methylated (100%) in hypermethylated/non-expressing cell lines, they were much less frequently methylated in divergently methylated lines (Fig. 20) even though these were found to be stably inactivated. Contrastingly, there was a significant drop in the frequency of methylation at CpG site #20, which is just 7 bp upstream of the RARE, and this drop is observed in *all* hyper- and divergently methylated lines in which RARβ2 is inactivated. We also found that the pattern of methylation observed at CpG sites #5 - 13 was shared by hypomethylated cell lines whether they express or not, as well as by divergently methylated cell lines that have a general level of methylation that is below the threshold level thought to be permissible for expression (38%).

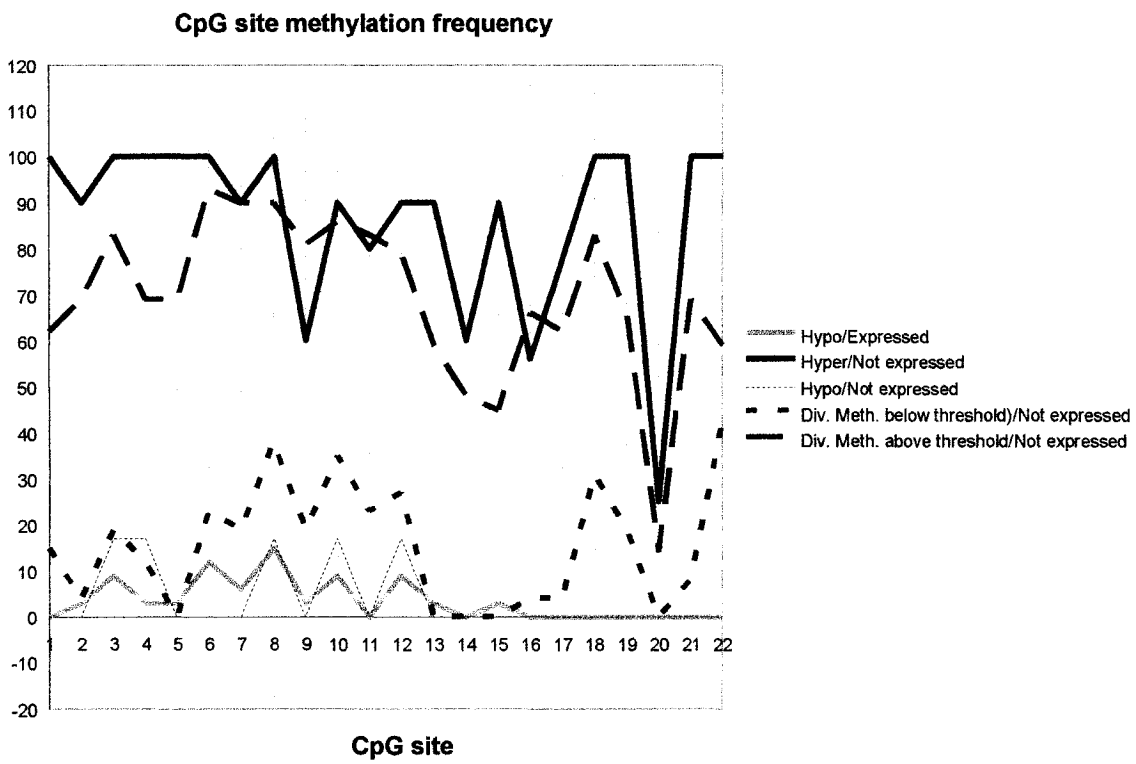


FIGURE 20: Line graph of methylation frequency at 22 individual CpG sites within the RARβ P2 promoter. Data sorted according to general level of RARβ2 methylation and expression statuses. Note that the threshold regarding the general level of methylation generally accepted for permitting expression ≥ 38% methylation. Hypo = hypomethylated; Hyper = hypermethylated; Div. Meth.= Divergently methylated.

3.5 ROH at THRβ2 analyses

In order to determine whether the observation of divergent methylation is correlated with retention of heterozygosity (ROH), which may reflect an allelic bias for methylation, we analyzed the linked site thyroid hormone receptor β2 (THRβ2), which is located at 3p24.3. Of the 21 different parental cell lines analysed, 6 of these showed ROH: CALU-1, HS-578T, CACO-2, SW-1222, LS-180 and NBE-E6E7 (Table XVII).

3.6 ROH at D3S1583, D3S2335, and rs755661 analyses

We also performed ROH analysis at two other sites located within or near the RARβ locus (D3S1583 and D3S2335), but obtained the same results as for THRβ2: CALU-1 and LS-180 showed ROH for D3S1583, and all other divergently methylated lines were non-informative at both sites (Table XVIII).

TABLE XVIII: List of the 5 divergently methylated cell lines and their ROH at D3S1583, D3S2335, and rs755661 statuses. ROH = Retention of heterozygosity; N/I = Non-informative.

CELL LINE	D3S1583	D3S2335	rs755661
CALU-1	ROH	N/I	N/I
SKMES	N/I	N/I	N/I
MB-231	N/I	N/I	N/I
ZR-75B	N/I	N/I	N/I
LS-180	ROH	N/I	ROH

It was determined that only 1 of these 5 divergently methylated cell lines, LS-180, a colorectal adenocarcinoma cell line, was also heterozygous for the rs755661 SNP (Tables XVIII and XVII).

In order to determine whether the tested phenomenon of methylation allelic bias was also present in other tumor samples, DNA samples from 18 available breast cancer tumors (T1-T18) kindly provided to us by D^r . Mark Basik (*32*) were tested in the same manner, and it was found that 10/18 samples, heretofore labeled T1 – T10, were also heterozygous for the rs755661 SNP (Table XIX).

TABLE XIX. List of the 18 breast cancer biopsies (*32*), their original code, new label and rs755661 C/T SNP status. ROH = Retention of heterozygosity; N/I = Non-informative.

	Code	New label	Rs755661
1	21	N/A	N/I
2	23BA1	N/A	N/I
3	26BA1	T1	ROH
4	34	N/A	N/I
5	36	T2	ROH
6	92	T3	ROH
7	41	T4	ROH
8	44	T5	ROH
9	140	N/A	N/I
10	64	N/A	N/I
11	98	T6	ROH
12	78	N/A	N/I
13	73	T7	ROH
14	80	T8	ROH
15	116	N/A	N/I
16	90	N/A	N/I
17	106	T9	ROH
18	353s	T10	ROH

3.7 DNMT-1, 2, 3A and 3B protein expression analyses

No deficiencies in DNMT expression were observed, other than lower levels of DNMT 3A in the lines that were uniformly hypomethylated (HS-578T and A-549), and no substantial changes in expression were seen in the presence of 1 uM ATRA (Fig. 21).

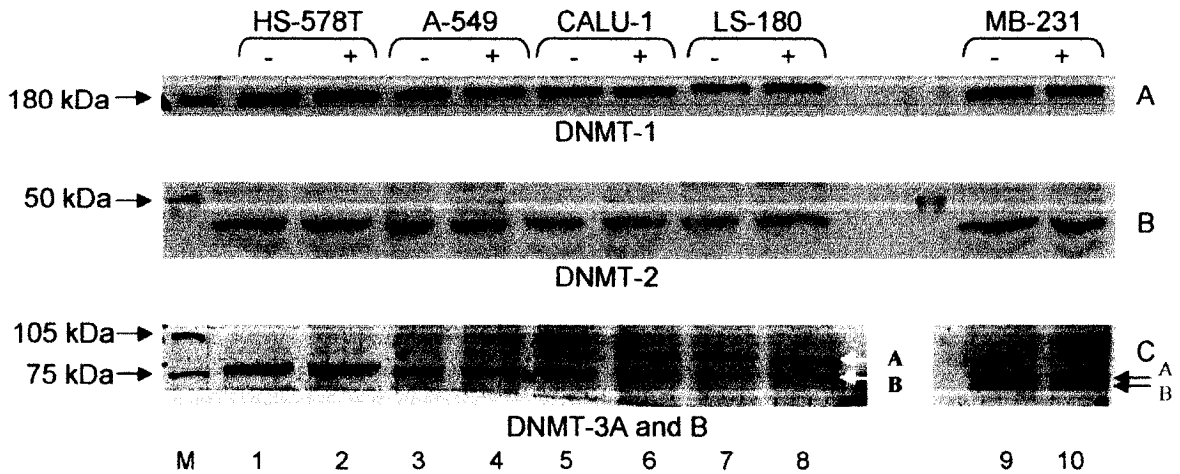


FIGURE 21. Representative DNMT-1, 2, 3A and 3B protein analyses in divergently methylated cell lines (Western blots). **A**. DNMT-1, 170-195 kDa; **B**. DNMT-2, 45 kDa; and **C**. DNMT-3A, ~90 kDa (band A) and DNMT-3B, ~80 kDa (Band B). Prepared from whole cell protein extracts from the divergently methylated cell lines CALU-1, LS-180, MDA-MB-231, Hs-578T or the RARβ2-expressing cell line A-549 cells treated or not with 1 uM ATRA for 24 hrs. M=Molecular weight markers; 1=HS-578T; 2=HS-578T + ATRA; 3=A-549; 4=A-549 + ATRA; 5=CALU-1; 6= CALU-1 + ATRA; 7=LS-180; 8= LS-180 + ATRA; 9=MB-231; 10=MB-231 + ATRA. - = No ATRA; + = 1 uM ATRA.

3.8 Methylation allelic bias assays

A database search showed that a high frequency (average heterozygosity = 0.499) C/T single nucleotide polymorphism (SNP), rs755661, exists near the RARβ P2 promoter. The rs755661 SNP (5'-aattaaccC/Ttccaaa-3') is situated at position chr3:25447005-25447005, band 3p24.2; it is located approximately 2.2 kb downstream from the RARβ2 promoter, and contains the Mnl I restriction enzyme site, $\begin{matrix}5'\text{-}CCTC(N)_7\ldots\text{-}3' \\ 3'\text{-}GGAG(N)_6\ldots\text{-}5'\end{matrix}$, in one of its two polymorphic forms (the C allele). Mnl I differential digestion was therefore used to test for a bias in allelic methylation in the five divergently methylated cell lines (CALU-1, SK-MES, MB-231, ZR-75B and LS-180). No other more proximal SNPs known at the time of this study were amenable to such an analysis. As indicated above (section 3.6), of the five divergently methylated cell lines, only LS-180 was heterozygous at this site. We therefore used rs755661 as a diagnostic marker to test for allelic identity following methylation-sensitive restriction enzyme digestion in this cell line, to determine whether divergent allelic methylation patterns were attributable to an allelic bias for methylation (see Fig. 22 for a schematic representation). We also tested the ten breast cancer biopsy DNA samples that tested positive for ROH at rs755661, T1-T10 (Table XIX).

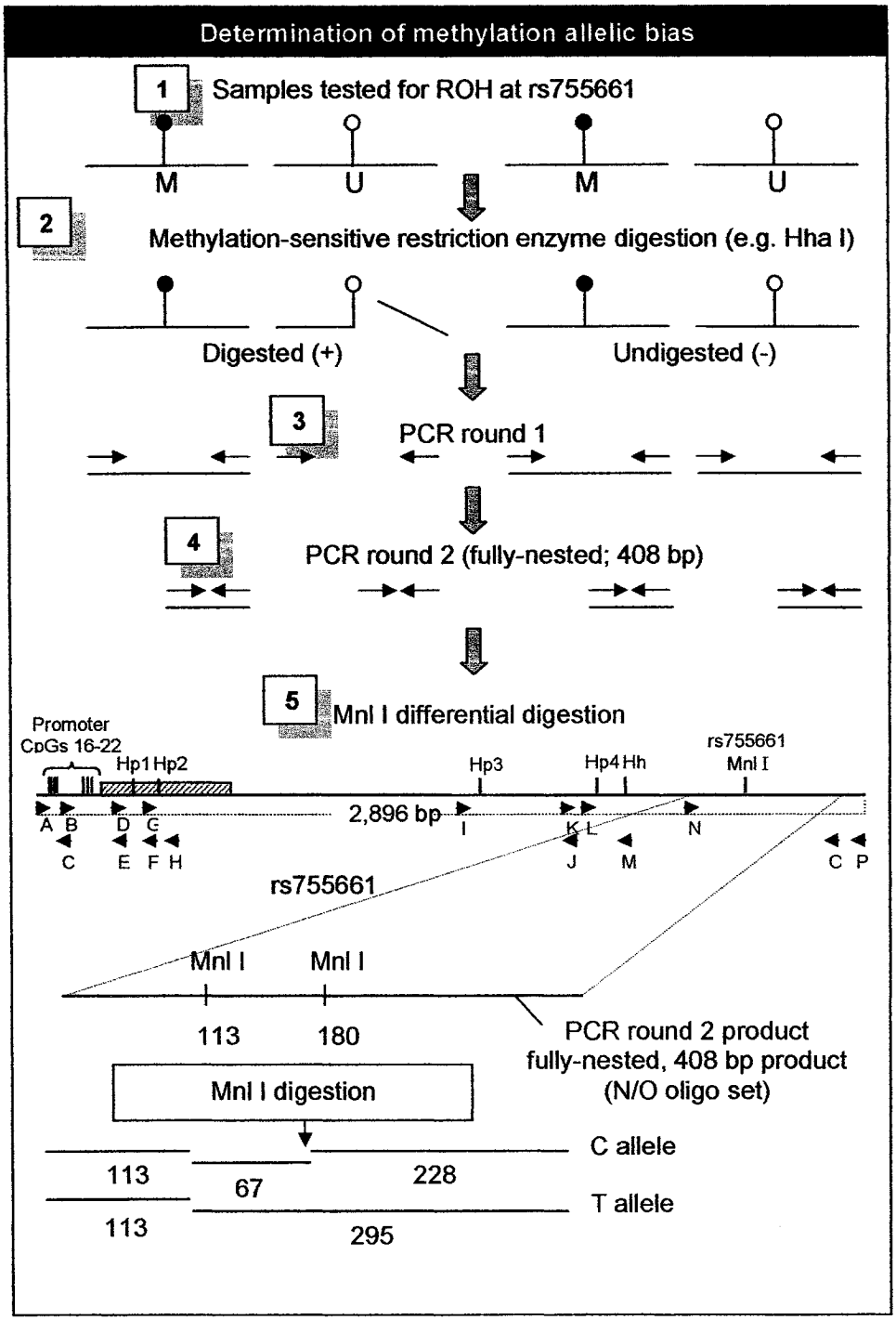

Determination of methylation allelic bias
1
Samples tested for ROH at rs755661
M
U
M
U
2
Methylation-sensitive restriction enzyme digestion (e.g. Hha I)
Digested (+)
Undigested (-)
3
PCR round 1
4
PCR round 2 (fully-nested; 408 bp)
5
Mnl I differential digestion
Promoter
CpGs 16-22
Hp1 Hp2
Hp3
Hp4 Hh
rs755661
Mnl I
2,896 bp
A B
C
D G
E F H
I
K L
J
M
N
C P
rs755661
Mnl I
Mnl I
113
180
PCR round 2 product
fully-nested, 408 bp product
(N/O oligo set)
Mnl I digestion
C allele
113
67
228
T allele
113
295

FIGURE 22: Principle of allelic identification using Mnl I differential digestion of the rs755661 SNP following methylation-sensitive restriction enzyme digestion. **1.** All samples are first selected for ROH at rs755661 (5'-aattaaccC/Ttccaaa-3'; located at 3p24), which consists of a C/T single nucleotide polymorphism (SNP), containing the Mnl I restriction enzyme site 5'-CCTC$(N)_7$ ...-3'
3'-GGAG$(N)_6$...-5' in one of its two alleles (the C allele). Note that methylated and unmethylated alleles are qualitatively represented but their proportions are not represented here. **2.** Samples are digested with a methylation-sensitive restriction enzyme such as Hha I: unmethylated alleles are sensitive to Hha I digestion (as shown by break), whereas methylated alleles are resistant. **3.** PCR round 1: unmethylated alleles, digested, can not be amplified (as shown by lack of PCR product), whereas methylated alleles, resistant to digestion, can be amplified (as shown by bands). **4.** PCR round 2 (fully nested; 408 bp): the oligo set N/O (see bottom of figure) is used to generate a shorter product amenable to Mnl I differential digestion. **5.** Mnl I differential digestion (used to assess the relative proportion of methylated C alleles versus methylated T alleles): Mnl I differential digestion yields either two fragments, 113 and 295 bp, for the allele carrying the T nucleotide within the polymorphism, or three fragments, 67, 113 and 228 bp, for the allele carrying the C polymorphism. Schematic diagram also represents the 2,896 bp (oligo set A/P) target region, which contains: (1) the three promoter CpG site(s) analyzed via Nae I, Hha I or Hpa II methylation-sensitive restriction enzymes (CpG #17, 18, and 20, respectively); (2) the five non-island CpG sites downstream from the promoter analyzed (Hpa II sites 1-4 and Hha I site); and (3) the rs755661 SNP/Mnl I site. Equally shown are the oligos used (see Materials and Methods, section 2.8). Please note that the 228 bp fragment was used to identify/semi-quantitatively measure the proportion of methylated C alleles; the 113 bp fragment was used as the constant band. Arrows A-P= 16 oligos used for the various assays (methylation allelic bias and methylation ratio assays). Also shown are the approximate locations of CpG sites #16-22 of 22 sites previously analyzed (see Fig.'s 17, 18 and 19) within the promoter. + = Digested; - = not digested. Hp1-Hp4 = Non-CpG island Hpa II sites #1-4 respectively; Hh = Non-CpG island Hha I site. Hatched rectangle = exon.

3.8.1 CpG sites downstream from the promoter; four Hpa II sites and one Hha I site

As previously mentioned, the rs755661 SNP is more than 2.2 kb downstream from the promoter-associated CpG island, and a number of non-island CpGs exist between this polymorphism and the promoter. These sites, known as bulk DNA CpGs, are normally hypermethylated, but in cancer cells may be hypomethylated (*33,34*). Hence, if any allelic bias existed at these sites, or if these sites were hypomethylated, then the interpretation of our results would become confounded. It was thus important to assure that there existed no such allelic bias and that these sites were not hypomethylated. The pertinent sites, Hp1 – Hp4, and Hh, as well as the other sites analyzed within the promoter, are shown in Fig. 22, step 5, and listed in Table XX.

Table XX: List of the 13 oligo sets used for the methylation allelic bias assays and the methylation ratio assays at various CpG sites within the 2,896 bp target region analyzed, and molecular weights of corresponding PCR products (see Fig.'s 16 and 22 for schematic diagrams). Hh= Non-CpG island Hha I site; Hp1-4= Non-CpG island Hpa II sites 1-4; M:U=methylated:unmethylated.

Experiment	Site/Sequence	Oligo set	Mol. Wt. (bp)
Full-length target	Nae I (CpG #17)	A/P	2896
	Hha I (CpG #18)		
	Hpa II (CpG #20)		
Nested product	For Mnl I differential digestion	N/O	408
Methylation ratio (M/U)	Hp1	D/F	194
	Hp2	G/H	135
	Hp3	I/J	520
	Hp4	K/M	202
	CpG #17	A/C	143
	CpG #20	B/E	167
Methylation allelic bias	Hp1	D/P	2637
	Hp2	G/P	2468
	Hp3	I/P	1368
	Hp4	K/P	873
	Hh	L/O	719

Amplification of DNA digested with Hha I or Hpa II using the appropriate primer pairs (D/F, G/H, I/J and K/M, for Hp1-Hp4, respectively, for methylation ratio; or D/P, G/P, I/P, K/P, and L/O, for Hp1-Hp4, and Hh, respectively, for methylation allelic bias), showed: first, that the level of methylation was usually near 100% at each site (Fig. 23, top); and second, that each allele was equally represented in the methylated and unmethylated populations (Fig. 23, bottom). Therefore, any bias that might or might not be detected in the promoter in later experiments (section 3.8.2) would not be influenced by a bias at these downstream (non-CpG island/bulk) sites.

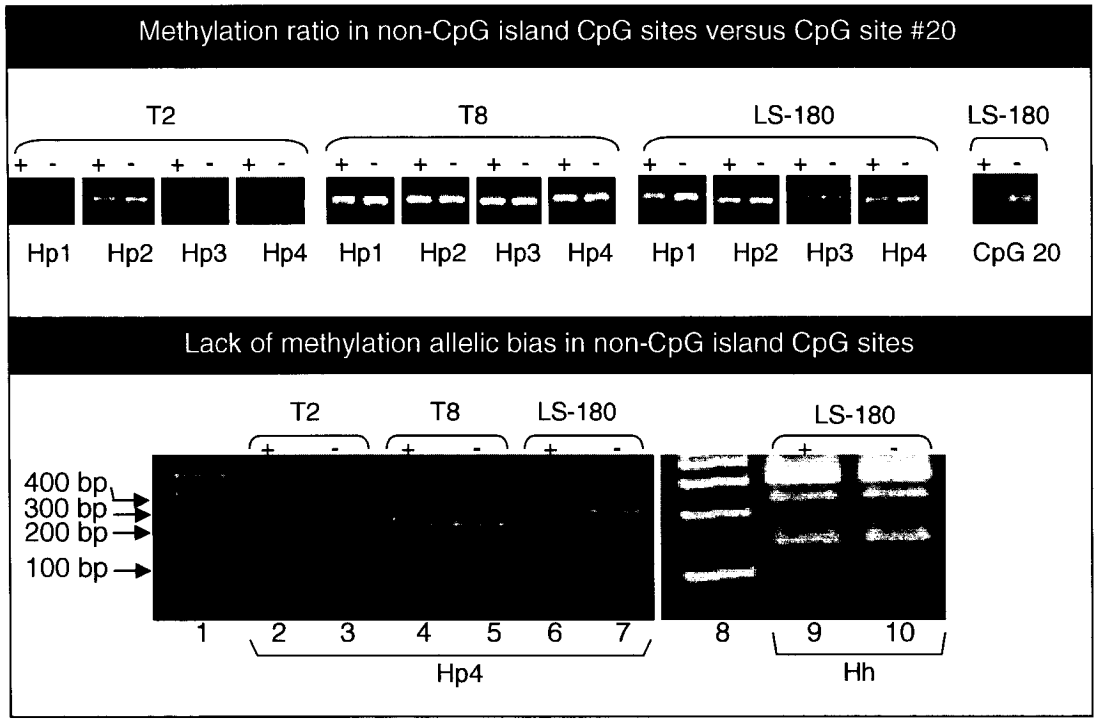


FIGURE 23: Representative methylation ratio assays and methylation allelic bias assays in four non-CpG island/bulk Hpa II sites and one Hha I site downstream of the RARβ2 P2 promoter. **Top**: Representative 2% agarose gels of PCR products amplified from oligos flanking the Hp1, Hp2, Hp3 and Hp4 test sites (external to the promoter) versus CpG site #20 (within the promoter) following Hpa II digestion as compared to undigested control. Note that the downstream CpG sites appear to be nearly 100% methylated, whereas CpG site 20, which is within the promoter, does not (about 25%). + = Digested; - = Undigested. **Bottom**: Representative 3% agarose gels of MnI I digestion products of the 408 bp nested PCR product amplified from the PCR N/O product. Note that the patterns are identical in the + and - lanes, reflecting the lack of methylation allelic bias present at sites Hp4 and Hh. Lanes : 1 = 1 kb Plus ladder; 2 = T2 + Hpa II; 3 = T2 undigested; 4 = T8 + Hpa II; 5 = T8 undigested; 6 = LS-180 + Hpa II; 7 = LS-180 undigested ; 8 = 1 kb Plus ladder; 9 = LS-180 + Hha I; 10 = LS-180 undigested. Hp1-Hp4 = Non-CpG island Hpa II sites #1-4 respectively; Hh = Non-CpG island Hha I site (see Fig. 22). M/U = methylated:unmethylated.

3.8.2 CpG sites within the promoter; Nae I, Hha I and Hpa II sites

We next analyzed for bias within the promoter. Digestion of genomic DNA of the LS-180 cell line was performed with one of three methylation-sensitive restriction enzymes: Hha I (GCG/C), Nae I (GCC/GGC) or Hpa II (C/CGG). Together, these restriction enzymes recognize 8 sites within the 2,896 bp target sequence.

The Hha I restriction enzyme has only one site in the promoter within the region amplified in these experiments (corresponding to CpG #18; Fig. 17). Identification of the alleles present at rs755661 after Hha I digestion consistently revealed that the T

allele was clearly more frequently methylated than the C allele in the LS-180 cell line (Fig. 24, middle; compare for example the 228 bp bands in lanes 7 versus 6, or 9 versus 8). Densitometry showed a difference of only 2:1 even though visual estimates showed a difference >4:1. This may be due to the fact that background noise reduces the accuracy of the densitometer sensor. These experiments were repeated six times with similar results.

The Nae I restriction enzyme has a unique site within the entire 2.9 kb region (corresponding to CpG site #17; Fig. 17). The results of these experiments also revealed a methylation allelic bias of approximately 2:1, but in the opposite direction (data not shown).

Since these experiments could only be performed on one cell line (LS-180), we sought to determine whether methylation allelic bias also existed in breast cancer samples, which are known to be frequently hypermethylated (>50%) at the RARβ2 promoter (*24,40*). Ten breast cancer biopsy samples that proved to be heterozygous at rs755661 (Table XIX) were available to us for this assay. Of these, two samples, T2 and T8, gave similar results as LS-180, when analyzed for both Nae I and Hha I enzymes, in that results showed that these samples were also subject to methylation allelic bias (results for T2/Hha I digestion shown in Fig. 24, middle, compare lanes 5 versus 4). There was insufficient material to carry out BGS on these samples.

The situation with Hpa II was more complicated than with Hha I and Nae I, because unlike the latter two, which recognize unique sites, the former recognizes four non-CpG island sites within the region amplified. We therefore chose to assess whether there may be an allelic bias over the average of these sites by performing a series of partial digestions at 0, 30, 90 and 300 seconds, and 60 minutes. Control experiments showed that 50% digestion for a given site was achieved with this enzyme within the 5 to 60 second range, whereas complete digestion was achieved by 60 minutes. Amplification and allele identification by Mnl I digestion always revealed the same tendency as was seen with Nae I (Fig. 24, bottom).

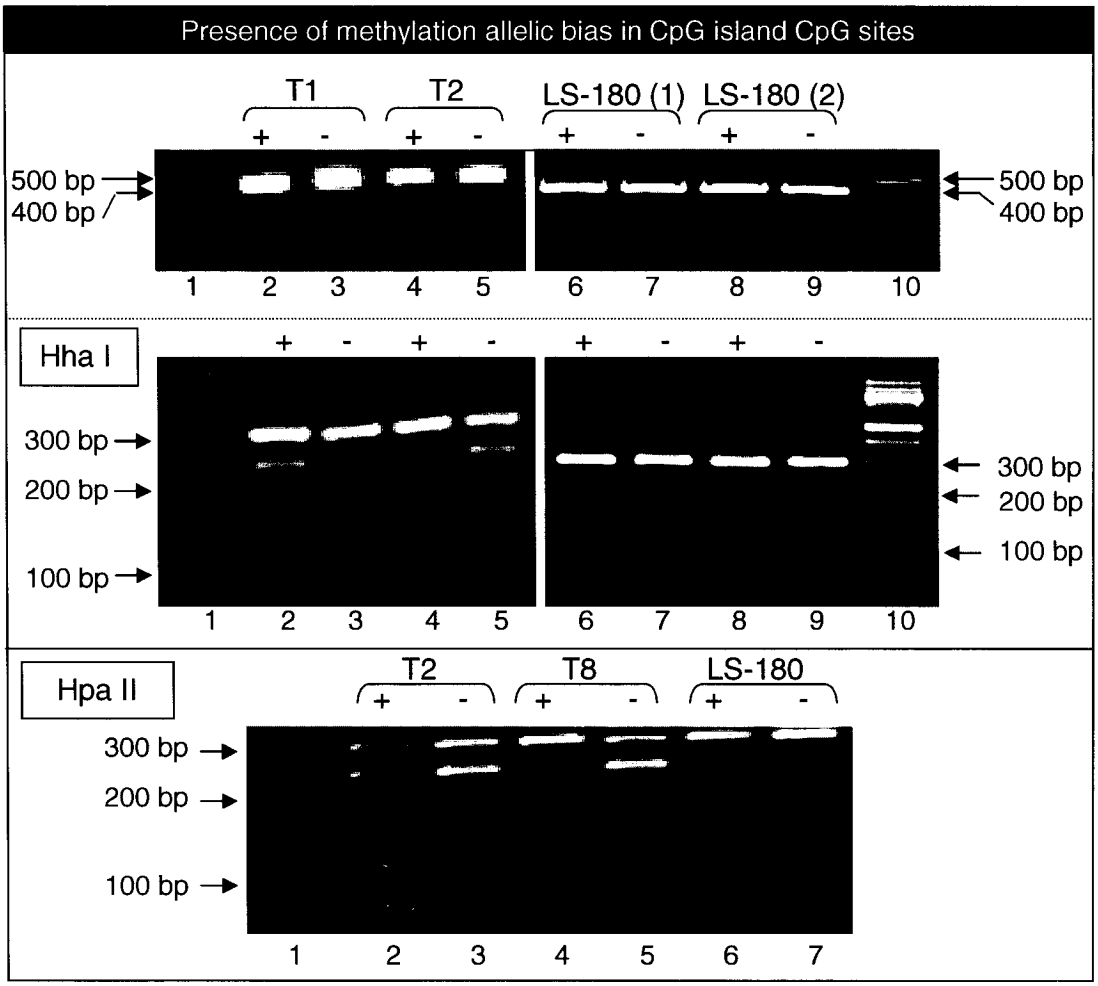


FIGURE 24: Representative methylation allelic bias assays at the Hha I site (CpG #18) and Hpa II site (CpG #20) within the RARβ2 P2 promoter. **Top:** Representative 1.5% agarose gel electrophoreses of the 408 bp products (oligo set N/O) amplified from Hha I–digested genomic DNA extracted from patient breast cancer biopsies (left) or from Hha I-digested genomic DNA extracted from the LS-180 colon adenocarcinoma cell line (right). **Middle:** Representative 3% agarose gel electrophoreses of the MnI I digestion products of the 408 bp PCR product seen in top gels (Hha I digestion). Note the near lack of the 228 bp band in the T2/Hha I sample (lane 4) as compared to the non-digested sample (lane 5). Also note the striking reduction of the 228 bp band in the LS-180 samples (lanes 6 and 8) as compared to the non-digested controls (lanes 7 and 9). Lanes (top and middle): 1 = 1 kb Plus ladder; 2 = T1 + Hha I; 3 = T1 undigested; 4 = T2 + Hha I; 5 = T2 undigested; 6 = LS-180 + Hha I; 7 = LS-180 undigested; 8 = LS-180 sample 2 + Hha I; 9 = LS-180 sample 2 undigested; 10 = 1 kb Plus ladder. LS-180 DNA samples, 1 and 2, were extracted from separately grown LS-180 cells. **Bottom:** Representative 3% agarose gel electrophoresis of the MnI I digestion products of the 408 bp PCR product (not shown) amplified from Hpa II-digested DNA. Note the striking reduction of the 228 bp band in the T8/Hha I sample (lane 4) as compared to the non-digested sample (lane 5). Also note near lack of the 228 bp band in the LS-180 sample (lane 6) as compared to the non-digested controls (lanes 7). Lanes (bottom): 1 = 1 kb Plus ladder; 2 = T2 + Hpa II; 3 = T2 undigested; 4 = T8 + Hpa II; 5 = T8 undigested; 6 = LS-180 + Hpa II; 7 = LS-180 undigested.

3.9 Determination of diploidy using G-banding and spectral karyotyping (SKY)

In order to perform fluorescence *in situ* hybridization (FISH) analyses for replication synchrony studies on the divergently methylated cell lines, it was essential to determine whether divergently methylated cell lines were diploid for chromosome 3 and whether or not this chromosome was subject to rearrangement. We tested 3 cell lines, CALU-1, MB-231 and LS-180, using G-banding and spectral karyotyping (SKY). Only LS-180 was found to have two normal (non-rearranged) chromosomes 3 (Fig. 25). MB-231 was triploid for chromosome 3 and CALU-1 was found to have a fraction of mitoses carrying a third abnormal copy of chromosome 3 (results not shown). Hence, FISH/replication timing experiments were carried out using LS-180 cells.

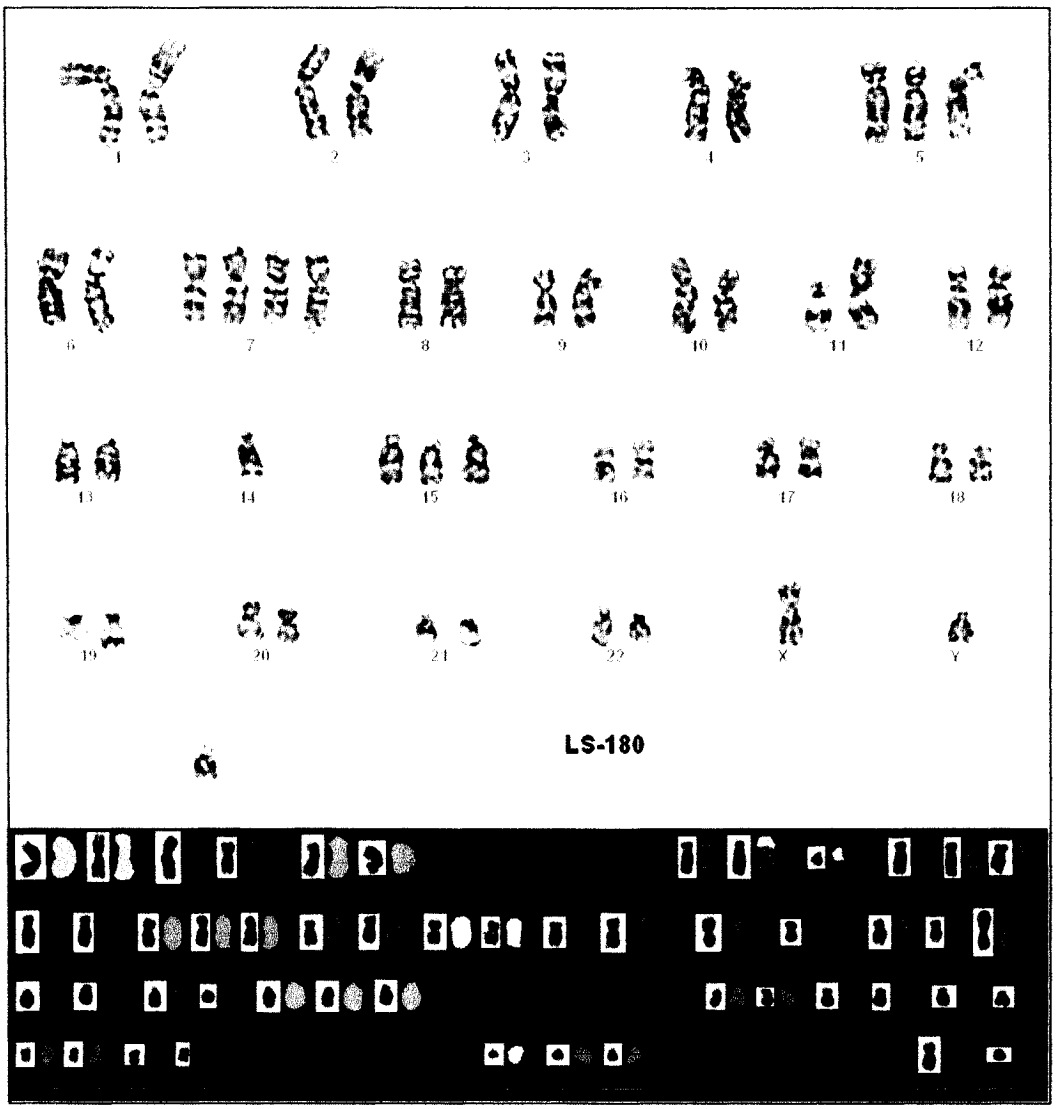
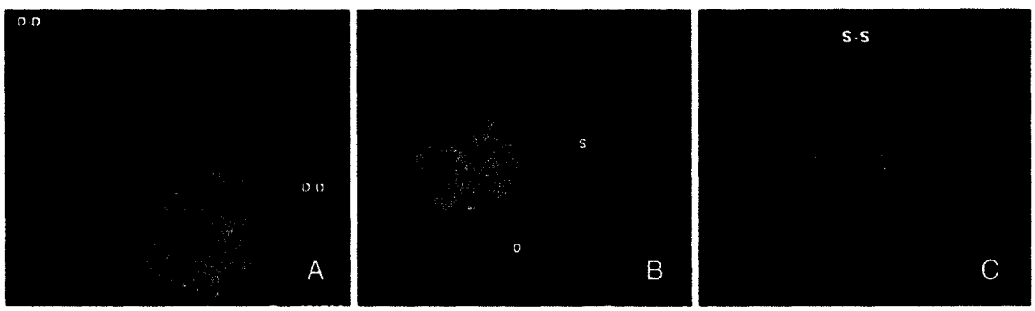


FIGURE 25: G-banding and spectral karyotyping analyses showing the diploid status of the LS-180 cell line. **Top**: G-banding of LS-180 metaphase spreads. **Bottom**: Spectral karyotyping (SKY) of LS-180 chromosomes.

3.10 Replication timing analyses using fluorescence *in situ* hybridization (FISH)

In order to determine whether methylation divergence was correlated with replication asynchrony, we performed FISH analysis of BrdU-pulse-labeled interphase nuclei, because this allows the selective examination of cells in S phase. When using a probe for a specific site, such as RARβ, cells that display two single hybridization signals (SS) indicate that neither allele has replicated, whereas cells displaying two double signals (DD) indicate that both alleles have replicated, and cells with one

single and one double signal (SD) indicate that only one allele has replicated. The normal cut-off level for asynchronously replicating loci is 20% in most published studies. We used the RP11-421F9 clone (see Materials and Methods, section 2.11) as the FISH probe to analyze the replication timing of the RARβ region in the LS-180 cell line (Fig. 26).



DD SD SS

FIGURE 26: Representative FISH analyses of BrdU-pulse-labeled LS-180 cells probed using BAC RP11-421F9 against RARβ2. **A.** Examples of cells with double hybridization signals (DD), indicating that both alleles have replicated; **B.** A cell with one single and one double hybridization signal (SD), indicating that only one allele has replicated; and **C.** A cell with two single hybridization signals (SS), indicating that neither allele has replicated. DD = doublet-doublet; SD = singlet-doublet; SS = singlet-singlet.

We found that the percentage of nuclei exhibiting the doublet pattern (DD) in each of the experiments indicated that the region complementary to the BAC RP11-421F9 region containing RARβ replicates in late S phase (DD=4%), whereas the regions complementary to the RARα and ETO control regions replicate in early to middle S phase (22% and 8%, respectively; Table XX). Analysis of the replication timing of the ETO and RARα revealed a relatively high percentage of SD nuclei (21.5% and 29%, respectively) compared with RARβ (11%). This high percentage of SD nuclei is probably due to an early–middle replication timing pattern of one of the alleles. In the literature, asynchronously replicating genes show an SD pattern in S phase cells between 30 to 40% (*41*). This is higher than what we observed for ETO and RARα loci. In any event, no suggestion of asynchronous replication was obtained for RARβ.

TABLE XXI: Frequency of the various replication timing patterns observed following BrdU-pulse-labeling and FISH using probes against BAC RP11-421F9, ETO or RARα, in BrdU-positive cells (n=200, each). Note that two different blinded observers scored 100 slides for replication status each. SS = singlet-singlet; SD = singlet-doublet; DD = doublet-doublet.

Pattern	Frequency (%)		
	RARβ2	ETO	RARα
SS	85	70	49
SD	11	22	29
DD	4	8	22

4. Discussion

4.1 RARβ P2 promoter methylation analyses show that divergent methylation, which affects nearly half of all cell lines with inactivated RARβ2 tested, is a genuine and novel phenomenon

On the one hand, some of our results confirm the well established correlation between promoter methylation and gene inactivation (*3*). In particular, the results from the 10 cell lines in which RARβ2 is expressed and the promoter is un- or hypo-methylated, and the 4 cell lines in which RARβ2 is inactivated and the promoter is hypermethylated, are consistent with the current literature (Fig. 18a and b top, respectively). The low density of methylation (14 to 36%) seen in NBE-E6E7 cells (Fig. 18a, top) is generally believed to be below a threshold level (35-38%) and is subsequently thought to permit gene expression (*39*). Moreover, this low density methylation does not appear to affect the three CpG sites near the RARE and TATA box, sites #20-22 (Fig. 17), which are sites that have previously been shown to be correlated with inactivation of RARβ2 expression when methylated [see Fig. 11 in Chapter 1, and references (*20,28*)]. These observations are therefore consistent with RARβ2 expression.

On the other hand, the results from the 5 cell lines in which we find both hyper- and hypomethylated alleles (CALU-1, SK-MES, ZR-75B, MB-231, and LS-180), and from the 5 subclones of 3 of these lines (Fig. 19), are inconsistent with the literature, and add to the current understanding of the role of methylation in gene inactivation. More precisely, they demonstrate the coexistence and transmissibility of hypermethylated and hypomethylated RARβ alleles in cancer-derived cell lines and suggest that a

previously undocumented event is responsible for inactivation of RARβ2 at the hypomethylated allele.

Different methylation patterns may arise in a single cell line in various ways: (1) from contamination of the cell line with normal (non-cancerous) cells; (2) from the potentially polyclonal origin of the original tumor resulting in the establishment of a cell culture with cancer cells of different lineages; (3) from the acquisition of methylation in only some of the cells in the culture, due to genomic or epigenomic instability, yielding a heterogeneous mix of cells with uniform methylation levels within each cell; or, (4) from the presence of differentially methylated alleles within a same cancer cell, and consequently, within its clonal progeny as well. The first possibility is highly unlikely since normal cells would have been lost due to senescence soon after the establishment of the culture. The second and third possibilities are also unlikely considering population dynamics of mixed cell cultures, but they remain formal possibilities. The last scenario can be distinguished from the others by methylation analysis of subclones, since each subclone is in theory generated from a single cell (see Material & Methods, section 2.2). More specifically, if different methylation patterns were derived from different cells (extracellular divergence) as in cases 1, 2 and 3, above, then divergent methylation should not be observed in any of the subclones. Several subclones of each of the three cell lines in which divergent methylation was observed were therefore analyzed, including CALU-1, MB-231 and LS-180 (Fig. 19c, d, and e, respectively), and divergent methylation was observed in all of them. The imperfect replication of methylation patterns may be explained by the fact that at least 22 generations of growth took place before the subclones were analyzed. The reduced relatedness observed between patterns of the CALU-1 subclones and the parental line (Fig. 19c) for example, may be attributable to epigenetic drift, since these cells had been passaged for several years after subcloning. And it is interesting to note that C19, which has acquired very low levels of expression from the endogenous gene, has reduced methylation near the RARE and TATA box sites, as compared to the parental cell line CALU-1, the very sites which have previously been shown to be specifically affected by methylation, as noted earlier (*20,28*). The increased homogeneity seen in MB-231 (Fig. 19d) may have been due to the daughter cell initially receiving only two of the three homologues of chromosome 3. This may be attributed to karyotypic instability, which frequently results in imperfect distribution of chromosome homologues to daughter cells (see section 3.10). In any event, if the source of differentially methylated alleles had been the heterogeneous nature of the parental cell culture, then none of the

subclones would have carried more than one pattern, yet all did. We therefore conclude that intracellular divergent methylation is genuine.

It is important to note that the proportion of cell lines in which we find divergent methylation is substantial: 5/11 (45%) of the cell lines with inactivated RARβ2 have both hypo- and hypermethylated alleles. But the real proportion may be even higher, since the data presented is based on an average of three sequencings per cell line. Additional sequencings may determine whether or not a similar lack of uniformity of methylation exists among alleles in the other cell lines.

This is the first demonstration of the coexistence of hypo- and hypermethylated alleles in cells in which the gene in question is completely inactivated. The existence of hypomethylated alleles in samples that demonstrate DNA hypermethylation has never before been reported *per se*, though it has been observed in cell lines and tumors at several other loci (*42-46*), as well as at the RARβ locus (*15*). However, these studies have generally used methylation-specific PCR (MSP), a powerful technique capable of identifying the presence of a single methylated allele among 1,000 (*47*). However, this technique is not as specific as BGS, since it does not provide information about all of the individual CpG sites in the amplified region of a *same allele*, since amplified products are usually distinguished on agarose gel rather than being subcloned and sequenced [following bisulfite conversion, MSP detects the presence of methylated CpG sites via PCR-amplification of bisulfite-treated DNA samples, using PCR primer sets capable of distinguishing between wild-type (W), methylated (M) and unmethylated (U) molecules]. Another pitfall of MSP is that it amplifies un/hypomethylated alleles indiscriminately, and in biopsy samples, these may arise from contaminating normal (non-cancerous) cells known to be present in as much as 10% of cells (*40*). In fact, Sirchia *et al.* state that *"it (is) very likely that the [unmethylated] products in the tumor samples were amplified either from residual normal epithelial cells, or stromal cells mixed to tumor cells" (40),* or that *"(the) presence of both unmethylated (U) and methylated (M) products likely reflects a mixture of normal and malignant cells in the tumor sample" (48).* As previously mentioned, we consider that normal cell contamination of the cancer cell lines analyzed herein is highly unlikely (see Discussion, section 4.1), since normal cells would have been lost due to senescence soon after the establishment of the culture, let alone following the great number of passages thereafter. However, a stronger point is that following bisulfite conversion, BGS uses sequencing of subcloned PCR

products to determine the status of *all* CpG sites on a *same* DNA template, and therefore provides information about single alleles, rather than pools of alleles. This may reflect an important difference when considering the fact that unmethylated and hypomethylated alleles, which may not be inactivated by methylation, may exist and hence be unaccounted. In addition, when used to analyze the methylation status of RARβ2, MSP has usually been based on oligos designed to target only 7 CpG sites (MSP upper and lower oligos; Fig. 17), and these are located 130 bases downstream of the transcription start site, in the 5' UTR and exon 3 (coding region A), of the RARβ2 protein (*49*). As has been discussed by Jones (*50*), downstream methylation may not actually inhibit the gene in question and may even be correlated with transcriptional activity. We therefore designed BGS oligos targeting 22 CpGs located in a region upstream of the transcription start site and within the P2 promoter proper. Also, we are aware of only one report in which individual allelic determinations were performed on cancer cells that were demonstrably uncontaminated by normal tissue (*45*). While these authors found some heterogeneity of methylation in the 5' region of the E-cadherin gene in two cancer cell lines, the overall levels of methylation within the analyzed sequences were similar. Consequently, the conclusion that a promoter methylation-independent event had contributed to inactivation could not be made. Our description of the coexistence of hypo- and hypermethylated alleles in the same cell, a phenomenon which we call "divergent methylation", is therefore a novel finding.

A precedent for methylation-independent inactivation events, called type II silencing, has been described in Chinese hamster ovary cells (*51*). Interestingly, this event resulted in the coordinated silencing at two linked genes, thymidine kinase and galactokinase in one case (*52*), and emtB and chr in the other (*53*).

4.2 The inactivating mechanism on the hypomethylated allele may be *cis*-acting

The observation of divergent methylation has certain interesting implications. Since co-repressive *trans*-acting factors would be expected to have equivalent effects on both alleles, it is difficult to rationalize how any additional selective advantage would arise from methylation of only one allele. It may thus be considered unlikely that inactivation of the unmethylated allele would be the consequence of co-repressive *trans*-acting factors, which in turn implies that the inactivating mechanism is *cis*-acting. An alternative interpretation is that one of the alleles is refractory to

methylation, and becomes inactivated by another mechanism, but we know of no report of this type of allelic preference. Furthermore, the available data indicate that at least some of these cell lines (e.g. CALU-1 and SK-MES) are capable of supporting transcription from an exogenous RARβ promoter (*11*), which argues against the involvement of *trans*-acting suppressive factors in these lines. We therefore favour the first interpretation. At the present time, the nature of such a *cis*-acting event is unknown.

As mentioned in the introduction, methylation is thought to occasion the binding of methyl CpG binding proteins (MeCPs), followed by the recruitment of histone deacetylases (HDACs) such as HDAC1, which results in the massive deacetylation and condensing of the local chromatin structure such that transcription does not occur. In this regard, it may be suggested that the effect of HDAC1 inhibition, via treatment with trichostatin A (TSA), for example, combined with stimulation of the RARE, via *all-trans* retinoic acid (ATRA) treatment, be tested to determine whether this treatment is sufficient to reactivate RARβ2. Arapshian *et al.* demonstrated that the combined treatment of ATRA + TSA + 5-azacytidine (an inhibitor of methylation) reactivated RARβ2 expression in the MCF-7 breast cancer cell line (*54*), but the level of expression following ATRA + TSA alone was not markedly superior to that of RA alone, signifying that demethylation of the hypermethylated allele was likely the major cause of re-expression. On the other hand, Sirchia *et al.* found that the combined treatment of ATRA + TSA significantly reactivated RARβ2 expression in MCF-7 and MDA-MB-231 breast cancer cells (*48*). However, these experiments would not reveal the identity of the methylation-independent mechanism leading to inactivation and would be difficult to interpret since reactivation of the hypermethylated allele would represent a confounding factor.

We postulate that the results from the 2 cell lines in which RARβ2 is inactivated but not hypermethylated (MM-1 and T47-D; Fig. 18b bottom) may be explained via transcription inhibition occurring through the binding of *trans*-acting co-repressor molecules, such as members of the silencing mediator (corepressor) for retinoid and thyroid-hormone receptor (SMRT) family (*3*). This would lead to the recruitment of histone deacetylases (HDACs) to the promoter complex according to the well established paradigm, and result in histone deacetylation, chromatin condensation and gene inactivation (*55*). It is also plausible that the mechanism (or one of the mechanisms) that may be operating to suppress RARβ2 in these hypomethylated,

non-expressing lines may be the same one that is operating in the 5 divergently methylated cell lines.

4.3 Methylation frequency analyses at individual CpG sites show that site #20, which is frequently unmethylated, may be of importance

Our results do not confirm that the methylation status of CpG sites #20-22 is particularly important with regard to gene inactivation as has been previously suggested (*54,56*). For example, CpG sites #21 and 22 (100% methylated) were not more frequently methylated than sites #18 and 19 (100% methylated) in hypermethylated non-expressing lines. Also, it is unlikely that the methylation of sites #21 and 22 play a role in sterically impeding access to transcription factors or other binding proteins, at least with regard to DNA primary structure, since sites #18 and 19, which are equally ubiquitously methylated, are at a considerable distance upstream from the RARE (i.e. approximately 124 and 128 bp, respectively). However, the significant drop in frequency of methylation at CpG site #20, which was 25% methylated in hypermethylated alleles, may indicate that this CpG is particularly important with regard to access to the RARE because this site is just 7 bp upstream from the RARE and because infrequency of methylation was observed in all hyper- and divergently methylated lines in which RARβ2 is inactivated. Then again, it is known that the rate of bisulfite conversion is <0.1% in double stranded DNA (*57,58*), and the formation of hairpin structures due to proximal methylated CpG sites, for example, significantly influences it (*59*). This is one of the reasons why multiple improvements to the BGS method have been sought and reported (*60-65*). Therefore, the possibility that this sudden drop in frequency is artifactual should not be excluded. Nonetheless, it is amusing to speculate that the absence of methylation at this site represents a selective advantage for the cell since RARβ2 expression via RA:RAR:RARE complex formation may be favoured at sterically unhindered sites. Increases in the levels of bioavailable RAs would therefore lead to reactivation of the RARβ2 promoter, and possibly even its demethylation. Though the region analyzed in this study contains several transcription factor binding elements, including a CREB site, an AP-1-like site, a RARE and a TATA box, results show that only this site (CpG #20) is strikingly differentially methylated (Fig. 20).

4.4 ROH at THRβ2, D3S1583 and D3S2335 analyses support the hypothesis that divergent methylation is correlated with ROH at 3p24 in LS-180

Retention of heterozygosity (ROH) studies revealed that of the five divergently methylated cell lines, only CALU-1 and LS-180 demonstrate ROH at THRβ2 and D3S1583, even though four polymorphisms, which each have frequencies of nearly 50% in the general population, were analyzed. The chances of ROH for a given one of these three cell lines is therefore approximately $(1/2)^4$. It is extremely likely therefore that at least one of these had experienced LOH, which suggests that ROH is not an obligatory condition for divergence of methylation. This in turn implies that an allele-specific mark, inherited from a given parent in a manner analogous to what is known to exist in imprinting does not likely play a role in the divergence of methylation patterns (see section 4.6.1, below).

4.5 DNMT protein levels do not show any deficiencies in expression

It may be hypothesized that divergent levels of methylation result in whole or in part from deficiencies in methylation. Consequently, we estimated the levels of the various DNA methylase isoforms (DNMT-1, 2, 3A and 3B) in three cell lines exhibiting divergent methylation levels (CALU-1, LS-180 and MDA-MB-231) and two control, unmethylated cell lines (Hs-578T and A-549). DNMT isoform expression appears to be uniform across all cell lines tested, whether exhibiting divergent or uniform methylation levels, except DNMT-3A, which was found to be expressed at a lower level in the hypomethylated cell lines (Fig. 21).

It is interesting to note that the DNMT-3A isoform is associated with *de novo* methylation (*66*); this may explain the hypomethylated state of the promoter in the hypomethylated cell lines. It is further possible that the hypomethylated allele in the lines with divergent methylation was resistant to DNMT-3A action at a critical moment when *de novo* methylation occurred. Although direct comparison of DNMT-3A levels with those in hypermethylated lines was not interpretable (data not shown), the explanation we favor suggests that there was a difference between the hypo- and hyper-methylated alleles with respect to their susceptibility to methylation. Further work would be necessary to test this possibility.

Though deficient DNMT-1, 2, 3A or 3B expression does not play a direct role in the presence of divergent methylation levels seen here, this does not exclude the possibility that deficiencies regarding the many other components of DNA methylation, such as methylation metabolism, including the enzymes methylene tetrahydrofolate reductase and methyl synthase, for example, may be involved.

DNMT levels have previously been shown to be upregulated in patients with certain forms of cancer and pre-cancerous conditions as compared to healthy individuals. Whether or not this is the case in these cell lines cannot be determined since the normal corresponding cell lines are not available.

4.6 Methylation at the RARβ P2 promoter appears to be subject to allelic bias

Our studies show that methylation at the RARβ P2 promoter appears to be subject to allelic bias. Our approach involved determining whether the alleles at rs755661 were present at an unequal proportion following digestion with methylation-sensitive restriction enzymes. If there had not been any allelic bias, then there should not have been any observable bias at *any* site by looking at the methylation status of DNA from the whole culture. In fact, we found bias at 3/3 sites we tested.

Our approach however did involve a certain number of assumptions and limitations. First, following testing [described in sections 2.8 (Materials and Methods) and 3.8 (Results)], downstream, non-island CpGs (Fig. 23) were assumed to be methylated, or, if incompletely methylated, then not in an allele-biased manner. Second, the number of CpG sites which were tested was necessarily limited, since there are a finite number of methylation-sensitive restriction enzyme sites within the promoter region and the non-island region upstream from the Mnl I site. Specifically, we were restricted to one Nae I site, one Hha I site, and one Hpa II sites. Results for the first two sites indicated bias, but with methylation on different alleles. Though this may seem counter-intuitive, it is in fact consistent with the hypothesis of allelic bias since the examination of detailed patterns (Fig. 19) shows many examples of individual sites which are methylated on a hypomethylated allele and not methylated on a hypermethylated allele. In particular, the adjacent CpG sites #17 (Nae I) and #18 (Hha I) were shown to be methylated and unmethylated, respectively, on different alleles (see Fig. 19). Third, because of the multiplicity of Hpa II sites (total of five), we were concerned that complete digestion would cause some loss of information,

because if any site was unmethylated, then the PCR product would not have been amplified. Our solution was to test partial digestions, and these experiments generated results consistent with those obtained above, supporting our hypothesis of methylation allelic bias. Nevertheless, experiments of this nature necessarily involve conditions in which the level of catalytic activity is difficult to quantify.

Also, the finding of methylation allelic bias was only found in three samples: 1/1 cancer cell lines (colon) and 2/10 cancer biopsies (breast) that tested positive for ROH at rs755661 (amenable to allelic identification through MnI I digestion). Also, since we do not have methylation data on the breast cancer biopsies due to insufficient material, it is difficult to assess the relevance of the methylation allelic bias data in these samples. However, since about half of all breast cancers do not show methylation at RARβ (at least not within the 5'UTR and coding region A of exon 3), then finding two cases, T2 and T8, is striking. In any event, our findings, including the presence of methylation allelic bias within the P2 promoter, as well as the control methylation ratio assays and the control methylation allelic bias assays downstream from the promoter, show that the two alleles are differently treated by the cell.

4.6.1 Methylation allelic bias at the RARβ2 P2 promoter is reminiscent of an aberrant form of *de novo* imprinting

Methylation allelic bias is reminiscent of an aberrant form of *de novo* imprinting, in which allelic inactivation occurs according to parent of origin and methylation patterns are re-established in the embryo following a wave of demethylation that takes place in the post-cleavage/pre-implantation embryo (67). This process is essential in defining which gene copy among meiotic paired alleles of sister chromatids is expressed. The processes and molecular signals responsible for these new patterns are not yet known. However, any similarity may have limitations, as suggested by the ROH studies herein (see section 4.4, above). This report is nevertheless the first to link hypermethylation in cancer to an imprinting-like mechanism; this finding must be verified further to establish whether a strong correlation exists and whether this correlation exists in other genes. This link is worth further investigation for two reasons: (1) many mechanisms crucial to embryonic cell development are known to be recapitulated (or blocked) in cancer, including proliferative, differentiative and apoptotic pathways, for example, and our findings suggest that the mechanisms responsible for the reprogramming of methylation patterns in the cells forming the

pre-implantation embryo may be recapitulated in the adult cancer cell, and hence support the recapitulation theory; (2) though DNA methylation in imprinting typically occurs at distant loci referred to as imprinting control elements or ICE (*68*), further studies may reveal any similarities between either of these and the *cis*-acting inactivation event described here, and may prove beneficial in developing strategies toward the reactivation of tumor suppressor genes in cancer.

4.7 Replication timing analyses at RARβ show that methylation allelic bias is not correlated with asynchronous replication

A widely accepted hallmark of preferential allelic inactivation, either on the inactive X chromosome, in imprinted genes, or in random allelic inactivation, is late replication of the inactive allele (*41*). The phenomenon we studied involved methylation allelic bias in which both alleles were inactivated and is therefore different than the scenario of the three cases above. Nevertheless, if one of the mechanisms responsible for inactivation of RARβ was analogous to imprinting or X-inactivation, then we may observe late replication of that allele, which would be detectable as asynchronous replication, if the other allele was replicated early. We found that methylation divergence was not correlated with replication asynchrony in the LS-180 cell line, but, interestingly, both alleles of RARβ were replicated in late S phase in the great majority of nuclei, whereas the alleles of RARα were both replicated in early to middle S phase (according to our results, the alleles of ETO appeared to be replicated late as well; Table XXI). Unfortunately, we do not know the methylation status of these genes at the present time.

It would be of interest to repeat the FISH experiments using LS-180 treated with a demethylating agent, such as 5-azacytidine or 5-aza-2'-deoxycytidine, to compare replication timing in divergently methylated alleles to replication timing in demethylated (non- or hypomethylated) alleles. However, demethylating agents are known to have genome-wide effects, which could potentially demethylate genes involved in cell cycling or DNA synthesis, and hence present confounding factors in such a study. A gene-specific method of demethylation is not yet known.

Two other possibilities are to repeat the experiments using: (1) a probe complementary to the same region (i.e. BAC RP11-421F9) but in two different cell lines, such as CACO-2, shown to have purely unmethylated copies of RARβ, or in

NCI-H596, shown to have purely methylated copies of RARβ (Fig. 18), these cell lines would first evidently have to be assessed for ploidy at chromosome 3; or (2) the same cell line (LS-180) but using a probe complementary to a region within a gene adjacent to RARβ which is not divergently methylated (i.e. whose alleles share the same status of methylation). Option 1 is currently underway in the laboratory.

5. Conclusion

Our descriptions of the coexistence, transmission and allelic bias of hypo- and hypermethylated alleles in the same cell are novel findings and pertinent to current research for numerous reasons. First, RARβ2 is one of the genes most frequently methylated in several cancers, including lung (*22*), esophageal (*23*), breast (*24*), cervical (*25*), ovarian (*26*) and urinary cancer (*27*), hence the RARβ promoter methylation-independent mechanisms herein described may be at play in several types of cancer. Second, methylation-independent mechanisms responsible for the inactivation of the RARβ2 gene may be responsible for the inactivation of other TSGs with similarly divergently methylated profiles. Third, DNA methylation analysis of single or multiple genes (methylation signatures) is presently being studied for its potential as an early and relatively non-invasive diagnostic tool in cancer [reviewed in (*69*)]. However, since lack of methylation does not necessarily equate with lack of inactivation, and presence of methylation does not negate lack of methylation at other allelic copies, it may be important to reassess our approaches to interpreting methylation data in order to fully seize the promise that methylation holds as a powerful and early diagnostic tool. Fourth, the finding of allelic bias in methylation suggests that mechanisms similar to those involved in imprinting may be involved in the faulty methylation of genes so frequently seen in cancer.

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References

(*1*) Knudson AG. Mutation and Cancer: Statistical study of retinoblastoma. Proc. Nat'l Acad. Sci. USA. 1971;68:820-3.

(*2*) Naylor SL, Johnson, B. E., Minna, J. D., Sakaguchi, A. Y. Loss of heterozygosity of chromosome 3p markers in small-cell lung cancer. Nature 1987;329:451-4.

(*3*) Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 2003;33 Suppl:245-54.

(*4*) Houle B, Rochette-Egly C, Bradley WE. Tumor-suppressive effect of the retinoic acid receptor beta in human epidermoid lung cancer cells. Proc Natl Acad Sci U S A 1993;90:985-9.

(*5*) Houle B, Leduc F, Bradley WE. Implication of RARB in epidermoid (Squamous) lung cancer. Genes Chromosomes Cancer 1991;3:358-66.

(*6*) Gebert JF, Moghal N, Frangioni JV, Sugarbaker DJ, Neel BG. High frequency of retinoic acid receptor beta abnormalities in human lung cancer. Oncogene 1991;6:1859-68.

(*7*) Nervi C, Vollberg TM, George MD, Zelent A, Chambon P, Jetten AM. Expression of nuclear retinoic acid receptors in normal tracheobronchial cells and in lung carcinoma cells. Exp Cell Res 1991;195:163-70.

(*8*) Roman SD, Clarke CL, Hall RE, Alexander IE, Sutherland RL. Expression and regulation of retinoic acid receptors in human breast cancer cells. Cancer Res 1992;52:2236-42.

(*9*) Geradts J, Chen JY, Russell EK, Yankaskas JR, Nieves L, Minna JD. Human lung cancer cell lines exhibit resistance to retinoic acid treatment. Cell Growth Differ 1993;4:799-809.

(*10*) Swisshelm K, Ryan K, Lee X, Tsou HC, Peacocke M, Sager R. Down-regulation of retinoic acid receptor beta in mammary carcinoma cell lines and its up-regulation in senescing normal mammary epithelial cells. Cell Growth Differ 1994;5:133-41.

(*11*) Moghal N, Neel BG. Evidence for impaired retinoic acid receptor-thyroid hormone receptor AF-2 cofactor activity in human lung cancer. Mol Cell Biol 1995;15:3945-59.

(*12*) Seewaldt VL, Caldwell LE, Johnson BS, Swisshelm K, Collins SJ, Tsai S. Inhibition of retinoic acid receptor function in normal human mammary epithelial cells results in increased cellular proliferation and inhibits the formation of a polarized epithelium in vitro. Exp Cell Res 1997;236:16-28.

(*13*) Cote S, Sinnett, D., Momparler, R. L. Demethylation by 5-aza-2'-deoxycytidine of specific 5-methylcytosine sites in the promoter region of the retinoic acid receptor beta gene in human colon carcinoma cells. Anticancer Drugs 1998;9:743-50.

(*14*) Bovenzi V, Le, N. L., Cote, S., Sinnett, D., Momparler, L. F., Momparler, R. L. DNA methylation of retinoic acid receptor beta in breast cancer and possible therapeutic role of 5-aza-2'-deoxycytidine. Anticancer Drugs 1999;10:471-6.

(*15*) Sirchia SM, Ferguson, A. T., Sironi, E., Subramanyan, S., Orlandi, R., Sukumar, S., Sacchi, N. Evidence of epigenetic changes affecting the chromatin state of the retinoic acid receptor beta2 promoter in breast cancer cells. Oncogene 2000;19:1556-63.

(16) Virmani AK, Rathi, A., Zochbauer-Muller, S.. Sacchi, N., Fukuyama, Y., Bryant, D., Maitra, A., Heda, S., Fong, K. M., Thunniss N, F., Minna, J. D., Gazdar, A. F. Promoter methylation and silencing of the retinoic acid receptor-beta gene in lung carcinomas. J Natl Cancer Inst 2000;92:1303-7.

(17) Bovenzi V, Momparler, R. L. Antineoplastic action of 5-aza-2'-deoxycytidine and histone deacetylase inhibitor and their effect on the expression of retinoic acid receptor beta and estrogen receptor alpha genes in breast carcinoma cells. Cancer Chemother Pharmacol 2001;48:71-6.

(18) Sirchia SM, Ren, M., Pili, R., Sironi, E., Somenzi,G., Ghidoni, R., Toma, S., Nicolo, G., Sacchi, N. Endogenous reactivation of the RARbeta2 tumor suppressor gene epigenetically silenced in breast cancer. Cancer Res 2002;62:2455-61.

(19) Cote S, Momparler, R. L. Activation of the retinoic acid receptor beta gene by 5-aza-2'-deoxycytidine in human DLD-1 colon carcinoma cells. Anticancer Drugs 1997;8:56-61.

(20) Arapshian A, Kuppumbatti, Y. S., Mira-y-Lopez, R. Methylation of conserved CpG sites neighboring the beta retinoic acid response element may mediate retinoic acid receptor beta gene silencing in MCF-7 breast cancer cells. Oncogene 2000;19:4066-70.

(21) Nakayama T, Watanabe, M., Yamanaka, M., Hirokawa, Y., Suzuki, H., Ito, H., Yatani, R., Shiraishi, T. The role of epigenetic modifications in retinoic acid receptor beta2 gene expression in human prostate cancers. Lab Invest 2001;81:1049-57.

(22) Toyooka S, Toyooka KO, Maruyama R, Virmani AK, Girard L, Miyajima K, et al. DNA methylation profiles of lung tumors. Mol Cancer Ther 2001;1:61-7.

(23) Kuroki T, Trapasso F, Yendamuri S, Matsuyama A, Alder H, Mori M, et al. Allele loss and promoter hypermethylation of VHL, RAR-beta, RASSF1A, and FHIT tumor suppressor genes on chromosome 3p in esophageal squamous cell carcinoma. Cancer Res 2003;63:3724-8.

(24) Fackler MJ, McVeigh M, Evron E, Garrett E, Mehrotra J, Polyak K, et al. DNA methylation of RASSF1A, HIN-1, RAR-beta, Cyclin D2 and Twist in in situ and invasive lobular breast carcinoma. Int J Cancer 2003;107:970-5.

(25) Narayan G, Arias-Pulido H, Koul S, Vargas H, Zhang FF, Villella J, et al. Frequent Promoter Methylation of CDH1, DAPK, RARB, and HIC1 Genes in Carcinoma of Cervix Uteri: Its Relationship to Clinical Outcome. Mol Cancer 2003;2:24.

(26) Evron E, Dooley WC, Umbricht CB, Rosenthal D, Sacchi N, Gabrielson E, et al. Detection of breast cancer cells in ductal lavage fluid by methylation-specific PCR. Lancet 2001;357:1335-6.

(27) Maruyama R, Toyooka S, Toyooka KO, Harada K, Virmani AK, Zochbauer-Muller S, et al. Aberrant promoter methylation profile of bladder cancer and its relationship to clinicopathological features. Cancer Res 2001;61:8659-63.

(28) Deng G, Peng, E., Gum J, Terdiman, J., Sleisenger, M. and Kim, Y.S. Methylation of hMLH1 promoter correlates with the gene silencing with a region-specific manner in colorectal cancer. Br. J Cancer 2002;86:574-9.

(29) Shen S, Kruyt FA, den Hertog J, van der Saag PT, Kruijer W. Mouse and human retinoic acid receptor beta 2 promoters: sequence comparison and localization of retinoic acid responsiveness. DNA Seq 1991;2:111-9.

(*30*) Clark SJ, Harrison, J., Paul, C.L. and Frommer, M. High sensitivity mapping of methylated cytosines. Nucleic Acids Res 1994;22:2990-7.

(*31*) Gareau J-L, P., Houle, B., Leduc, F., Bradley, W.E.C. and Dobrovic, A. A frequent HindIII RFLP on chromosome 3p21-25 detected by a genomic erbAb sequence. Nucl. Acids Res 1988;16:201.

(*32*) Przybytkowski E, Girouard S, Allard B, Lamarre L, Basik M. Widespread bimodal intrachromosomal genomic instability in sporadic breast cancers associated with 13q allelic imbalance. Cancer Res 2003;63:4588-93.

(*33*) Gama-Sosa MA, Slagel VA, Trewyn RW, Oxenhandler R, Kuo KC, Gehrke CW, et al. The 5-methylcytosine content of DNA from human tumors. Nucleic Acids Res 1983;11:6883-94.

(*34*) Feinberg AP, Gehrke CW, Kuo KC, Ehrlich M. Reduced genomic 5-methylcytosine content in human colonic neoplasia. Cancer Res 1988;48:1159-61.

(*35*) Smith ZE, Higgs DR. The pattern of replication at a human telomeric region (16p13.3): its relationship to chromosome structure and gene expression. Hum Mol Genet 1999;8:1373-86.

(*36*) Houle B, Pelletier, M., Wu, J., Goodyer, C., Bradley, W. E. Fetal isoform of human retinoic acid receptor beta expressed in small cell lung cancer lines. Cancer Res 1994;54:365-9.

(*37*) Widschwendter M, Berger J, Hermann M, Muller HM, Amberger A, Zeschnigk M, et al. Methylation and silencing of the retinoic acid receptor-beta2 gene in breast cancer. J Natl Cancer Inst 2000;92:826-32.

(*38*) Boyes J, Bird A. Repression of genes by DNA methylation depends on CpG density and promoter strength: evidence for involvement of a methyl-CpG binding protein. Embo J 1992;11:327-33.

(*39*) Pao MM, Tsutsumi M, Liang G, Uzvolgyi E, Gonzales FA, Jones PA. The endothelin receptor B (EDNRB) promoter displays heterogeneous, site specific methylation patterns in normal and tumor cells. Hum Mol Genet 2001;10:903-10.

(*40*) Sirchia SM, Ferguson AT, Sironi E, Subramanyan S, Orlandi R, Sukumar S, et al. Evidence of epigenetic changes affecting the chromatin state of the retinoic acid receptor beta2 promoter in breast cancer cells. Oncogene 2000;19:1556-63.

(*41*) Singh N, Ebrahimi FA, Gimelbrant AA, Ensminger AW, Tackett MR, Qi P, et al. Coordination of the random asynchronous replication of autosomal loci. Nat Genet 2003;33:339-41.

(*42*) Cameron EE, Baylin SB, Herman JG. p15(INK4B) CpG island methylation in primary acute leukemia is heterogeneous and suggests density as a critical factor for transcriptional silencing. Blood 1999;94:2445-51.

(*43*) Song SH, Jong HS, Choi HH, Kang SH, Ryu MH, Kim NK, et al. Methylation of specific CpG sites in the promoter region could significantly down-regulate p16(INK4a) expression in gastric adenocarcinoma. Int J Cancer 2000;87:236-40.

(*44*) Kinoshita H, Shi Y, Sandefur C, Meisner LF, Chang C, Choon A, et al. Methylation of the androgen receptor minimal promoter silences transcription in human prostate cancer. Cancer Res 2000;60:3623-30.

(45) Koizume S, Tachibana K, Sekiya T, Hirohashi S, Shiraishi M. Heterogeneity in the modification and involvement of chromatin components of the CpG island of the silenced human CDH1 gene in cancer cells. Nucleic Acids Res 2002;30:4770-80.

(46) Tan LW, Bianco T, Dobrovic A. Variable promoter region CpG island methylation of the putative tumor suppressor gene Connexin 26 in breast cancer. Carcinogenesis 2002;23:231-6.

(47) Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 1996;93:9821-6.

(48) Sirchia SM, Ren M, Pili R, Sironi E, Somenzi G, Ghidoni R, et al. Endogenous reactivation of the RARbeta2 tumor suppressor gene epigenetically silenced in breast cancer. Cancer Res 2002;62:2455-61.

(49) Cote S, Sinnett D, Momparler RL. Demethylation by 5-aza-2'-deoxycytidine of specific 5-methylcytosine sites in the promoter region of the retinoic acid receptor beta gene in human colon carcinoma cells. Anticancer Drugs 1998;9:743-50.

(50) Jones PA. The DNA methylation paradox. Trends Genet 1999;15:34-7.

(51) Dobrovic A, Gareau, J. L., Ouellette, G., Bradley, W. E. DNA methylation and genetic inactivation at thymidine kinase locus: two different mechanisms for silencing autosomal genes. Somat Cell Mol Genet 1988;14:55-68.

(52) Bradley WE. Mutation at autosomal loci of Chinese hamster ovary cells: involvement of a high-frequency event silencing two linked alleles. Mol Cell Biol 1983;3:1172-81.

(53) Grant SG, Campbell, C. E., Duff, C., Toth, S. L., Worton, R. G. Gene inactivation as a mechanism for the expression of recessive phenotypes. Am J Hum Genet 1989;45:619-34.

(54) Arapshian A, Kuppumbatti YS, Mira-y-Lopez R. Methylation of conserved CpG sites neighboring the beta retinoic acid response element may mediate retinoic acid receptor beta gene silencing in MCF-7 breast cancer cells. Oncogene 2000;19:4066-70.

(55) Xu L, Glass CK, Rosenfeld MG. Coactivator and corepressor complexes in nuclear receptor function. Curr Opin Genet Dev 1999;9:140-7.

(56) Nakayama T, Watanabe M, Yamanaka M, Hirokawa Y, Suzuki H, Ito H, et al. The role of epigenetic modifications in retinoic acid receptor beta2 gene expression in human prostate cancers. Lab Invest 2001;81:1049-57.

(57) Hayatsu H. Bisulfite modification of nucleic acids and their constituents. Prog Nucleic Acid Res Mol Biol 1976;16:75-124.

(58) Shortle D, Nathans D. Local mutagenesis: a method for generating viral mutants with base substitutions in preselected regions of the viral genome. Proc Natl Acad Sci U S A 1978;75:2170-4.

(59) Rother KI, Silke J, Georgiev O, Schaffner W, Matsuo K. Influence of DNA sequence and methylation status on bisulfite conversion of cytosine residues. Anal Biochem 1995;231:263-5.

(60) Tasheva ES, Roufa DJ. Deoxycytidine methylation and the origin of spontaneous transition mutations in mammalian cells. Somat Cell Mol Genet 1993;19:275-83.

(*61*) Feil R, Charlton J, Bird AP, Walter J, Reik W. Methylation analysis on individual chromosomes: improved protocol for bisulphite genomic sequencing. Nucleic Acids Res 1994;22:695-6.

(*62*) Reeben M, Prydz H. An improved method for detection of 5-methylcytosine by PCR-based genomic sequencing. Biotechniques 1994;16:416-7.

(*63*) Raizis AM, Schmitt F, Jost JP. A bisulfite method of 5-methylcytosine mapping that minimizes template degradation. Anal Biochem 1995;226:161-6.

(*64*) Grigg GW. Sequencing 5-methylcytosine residues by the bisulphite method. DNA Seq 1996;6:189-98.

(*65*) Paulin R, Grigg GW, Davey MW, Piper AA. Urea improves efficiency of bisulphite-mediated sequencing of 5'-methylcytosine in genomic DNA. Nucleic Acids Res 1998;26:5009-10.

(*66*) Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 1999;99:247-57.

(*67*) Trasler JM. Origin and roles of genomic methylation patterns in male germ cells. Semin Cell Dev Biol 1998;9:467-74.

(*68*) Smith-Ferguson AC, Surani. Imprinting and the epigenetic asymmetry between parental genomes. Science 2001;293:1086-9.

(*69*) Tsou JA, Hagen JA, Carpenter CL, Laird-Offringa IA. DNA methylation analysis: a powerful new tool for lung cancer diagnosis. Oncogene 2002;21:5450-61.

EXPLANATION REGARDING THE SEQUENCE OF MANUSCRIPTS

Several lines of evidence suggest that the retention of RARβ2 expression represents a selective advantage for cancer cell growth and oncogenic character (see section 6. *Evidence contradicting the postulate that RARβ2 acts solely as a TSG*). And, among other findings, the results from the previous manuscript (Chapter 2) suggest that the potential for RARβ2 expression exists from the unmethylated allele. Two questions naturally arise: (1) *Are there functional reasons why one RARβ2 allele is never genetically altered in cancer cells?* That is to ask, *Is methylation, through its reversibility, an epigenetic aberration reserved for genes whose expression is required during carcinogenesis?; and (2) What are the effects of knocking down RARβ2 expression in cancer cell lines having retained RARβ2 expression?* The main objectives of the following manuscript (Chapter 3) are to determine the effect of knocking down RARβ2 expression in cancer cell lines *having retained RARβ2 expression* on cancer cell growth and to elucidate and compare the gene expression profiles in cancer cells following retinoid stimulation/RARβ2 activation versus RARβ2 inhibition. This is all the more a timely and appropriate approach considering the fact that the great majority of studies to date have tested the effects of expressing RARβ2 constructs in cancer cells that have *lost* RARβ2 expression, rather than the effects of inhibiting or reducing RARβ2 expression in cells that have *retained* RARβ2 expression.

CHAPTER 3

MANUSCRIPT 2

Antisense oligonucleotides against RARβ2 reduce proliferation and oncogenic phenotype of lung cancer cell lines

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ABSTRACT

INTRODUCTION: Retinoic acid receptor β2 (RARβ2) is a putative tumor suppressor gene that undergoes loss of expression in ~50% of all lung cancers, and most studies suggest that the mechanism of inactivation occurs through methylation, which is a reversible process. The majority of lung cancers therefore retain the potential for RARβ2 expression. Moreover, a recent finding unexpectedly showed that RARβ2 expression was correlated with worse prognosis in patients with non-small cell lung carcinoma (NSCLC), and two major chemoprevention trials showed that retinoic acid (RA), the activating ligand of RARs, may not have uniquely protective effects in patients at risk for developing lung cancer, but may actually be harmful. **HYPOTHESIS:** Based on these findings, we postulated that RARβ2 expression is necessary for cancer cell growth and survival in cancer cells having retained RARβ2 expression. **METHODS:** We therefore designed and tested 18 antisense oligonucleotides (oligos) against RARβ2 in various cancer cell lines, and used microarray technology to elucidate and compare the patterns of gene expression upon treatment with antisense oligo or RA. **RESULTS:** Upon testing their ability to reduce RARβ2 mRNA expression in A-549 lung cancer cells, we found that cellular proliferation was correlated with the level of RARβ2 expression following antisense oligo treatment. We then found that the most potent oligo, JP-2, altered the level of RARβ2 mRNA expression in a dose-dependent fashion. The most effective oligos, including JP-2, both reduced cellular proliferation and caused apoptosis in multiple cancer cell lines in which RARβ2 expression has been retained, whereas the same oligos had no apparent effects in three cancer cell lines in which it has been lost. Finally, we performed paired microarray experiments in which we compared mRNA expression in A-549 cells following JP-2 treatment versus cells treated with ATRA. Results showed a striking decrease in the expression of multiple genes contributing to carcinogenesis and/or resistance to chemotherapeutic compounds in cells treated with JP-2, and some of these genes were up-regulated upon treatment with ATRA. **CONCLUSION:** Our results show that RARβ2 expression is necessary for growth and maintenance of the oncogenic phenotype in certain RARβ2-expressing cancer cell lines. This is a novel finding since exogenous RARβ2 expression has been shown to have tumor suppressive effects in multiple cancer cell lines having lost endogenous RARβ2 expression. This duality of function demonstrated for the first time here may have wide

implications in cancer research since it helps explain one potential source of the inconsistent findings surrounding both experimental research on RARβ2 and chemoprevention trials on the use of retinoids in patients suffering from or at risk for lung cancer.

1. Introduction

The retinoic acid receptor β2 (RARβ2) is a nuclear transcription factor (1) that has been studied for its tumor suppressive activities since 1987 when it was initially cloned (*2,3*). In 1982, its chromosomal locus, 3p24, was shown to be genetically altered by loss of heterozygosity (LOH) in 90% of all non-small cell (NSCLCs) and 100% of all small cell lung carcinomas (SCLCs) (4), and RARβ2 is known to undergo loss of mRNA expression in ~50% of all lung cancers, including both NSCLCs and SCLCs. RARβ2 has been shown to be inactivated and to have tumor suppressive properties in numerous cancer-derived cell lines, including those derived from lung, breast and colon cancers (5-12). In addition, modulation of RARβ2 expression in transgenic mice by dominant-negative or antisense constructs results in the appearance of tumors in which endogenous RARβ2 expression is greatly reduced (13).

Nevertheless, several lines of evidence cast doubt on the status of this gene as a tumor suppressor. First, the appearance of point mutations in tumors, a hallmark of tumor suppressor genes, has not been reported. RARβ2 promoter methylation, which has been widely reported as an important contributor to inactivation of tumor suppressor genes, may serve as the second hit at this locus, replacing point mutation in the two-hit model of Knudson (14), but the complete lack of mutations remains unexplained. Second, not only is methylation reversible, but numerous groups including our own have analyzed the RARβ2 promoter and all report the existence of cell lines where inactivation is not correlated with methylation (*15-23*). We have also found that several cell lines exhibit *divergent methylation*, where differentially methylated alleles (both hypo- and hyper-methylated) coexist within the same cell, consequently suggesting the existence of unmethylated alleles (*23*). Third, NSCLC tumors expressing RARβ2 are associated with poorer prognosis than those in which the gene is inactivated, the opposite of what is expected for a tumor suppressor (*24*). Fourth, a further difficulty arises from the results of expression studies of RARβ2 in normal bronchial tissue of patients at risk for - or already diagnosed with - lung cancer. Both our results (*25*) and those of others (*26*) suggest that as much as half of the respiratory epithelium of patients at risk comprises foci of normal cells which are deficient in RARβ2 expression; this should provide an enormous pool of cells predisposed to the carcinogenic process. The fact that at the

end of this process half of all tumors express RARβ2 suggests that the initial turn-off did not provide the advantage to the cells that we would expect from the inactivation of a tumor suppressor. Finally, the disappointing results of several major chemoprevention trials with beta-carotene and retinoic acid (RA) are also inconsistent with a tumor suppressive effect of RARβ2 (*27,28*). In individuals at high risk of developing lung cancer, beta-carotene actually raised the relative risk by 16 to 29% compared to placebo (*27*). RA or retinyl palmitate were used in three of these interventions, and again, smokers on the intervention arm fared worse than those on the placebo, even though it is known from other studies that RA administration to this high-risk group results in the reactivation of RARβ2.

While many of the genes normally controlled by RARβ2 (and possibly other nuclear receptors) would be associated with maintenance of a normal, stable epithelial layer, we propose that under altered conditions which may occur during the early stages of tumor progression, retinoid signaling via RARβ2 may be altered such that genes under its control in tumor cells which express RARβ2 may actually contribute to further progression.

We have tested this hypothesis using antisense oligonucleotides (oligos) to knock down RARβ2 mRNA levels in several tumor-derived cell lines and we found a relation between the extent of knock-down and the inhibition of cell growth. Using microarray technology we were able to attribute this effect to altered patterns of gene control and found that some of the oncogenes most heavily inhibited following RARβ2 knockdown were some of the same genes most heavily upregulated upon treatment with RA.

2. Materials and Methods

2.1 Cell culture

Cells were grown in RPMI-1640 medium (Wisent Inc., St-Bruno, Quebec, Canada) supplemented with 5% heat-inactivated fetal calf serum (Wisent Inc., St-Bruno, Quebec, Canada) and 1% penicillin-streptomycin (LifeTechnologies, Burlington, Canada). Cells

were maintained in a 37°C/5% CO_2 incubator in a humidified atmosphere and were regularly passaged upon attaining 80-90% confluence.

2.2 Synthesis of antisense oligos against RARβ2

Phosphorothioate-modified 20-mer oligos complementary to the RARβ2 mRNA sequence were designed and synthesized (JP-1 to JP-18, and a scrambled control oligo, SCR; Table XXII). The phosphorothioate modifications were made using the Beaucage reagent for sulfurization and were incorporated via oxidation with a 10% solution of *t*-butylhydroperoxide in acetonitrile. Oligodeoxynucleotides were deprotected in concentrated ammonium hydroxide at 55°C for 24 hrs and purified by reversed-phase high performance liquid chromatography on a C-18 column using an acetonitrile gradient.

TABLE XXII. DNA sequences of antisense oligos JP-1 to JP-18, and SCR.

Oligo	Sequence 5'-3'	Oligo	Sequence 5'-3'
JP-1	GGTGTGGAGGCAAATGGCAT	JP-11	AAACATGTGAGGCTTGCTGG
JP-2	AAACATGATCTCCCTTGCAC	JP-12	GGACTGTGCTCTGCTGTGTT
JP-3	CCAGGATTTGCCCAGGACTC	JP-13	GGTTGCATGAAATGGCTGAT
JP-4	CTGCCATTCGGTTTGGGTCA	JP-14	CCTTGGAGGCTATCATTACT
JP-5	CACAGGCGCTGACCCCATAG	JP-15	TGGCATCAAGAAGGGCTGGA
JP-6	TTGGCATCGATTCCTGGTGA	JP-16	CTTTGCCAGGAGAGTCCATG
JP-7	GGAAAGTTTCCTGGTGAGCT	JP-17	GTCTTTGCCATGCATCTTGA
JP-8	TCGATTTAGGGTAAGGCCGT	JP-18	TACACGCTCTGCACCTTTAG
JP-9	AAGGCAGGAGCTGGTTGGCA	SCR	CGAACGCCATACCGAGCTCG
JP-10	TTCCTCAAGGTCCTGGCGGT		

2.3 Antisense oligo transfections

Cells (1.0 -2.0 x 10^6) were plated in 100 mm dishes and transiently transfected upon reaching 70% confluence. A final concentration of 400 nM was used unless otherwise indicated and the treatment was allowed to proceed for 4 hrs in a solution of Dulbecco's Modified Eagle's Medium (DMEM) and lipofectin Reagent (Life Technologies, Burlington, Canada). Care was taken to ensure that high doses known to cause non-specific effects were not used (*29*). The transfection solution contained 2.5 ug lipofectin/ml DMEM/100 nM antisense oligo. This solution was removed and replaced with complete medium for 24 hrs. Control (antisense oligo-negative) samples were treated with a solution of DMEM/lipofectin.

2.4 RARβ2, GAPDH and RARα mRNA expression analyses

mRNA expression was estimated by reverse transcription using Moloney Murine Leukemia virus (Life Technologies, Burlington, Canada) followed by polymerase chain reaction (PCR) using Taq DNA polymerase (Pharmacia). The number of PCR cycles was calibrated such that the optimal number was used. RARβ2 (31 cycles) was estimated using the oligos 5'-GGG-TAG-GAT-CCG-GAA-CGC-ATT-3' and 5'-GAC-GAG-TTC-CTC-AGA-GCT-GG-3'. The control gene GAPDH (23 cycles), which is constitutively expressed, was amplified at the annealing temperature of 51°C using the oligos 5'-TCA-TCA-TCT-CTG-CCC-CCT-CT-3' and 5'-TCC-GAC-GCC-TGC-TTC-ACC-AC-3'. RARα (35 cycles), also constitutively expressed, was amplified at the annealing temperature of 48°C using the oligos 5'-GAG GAC TTG TCC TGA CAG A-3' and 5'-CTT CTG ACT GTG GCC GCT-3' (not shown). The PCR reaction conditions were 5 uL RT product, 100 pmoles each primer, 0.2 mM dNTPs, 2.5 units Taq DNA polymerase in 1x Taq buffer. 20% of the total reaction was loaded onto a 1.5% agarose gel and band signal intensities were estimated using spot densitometry (Alphalmager).

2.5 Cell counting

24 hrs following transient transfection (4 hrs), the remaining adherent cells were trypsinized, stained with Trypan Blue (Life Technologies, Burlington, Canada) and counted using a Neubauer brightline hemacytometer. All cell counts were performed in

duplicate, minimum. In some experiments, RNA yield was used to reflect cell culture mass.

2.6 Programmed cell death analyses

Floating cells were discarded and the remaining adherent cells were rinsed with PBS, trypsinized, and rinsed again twice. Cells were then incubated with or without FITC-labeled anti-annexin V antibody (PharMingen, Becton Dickinson), conjugated to FITC-A, in 100 ul of buffer for 30 minutes on ice in the dark. The cells were rinsed twice and resuspended in 0.5 ug/ml propridium iodide. Cells were gated, analyzed, and counted by fluorescence-activated cell sorting (FACS).

2.7 ATRA and JP-2 microarray experiments

2.7.1 Cell treatments

Two paired microarray experiments were performed: (1) A-549 human lung adenocarcinoma cells were treated with *all-trans* retinoic acid (ATRA; final ATRA concentration = 1 uM) dissolved in ethanol or with ethanol alone (control) in plain DMEM medium for 4 hours ("ATRA microarray"); or with (2) JP-2 antisense oligo dissolved in lipofectin (final JP-2 concentration = 400 nM) or with lipofectin alone (control) in plain DMEM medium for 4 hours ("JP-2 microarray"). To ensure that extraneous factors were held constant, cells treated with ATRA or ethanol were equally treated with lipofectin, and cells treated with JP-2 or lipofectin were equally treated with ethanol. Treatments proceeded for 4 hours and respective solutions were removed and replaced with RPMI supplemented with 5% FBS and 1% penicillin-streptomycin for 24 hrs.

2.7.2 Poly A RNA extraction, reverse transcription and fluorescent labeling

RNA was extracted using Trizol and subsequently purified to enrich for the poly A-containing fraction using the PolyA RNA Midi Kit (Qiagen, Mississauga, Ontario). Poly A RNA was then reverse transcribed using oligo-(dT) with Superscript II (Stratagene, La Jolla, CA) and then fluorescently labeled with Cy5-dCTP and Cy3-dCTP (Pharmacia).

2.7.3 cDNA microarrays

14,080 genes, established sequence tags (ESTs), and hypothetical proteins were represented on each of two microarrays, which were printed at the Microarray Core Facility at the National Human Genome Research Institute (NHGRI), under the direction of Abdel G. Elkahloun. Spots consisted of I.M.A.G.E. consortium clones that were PCR-amplified into DNA fragments of approximately 150 bp.

2.7.4 cDNA and microarray hybridization

The fluorescently-labeled cDNAs were hybridized overnight at 60°C in TE pH7.4, Cot-1 DNA, polydA, yeast tRNA and Denhardt's, SSC (2X), SDS (0.25%).

2.7.5 Image detection and analysis using DeArray software

Fluorescence intensities of the hybridized spots were measured using a laser confocal scanner (Agilent Technologies, Palo Alto, CA) and the data for each microarray were analyzed using DeArray software (*30*), developed within the NHGRI. After background subtraction, average intensities at each clone in the treated hybridization were divided by the average intensity of the corresponding clone in the untreated hybridization. Ratios were normalized based on the distribution of all targets on the array. A ratio of quality was calculated for each spot on the microarray according to several parameters (*31*), including spot morphology, uniformity, intensity, and background. Low quality measurements (e.g. mean fluorescence intensities < 50 for both channels and ratio of quality < 0.2) were excluded from further analysis and were treated as missing values (units are arbitrary and reflect scanner fluorescence output).

2.7.6 Selective and statistical treatment of microarray fluorescence intensities

After background subtraction, average intensities at each clone in the treated hybridization were divided by the average intensity of the corresponding clone in the untreated hybridization. Ratios were normalized based on the distribution of all targets on the array. Low quality measurements (e.g. mean fluorescence intensities < 50 for

both channels and ratio of quality<0.2) were excluded from further analysis and were treated as missing values. Overall quality of the microarray experiment was evaluated based on the number of high quality measurements. Statistical significance of the normalized ratios was calculated based on 99% confidence intervals of the distribution of all targets prior to exclusion. Only high quality values were taken into consideration for further analysis. For each clone, the calibrated treated/untreated ratio was calculated and normalized for each experiment. Of note is that 88 clones were spotted 8 times, allowing for internal quality control of the microarrays.

3. Results

3.1 Transfection of antisense oligos against RARβ2 decreases RARβ2 mRNA expression in A-549 lung cancer cells

The efficiency of each of the 18 antisense oligos (JP-1 to 18) to decrease the level of RARβ2 mRNA in A-549 lung adenocarcinoma cells was tested 24 hrs following a 4-hr transient transfection using semi-quantitative RT-PCR (Fig. 27). One of the 18 antisense oligos, JP-2, was found to be particularly effective in reducing RARβ2 mRNA (reaching 60-65% inhibition). The control genes, GAPDH and RARα (results not shown) were not affected. Transfections were repeated in quadruplicate.

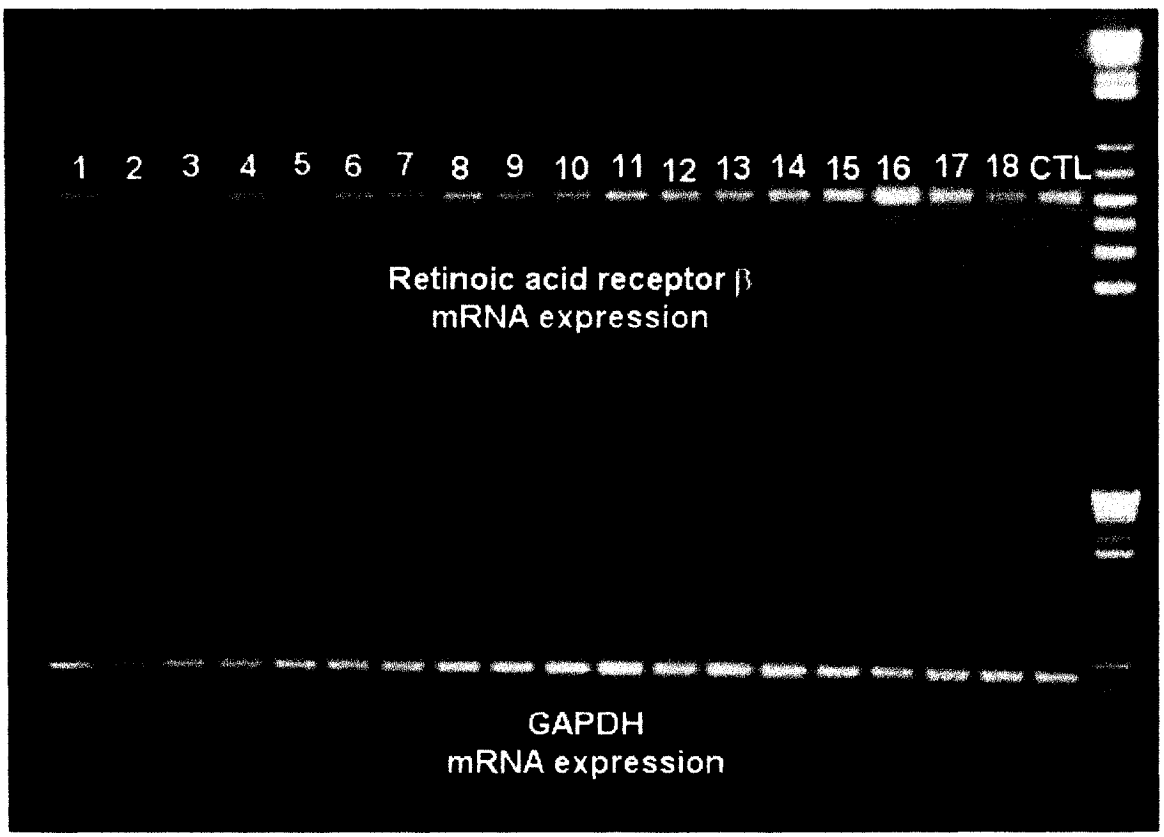

FIGURE 27: Representative 2% agarose gel of RARβ2 versus GAPDH mRNA expression (RT-PCR) 24 hrs following a 4-hr transient transfection with antisense oligos JP-1 – JP-18 against RARβ2 mRNA (final concentration = 400 nM), or with lipofectin alone, in A-549 lung adenocarcinoma cells. **Top**: RARβ2; **Bottom**: GAPDH control. Loaded were 20% of total reaction volume of each sample and 700 ng of the 1 kb ladder. 1 - 18 = JP-1 to JP-18; CTL = Lipofectin alone.

3.2 The effect of JP-2 to inhibit RARβ2 mRNA expression is dose-dependent

The level of inhibition of RARβ2 mRNA expression was tested using the most efficient antisense oligo, JP-2, at various concentrations ranging from 0 to 1200 nM, 24 hrs following a 4-hr transient transfection. The decrease in RARβ2 mRNA was found to be dependent on the dose of JP-2 used, and reached a maximum inhibition of approximately 70% at 1250 nM (Fig. 28). The dose used for all following experiments was 400 nM, since higher doses were not much more effective, and since higher concentrations are associated with non-specific toxicity due to aptameric effects (*29*). This dose caused near maximal inhibition, ranging from 60 to 65%. Transfections were repeated in quadruplicate.

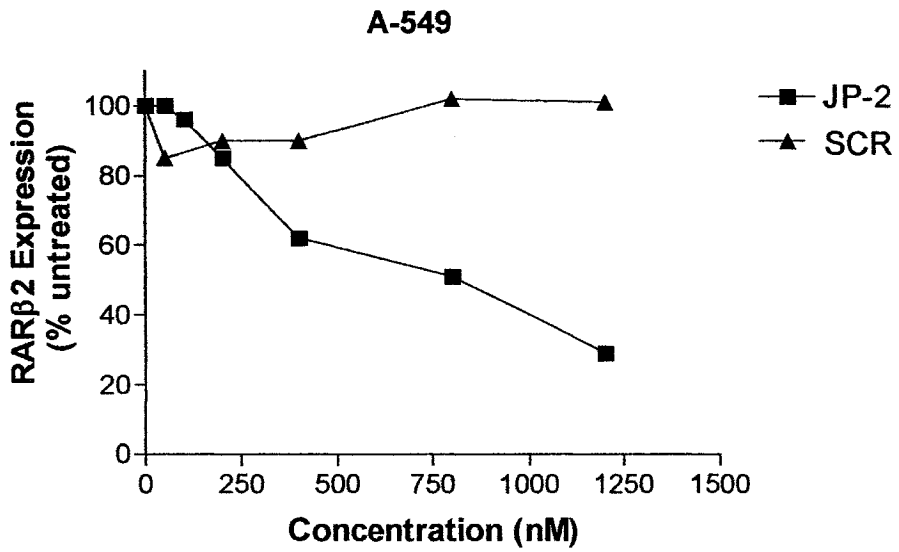

FIGURE 28: Graph illustrating the dose-dependent reduction of RARβ2 mRNA expression 24 hrs following a 4-hr transient transfection with the JP-2 antisense oligo against RARβ2 mRNA or the control SCR antisense oligo (final concentrations = 0, 50, 100, 200, 400, 800, 1200 nM) in A-549 cells.

3.3 A-549 cell growth is direcly correlated with the level of RARβ2 mRNA expression

A-549 cell growth was plotted against the level of RARβ2 mRNA expression 24 hrs following a 4-hr transient transfection with each of the oligos (JP-1 to JP-18). Growth and RARβ2 mRNA expression were directly correlated (Fig. 29), with $p<0.0005$.

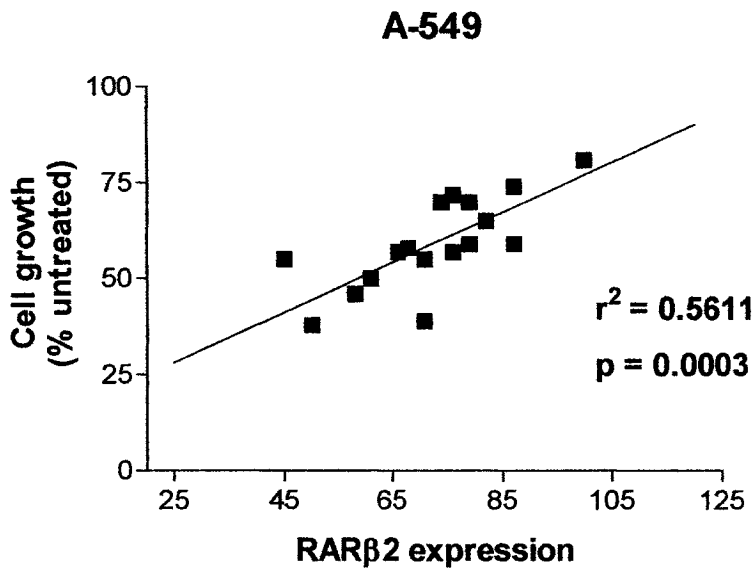

FIGURE 29: Scatter plot illustrating the correlation between the effect of antisense oligos JP-1 to JP-18 (final concentration = 400 nM) on RARβ2 mRNA expression and growth of A-549 cells.

3.4 JP-2 reduces cell growth by up to 80% in RARβ2-expressing cells

The most effective antisense oligo, JP-2, was tested in 6 cancer cell lines that either had maintained or lost endogenous RARβ2 expression. JP-2 significantly reduced cell growth in RARβ2-expressing cancer-derived cell lines such as NCI-H125, NCI-H23 and A-549 (Fig. 30, top), whereas the control oligo SCR or the lipofectin reagent alone had no apparent effect. JP-2 did not affect cell growth in cell lines where RARβ2 has been lost, such as NCI-H596 or CALU-1 (Fig. 30, bottom left) and SK-MES (data not shown). Normal bronchial epithelial cells (NBE-E6E7) were not affected by either JP-2 or SCR (Fig. 30, bottom right).

EFFECT OF ANTISENSE OLIGO TREATMENTS ON VARIOUS CELL TYPES

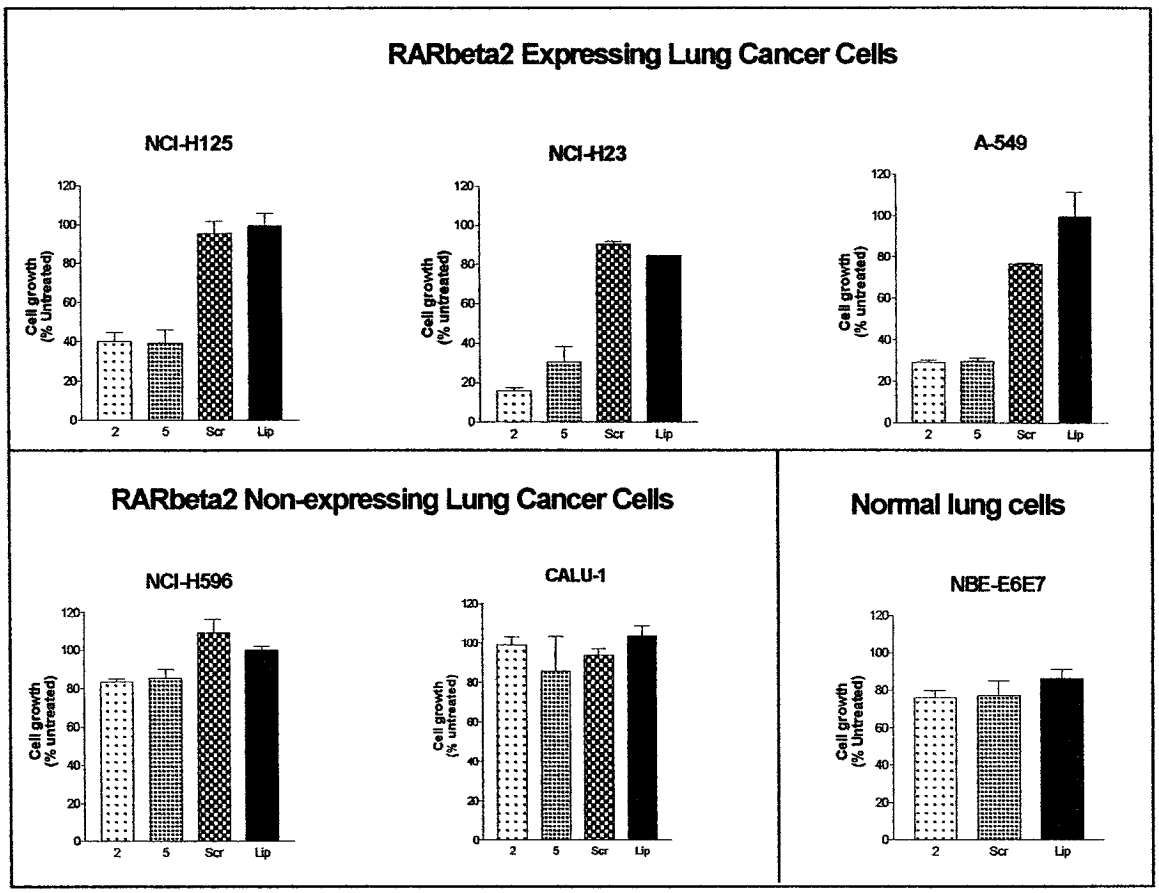

FIGURE 30: Composite bar graph illustrating the effects of JP-2 compared to the control oligo SCR or the lipofectin reagent alone, on RARβ2-expressing cancer cells, versus their lack of effect on RARβ2-non-expressing cancer cells. JP-5, the second most effective oligo in the genewalk experiment, had somewhat similar results as JP-2. **Top**: RARβ2-expressing NCI-H125 adenosquamous cells, NCI-H23 adenocarcinoma cells, and A-549 adenocarcinoma cells; **Bottom left**: RARβ2-non-expressing NCI-H596 adenosquamous cells and CALU-1 epidermoid cells; **Bottom right**: NBE-E6E7 normal bronchial epithelial cells.

3.5 Phase contrast micrography

The RARβ2-negative cell line CALU-1 remained apparently unaffected when treated with JP-2 or the control oligo SCR (Fig. 31a and b, respectively). However, the RARβ2-expressing cell line NCI-H125 showed significant proliferation block and death when treated with JP-2 (Fig. 31c), but remained apparently unaffected when treated with control oligo SCR (Fig. 31d).

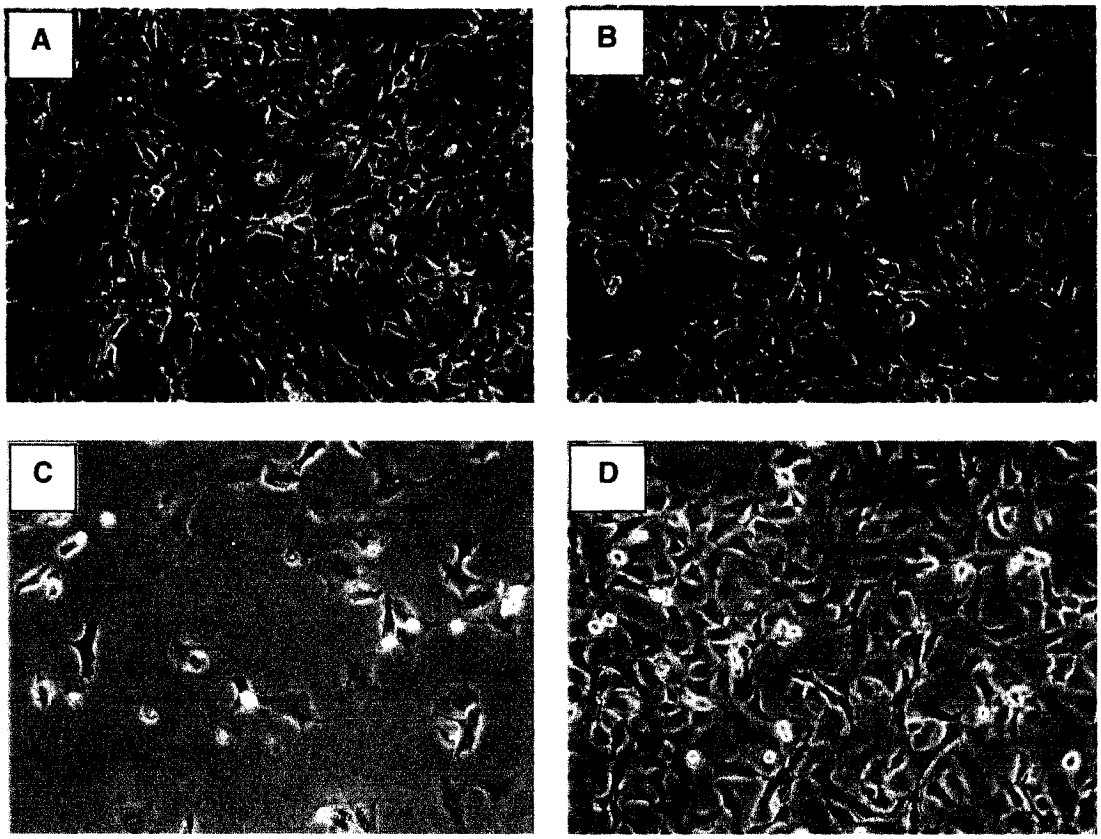

FIGURE 31: Composite micrograph illustrating the killing effects of the antisense oligo JP-2 against RARβ2-expressing cells, 24 hrs following a 4-hr transient transfection with 400 nM of antisense oligo. **A**. JP-2-treated CALU-1 RARβ2-negative cancer cells; **B**. SCR-treated CALU-1 RARβ2-negative cancer cells; **C**. JP-2-treated NCI-H125 RARβ2-expressing cancer cells; **D**. SCR-treated NCI-H125 RARβ2-expressing cancer cells (control oligo).

3.6 The reduction in cell growth following JP-2 treatment is at least partially due to activation of programmed cell death

Annexin V staining was used to test NCI-H23 and NCI-H125 adenocarcinoma cells as early as 4 hrs following a 4-hr transient transfection with JP-2 or SCR. This assay tests for the presence of phosphatidylserine on the outer leaflet of the plasma membrane, resulting from its translocation from the inner leaflet, one of the earliest events that occur during apoptosis. The results repeatedly showed that NCI-H23 cells for example, have a baseline rate of apoptosis of approximately 10.8%, but this increases to about 28.2% in the presence of JP-2, and 19.5% in the presence of the control oligo SCR (Table XXIII,

representative experiment shown in Fig. 32). Similar results were observed in the H-125 cell line (Table XXIII).

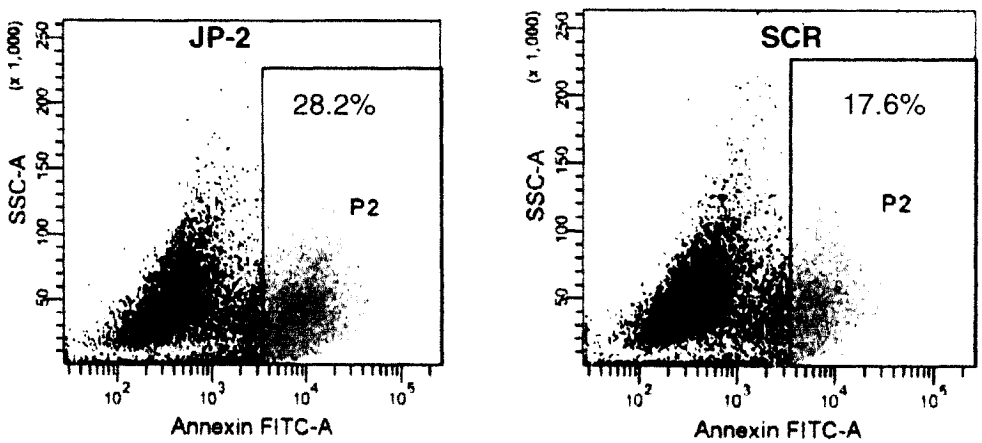

FIGURE 32: Graphs illustrating early apoptotic effects (Annexin V staining and fluorimetric analyses), 4 hrs following a 4-hr transient transfection with 400 nM of antisense oligo JP-2 versus SCR control oligo, in human NCI-H23 lung adenosquamous cells that express the RARβ2 gene. **Left**: JP-2; **Right**: SCR.

Importantly, these figures are conservative estimates since gating was set using cells with 99% viability following trypan blue staining. These results suggest that the process responsible for death of the cells such as those observed above is apoptosis. These effects were not seen in the cell line CALU-1, which does not express RARβ2 (results not shown).

TABLE XXIII. Programmed cell death analyses using Annexin V-FITC-A labeling of NCI-H23 and NCI-H125 cells 4 hrs following a 4-hr treatment with JP-2 or SCR antisense oligo (final concentration = 400 nM). Average errors shown. Baseline = baseline rate of apoptosis observed in untreated cells.

Cell line	JP-2	SCR	Baseline
NCI-H23	28.2 ± 0.1	19.5 ± 1.9	10.8
NCI-H125	23.2 ± 0.2	12.3 ± 0.3	10.1

3.7 Inhibition of RARβ2 mRNA expression reduces the expression of important downstream genes involved in tumor promotion

14,080 genes, ESTs, and hypothetical proteins were represented on the microarrays. Of these, 13,547 were of sufficient quality on the antisense JP-2 array while 8,728 clones were of adequate quality on the ATRA treatment array. Indeed, the JP-2 microarray was of high quality while the ATRA microarray was of moderate quality (only 62% of the clones were available for analysis by our criteria). Since the microarrays have 88 clones which were spotted 8 times (4 spots in 2 different zones of the microarray slide each), we were able to calculate a measure of internal quality control. Standard deviations of the calibrated ratios were calculated for each clone as a percentage of the mean ratio. Median percent standard deviation of the mean for the entire JP-2 set for all 88 clones was only 4%, while that for the ATRA set was 9%, reflecting the superior quality of the JP-2 microarray.

Using a very stringent cut-off of the 99% confidence interval for under or over-expression for the microarrays, we found that for the JP-2 array, clones with values <0.625 or >1.60 were significantly different from the entire set ($p<0.0001$), while for the ATRA array, clones with values <0.69 or >1.44 were significantly different ($p<0.001$). For the JP-2 array, this meant that 901 (6.7%) of the high quality clones showed relative over-expression compared to untreated cells, while for the ATRA array, 199 (2.2%) were over-expressed compared to untreated cells. Similarly, 326 (2.4%) of the high-quality clones were down-regulated in the JP-2 array while 188 (2.2%) of the high-quality clones were down-regulated in the ATRA microarray. Interestingly, only 6 over-expressed clones and 5 down-regulated clones were common to the two experiments, reflecting a distinct biological effects of the two treatments.

The second most down-regulated gene in the JP-2 microarray was the RARβ (0.35 ratio), thus validating the microarray experiment. Analysis of the gene expression data from the JP-2 microarray using the SOURCE database (*32*) (http://source.stanford.edu) effectively categorized the genes in seven categories, including: growth, apoptosis, cytoskeleton, biosynthesis, immune function, metabolism, and signal transduction. Analysis revealed that the majority of genes that were down-regulated in the JP-2-treated cells are involved in many aspects of tumorigenicity and tumor promotion.

Presented here are the top 5 down-regulated and top 5 up-regulated genes by JP-2 per category, for the JP-2 microarray (Tables XXIV and XXV, respectively).

TABLE XXIV. Top 5 down-regulated genes by JP-2 per category, including: up-regulated by ATRA; growth; apoptosis; cytoskeleton; biosynthesis; immune; metabolism; and signal transduction.

TOP 5 DOWNREGULATED GENES BY JP-2 PER CATEGORY					
CATEGORY	Rank	GENE NAME	GENBANK ID	JP-2	ATRA
Up-regulated by ATRA	1	retinoic acid receptor, beta	AA419238	0.35	1.10
	2	cytochrome P450, subfamily XXIV	N30976	0.38	4.41
	3	HSPC037 protein	AA961752	0.40	1.61
	4	ribonucleotide reductase M2 polypeptide	AA187351	0.41	1.52
	5	Homo sapiens endothelin-1 (EDN1)	H11622	0.49	2.10
Growth	1	fibroblast growth factor receptor 4	AW025920	0.49	0.94
	2	leukemia-associated phosphoprotein p18	AA873060	0.49	1.49
	3	transforming acidic coiled-coil containing protein 3	AA279990	0.53	1.15
	4	insulin-like growth factor binding protein 3	AA598601	0.56	2.56
	5	mothers against decapentaplegic, Dros. hom. 6	AA007518	0.56	0.76
Apoptosis	1	apoptosis inhibitor 4 (survivin)	AA460685	0.46	1.02
	2	E2F transcription factor 1	AA424950	0.52	1.10
	3	NCK-associated protein 1	AW440540	0.58	0.92
	4	dedicator of cyto-kinesis 1	W02657	0.63	0.88
Cytoskeleton	1	tubulin, beta, 2	AA888148	0.52	0.99
	2	tubulin, beta, 5	N74524	0.55	0.98
	3	actinin, alpha 4	H51645	0.61	0.92
	4	ser/thr kinase with Dbl- and pleckstrin hom. domains	AI368219	0.66	0.92
	5	capping protein (actin filament), gelsolin-like	s	0.66	0.70
Biosynthesis	1	eukaryotic translation initiation factor 5A	AA878570	0.51	0.73
	2	ATP citrate lyase	AA136054	0.51	0.96
	3	Human acidic ribosomal phosphoprotein P0 Mrna	R42581	0.55	1.22
	4	farnesyl diphosphate synthase	T65790	0.58	1.05
	5	1-acylglycerol-3-phosphate O-acyltransferase 2	AA938623	0.61	1.00
Immune	1	parathymosin	R11526	0.57	0.68
	2	CCAAT/enhancer binding protein (C/EBP), delta	AA043494	0.63	0.85
	3	thymosin, beta 4, X chromosome	AA634103	0.63	1.14
	4	annexin A11	AA464982	0.64	1.18
	5	prothymosin, alpha (gene sequence 28)	R89610	0.65	2.42
Metabolism	1	fatty acid synthase	H50323	0.39	1.06
	2	thymidine kinase 1, soluble	AA778098	0.41	0.89
	3	pyruvate dehydrogenase kinase, isoenzyme 4	AA169469	0.48	0.72
	4	aldehyde dehydrogenase 1, soluble	AA664101	0.50	1.92
	5	aldo-keto reductase family 1, member C2	H91078	0.51	1.11
Signal transduction	1	retinoic acid receptor, beta	AA419238	0.35	1.10
	2	calcium-regulated heat-stable protein (24kD)	W72753	0.47	0.96
	3	leukemia-associated phosphoprotein p18 (stathmin)	AA873060	0.49	1.49
	4	inositol(myo)-1(or 4)-monophosphatase 2	R42685	0.54	0.85
	5	LIM protein (similar to rat PKC-binding enigma)	H12519	0.55	1.28

TABLE XXV. Top 5 up-regulated genes by JP-2 per category, including:down-regulated by ATRA; growth; apoptosis; cytoskeleton; biosynthesis; immune; metabolism; and signal transduction.

TOP 5 UPREGULATED GENES BY JP-2 PER CATEGORY					
CATEGORY	Rank	GENE NAME	GENBANK ID	JP-2	ATRA
Down-regulated by ATRA	1	membrane protein, palmitoylated 3	W44685	1.69	0.64
	2	similar to APOBEC1	AA864496	1.68	0.66
	3	BCL2-like 1	AA931820	1.58	0.58
	4	RAD51 (S. cerevisiae) homolog C	T64278	1.57	0.64
	5	chymotrypsinogen B1	AA845168	1.51	0.63
Growth	1	growth arrest and DNA-damage-inducible, beta	AA504354	8.35	0.81
	2	growth arrest and DNA-damage-inducible, beta	AA404666	8.10	1.11
	3	protein phosphatase 1D mg-dependent, delta	N33955	7.34	0.85
	4	lectin, galactoside-binding, soluble, 4	AA130579	5.52	1.07
	5	GTP-binding protein overexpressed in skeletal muscle	AA418077	5.38	1.27
Apoptosis	1	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	AI952615	12.08	1.18
	2	copine V	AW341769	11.14	0.96
	3	growth arrest and DNA-damage-inducible, beta	AA504354	8.35	0.81
	4	growth arrest and DNA-damage-inducible, beta	AA404666	8.10	1.11
	5	lectin, galactoside-binding, soluble, 4 (galectin 4)	AA130579	5.52	1.07
Cytoskeleton	1	MCF.2 cell line derived transforming sequence	H05800	12.19	0.85
	2	epithelial protein lost in neoplasm beta	AA487557	4.14	1.21
	3	ras homolog gene family, member E	AA443302	3.95	1.06
	4	advillin	AA427733	3.82	0.87
	5	rho/rac guanine nucleotide exchange factor (GEF) 2	AA464578	3.06	0.76
Biosynthesis	1	cytochrome P450, subfamily XXIA	AI972232	3.13	0.94
	2	acetylserotonin O-methyltransferase	AW003492	2.52	0.83
	3	UDP-glucose ceramide glucosyltransferase	AA456051	2.08	0.99
	4	tyrosyl-tRNA synthetase	AA486761	2.03	1.04
	5	glycogen synthase 2 (liver)	N52282	1.96	0.91
Immune	1	GTP-binding protein overexpressed in muscle	AA418077	5.38	1.27
	2	small inducible CK subfamily A (Cys-Cys), member 20	AI285199	4.55	0.90
	3	MHC1-like sequence	AA873499	4.03	1.01
	4	leukocyte-associated Ig-like receptor 1	AA991196	2.83	1.14
	5	X-box binding protein 1	W90128	2.76	1.11
Metabolism	1	phospholipase A2, group IVC	AW236676	7.82	1.32
	2	amine oxidase, copper containing 3	AI871588	5.19	1.14
	3	amine oxidase, copper containing 3	AA036975	4.04	1.18
	4	aldehyde oxidase 1	AI343711	3.75	1.15
	5	prostaglandin-endoperoxide synthase 2	AA644211	3.69	1.03
Signal transduction	1	interleukin 8	AA102526	13.60	1.18
	2	prostate differentiation factor	AI982657	5.91	1.04
	3	Ras-related associated with diabetes	W84445	5.64	0.84
	4	GTP-binding protein (skeletal muscle)	AA418077	5.38	1.27
	5	small inducible cytokine subfamily A (Cys-Cys), member 20	AI285199	4.55	0.90

Of all of the down-regulated genes, about half have a known biological function. Of these, several have well defined roles in carcinogenesis, and 17 are listed here (Table XXVI), including Cyp24 (vitamin D3 hydroxylase), aldehyde dehydrogenase-1 (ALDH1), alpha-tubulin (TUBA3), insulin-like growth factor binding protein-3 (IGFBP-3), survivin, cyclin D3, and v-Myb homology-like 2 (MYBL2).

TABLE XXVI. Relative expression levels of 17 selected genes for further study on A-549 ± JP-2 microarray and A-549±ATRA. Onc = known oncogenic activity; TSG = known tumor suppressor activity.

Gene	JP-2	RA	Gene product function	Onc	TSG
RARβ2	0.35	1.10	Nuclear transcription factor	●	●
Cyp24	0.38	4.41	Vit. D3 hydroxylase	●	○
NTRK3	0.36	0.99	Marker of cell proliferation and differentiation	●	●
Cyclin D3	0.42	N/A	Promotes cell cycle	N/A	N/A
Survivin	0.46	1.02	Inhibits of apoptosis	●	○
FGFR4	0.49	0.94	Growth factor	●	○
cyclin A2	0.49	0.95	Promotor of G1/S and G2/M	●	○
ET-1	0.49	2.1	Neoangiogenic and mitogenic	●	○
ALDH1	0.50	1.92	Metabolizes chemotherapeutic agents; synthesizes RA	●	○
TUBA3	0.51	2.04	Involved in mitosis	N/A	N/A
MYBL2	0.51	1.04	Regulates G1/S, highly expressed in proliferating cells	●	○
E2F1	0.52	1.10	Binds Rb, induces apoptosis	●	●
IGFBP3	0.56	2.56	Modulates IGF signaling; carrier of IGF1/2 in circulation	○	●
cyclin B1	0.57	1.02	Promotes G2/M	N/A	N/A
MHC I	2.10	N/A	Presents tumor antigen to CD8 cells	○	●
ICAM-1	2.99	N/A	Correlated with invasion/metastasis	●	●
COX-2	3.69	N/A	Mitogenic	●	○
CDKN1a	12.08	N/A	Inhibits cyclin kinase activity, induces G1/S arrest and apoptosis	○	●

In particular, the mRNA expression of Cyp24, a cytochrome-containing enzyme involved in reducing the efficiency of certain chemotherapeutic agents, such as vitamin D (calcitriol) or vitamin D analogs (*33*), was decreased by 62%. ALDH1, a key metabolic enzyme involved in the degradation of chemotherapeutic compounds such as nitrogen mustards and cyclophosphamides (*34*) was decreased by 50%.

Also, survivin, a dominant factor in apoptotic cascades capable of overriding pro-apoptotic signals and thereby rescuing cells from programmed cell death (*35*) was decreased by 54%. Similarly, cyclin D3 mRNA was reduced by 58%, and that of another cyclin, A2, was decreased by 51%. These genes code for proteins necessary for advancing the cell division process at various checkpoints throughout the cell cycle. In addition, the mRNA level of MYBL2, the most potent and highly-expressed of the myb family of oncogenes, and which promotes cell growth by activating CDC2 and cyclin D1, was reduced by 49%. The reduction of RARβ2 mRNA expression by antisense oligos thus reduced the expression of important downstream genes which can contribute in various ways to cancer progression.

Some of the genes down-regulated with JP-2 were up-regulated with ATRA, including, ALDH1, cyp24, and TUBA3. These genes are known to have oncogenic activity and are also known to be regulated by RA. [ALDH1 is definitely regulated through RA, Cyp24 is thought to be regulated through RA but additional proof is required (*36*)].

We chose six genes for further validation via semi-quantitative RT-PCR and confirmed the findings from the microarrays. Shown are the results from four of these genes (ALDH1, cyp24, survivin and COX-2; Fig. 33). Semi-quantitative RT-PCR results from rescue-type experiments in A-549 cells showed that the effects of JP-2 neutralized the effects of ATRA. The genes assayed were ALDH1 and cyp24 (not shown).

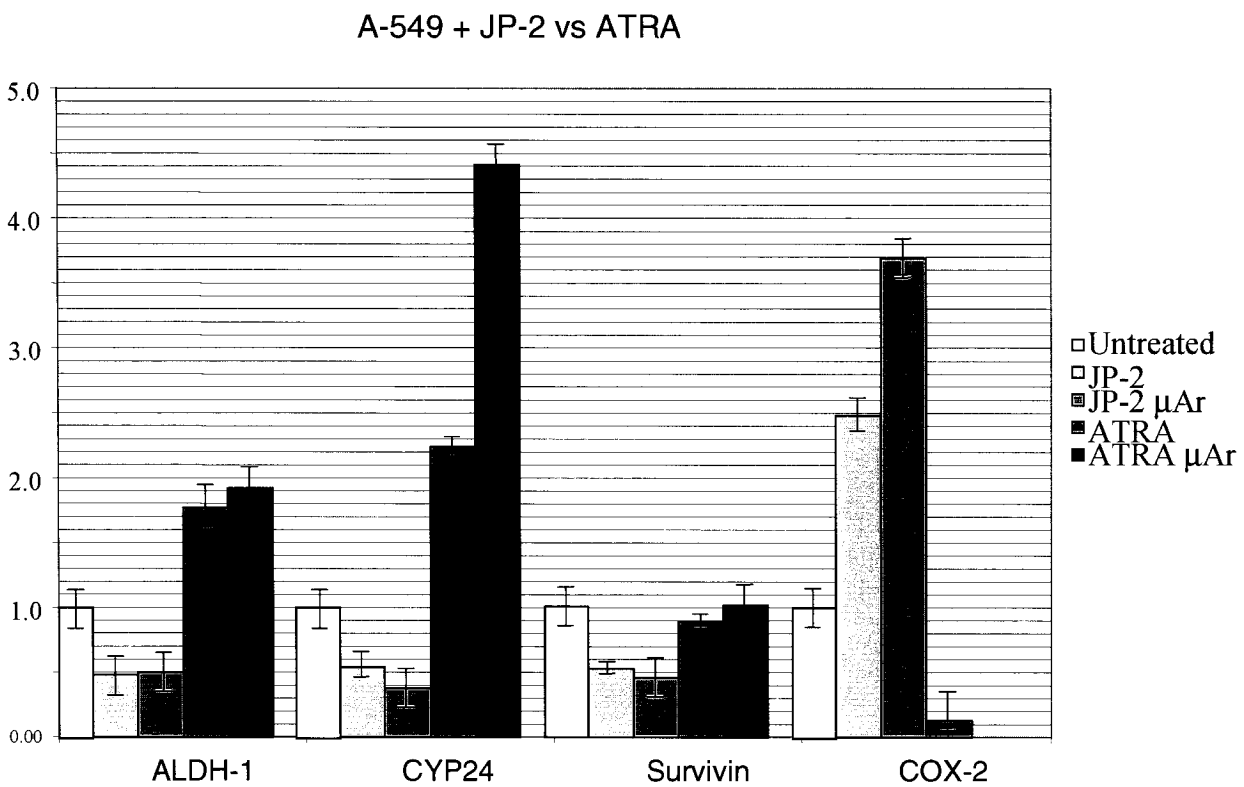

FIGURE 33: Representative graph illustrating the changes in mRNA expression as reflected by RT-PCR assays, normalized to untreated cells, of the JP-2 antisense oligo designed against RARβ2 mRNA, versus the effect of ATRA, on the genes ALDH-1, CYP24, survivin and COX-2, 24 hrs following a 4-hr treatment in A-549 cells. Final concentrations are 400 nM JP-2 and 1 uM ATRA. Also included for comparison are the corresponding results from the microarray experiments. The result for COX-2 + ATRA from the microarray is not available. Experiments were done in triplicate. µAr = microarray.

We previously found that RARβ2 differentially regulates the expression of several genes involved in the immunomodulation of cancer cells, including MHC Class I and ICAM-1, in particular (*37*). In particular, we found that RARβ2 upregulates MHC Class I and ICAM-1 in cells having lost RARβ2 expression but transfected (and expressing) exogenous RARβ2, such as CALU-1. These same genes were found to be up-regulated in A-549 cells treated with JP-2, at an average of 2.82 and 1.52, but not affected in cells treated with ATRA. We set out to determine the ability of the natural compound ATRA to differentially regulate other immunomodulatory genes, such as COX-2 and IL-1β, as well as the vascular gene endothelin-1 (ET-1). We used the C64 and C30 subclones of the CALU-1 cell line (Table XXVII), which were transfected with an exogenous RARβ2 gene

construct and the G418 resistance gene, respectively (*38*). We found that ATRA was able to up-regulate ET-1 and IL-1β, but not COX-2, in C64 cells treated with ATRA.

TABLE XXVII. COX-2, IL-1β and ET-1 mRNA expression analyses (RT-PCR) in CALU-1 transfectants. Values normalized according to GAPDH controls and C30 + EtOH.

Subclone	Treatment	COX-2	IL-1β	ET-1
C30	EtOH	1.0	1.0	1.0
	ATRA	0.7	1.3	0.8
C64	EtOH	0.6	1.5	0.7
	ATRA	0.9	3.5	1.3

4. Discussion

In using antisense oligos to inhibit the expression of the putative tumor suppressor gene RARβ2, we found that proliferation was correlated with the level of RARβ2 mRNA expression in A-549 lung cancer cells. We also found that the most effective antisense oligo, JP-2, specifically and rapidly decreased the level of mRNA expression by approximately 60-65%, and caused decreased cell growth as well as apoptosis in several RARβ2-expressing cancer cell lines (3 shown here). Control oligos, such as SCR, had no apparent effect, and 4 control cell lines (3 cancer-derived lines that had lost RARβ2 expression and 1 normal bronchial epithelial cell line) were not affected by test or by control oligos. These results support the hypothesis that RARβ2 is required for cancer cell growth, at least under certain circumstances, such as in cells having retained RARβ2 expression.

Previous transient transfection experiments against other genes, such as protein kinase C α (PKCα) or survivin, have shown that oligo uptake ranges from 70 to 90% [L.L. unpublished and reference (*35*)]. Our results show that the maximal inhibition of RARβ2 was approximately 70% at 1250 nM, which is only 5% more than at 400 nM, and this likely reflects sub-optimal oligo uptake. Since cells that were impervious to transfection were necessarily included, but dead floating cells which were likely permeable to transfection were not, then the estimate of 60 to 65% inhibition of RARβ2 expression is

probably negatively biased. Thus our results are consistent with RARβ2 knock-down having an even greater effect on cell growth and viability than is shown here.

The A-549/JP-2 microarray revealed that RARβ2 was the gene most heavily inhibited, with a decrease in mRNA of 65% (Table XXIV), which is equal to the level of inhibition determined by RT-PCR expression analysis. In fact, RARβ2 was the most reduced gene of all the 14,081 genes on the microarray. These results reflect the reliability of the microarray technology used.

The A-549/ATRA microarray revealed that RARβ2 was up-regulated by only 10%. This was not unexpected: it has previously been shown that A-549 cells are RA-resistant [they are refractory to RA-induced differentiation and proliferation block (*39*), as well as apoptosis (*40*)]; and we have previously observed lack of RARβ2 up-regulation in A-549 cells following stimulation with ATRA (Pappas and Bradley, unpublished results). However, one study has shown that A-549 cells are sensitive to the effects of certain retinoids, including ATRA (*41*). Nonetheless, if RARβ2 is a key player in the tumor suppressive effects of RA (including stimulation of differentiation and proliferation block), then the finding that RARβ2 is only up-regulated by 10% is consistent with the finding that A-549 cells are RA-resistant. It is important to clarify that though RARβ2 mRNA expression is not significantly up-regulated by ATRA in A-549 cells, it is RARβ2's overall activity following hetero-dimerization with RXR partners and subsequent activation of downstream genes that is ultimately targeted by the paired microarray experiments. RXR-RARβ2 activity in JP-2-treated A-549 cells may be assumed to be reduced by loss of the RARβ2 partner, whereas RXR-RARβ2 activity in ATRA-treated A-549 cells may be thought to be increased through increased ligand-dependent activation. Increased ATRA uptake is also expected to up-regulate RARβ2 mRNA expression and consequent activity through the retinoic acid response element (RARE), which is located in the RARβ2 P2 promoter (*42*), provided that the ATRA is bioavailable at the intracellular level. It is not yet known why certain cell lines, such as A-549, do not up-regulate RARβ2 significantly upon stimulation with ATRA. However, we have confirmed that lack of inducibility in A-549 cells does not occur through promoter methylation, since none of the 22 CpGs in a 541 bp region of the P2 promoter (containing several promoter elements including a RARE) are methylated (*23*). One potential explanation may lie in

the observation that soluble ALDH1, a key metabolic enzyme involved in the degradation of retinoids, including ATRA, is significantly up-regulated upon ATRA treatment (92%). Evidently, the unidentified *cis*-acting mechanism affecting the hypomethylated alleles in the non-expressing lines may also be affecting unmethylated alleles in expressing lines such as A-549, albeit only partially since basal expression is permitted.

Also, in a few "rescue"-type experiments conducted on A-549 cells to determine whether the effects of JP-2 were capable of neutralizing the effects of ATRA, results indicated that this was indeed the case (not shown). The genes assayed were ALDH1 and cyp24. Hence, the specificity of JP-2 for ATRA-stimulated increases in RARβ2-regulated genes was further demonstrated.

Of the top repressed genes [with a calibrated ratio of <62% control (lipofectin alone) and with an adequate spot quality], 14 genes are of particular interest in the work presented here (Table XXVI). First, four genes, Cyp24, ALDH1 (mentioned above), TUBA3, and IGFBP3, were strongly inhibited by JP-2, and up-regulated by ATRA (and may therefore represent important effectors of RARβ2- and/or ATRA-mediated tumor promotion, in light of their pivotal response to RARβ2-dependent regulation). Cyp24 (*33*) and ALDH1 (*34*) play roles in the catabolism of certain chemotherapeutic agents (such as vitamin D (calcitriol) andcyclophosphamides, respectively). TUBA3 has been shown to confer resistance against several antimitotic agents (*43*), and IGFBP3 is a mediator of cell cycle blockade and apoptosis (*44*). Second, nine other genes, NTRK3, cyclins A2, B1 and D3, survivin, FGFR4, v-Myb homology-like 2, E2F1 and CDKN1, are involved in the regulation of the cell cycle or apoptosis, they are known to be abnormally regulated in cancer, and they are down-regulated by JP-2. It is interesting to note that ALDH1, which was shown to oxidize all-trans, 9-*cis*, and 13-*cis* retinal to RA (*45*), was upregulated in the presence of ATRA.

Three other genes are of interest with regard to projects having begun in our laboratory: intercellular adhesion molecule-1 (ICAM-1), major histocompatibility complex class I (MHC I) and cyclooxygenase-2 (COX-2) (Table XXVI). We have previously shown that ICAM-1 and MHC I are up-regulated by as much as 2-3 times by RARβ2 transfection in CALU-1 and SK-MES RARβ2-negative lung cancer cells [(*37*); Appendix B]. These

molecules play central roles in the immune surveillance of cancer cells, more specifically the presentation of tumor antigens on (or derived from) tumor cells to cytotoxic T-lymphocyte precursors (CD8 cells) and their consequent activation and cytotoxic response. Consequently, loss of RARβ2 function has been associated with immune evasion. In the paper referenced above, we demonstrated that increased ICAM-1 and MHC Class I expression specifically increases the immunogenicity of RARβ2-negative lung cancer cells. Also, it has been found that ICAM-1 and MHC Class I expression levels are inversely correlated with metastasis and poor prognosis in NSCLC, and are associated with the early lymphatic spread of tumor cells (*46*). Consistent with our hypothesis of duality of roles conducted by RARβ2 in different contexts (one of which is the retained expression of RARβ2), we found that these molecules were up-regulated in JP-2-treated A-549 cells, at an average of 2.82 and 1.52, respectively, rather than down-regulated. A-549 cells treated with RA (A-549±ATRA microarray) did not show up-regulation of these genes, however, A-549 cells retrovirally transduced with the RARβ2 gene and treated or not with ATRA (-ATRA/+ATRA) up-regulated both ICAM-1 by and MHC Class I by 2.0/3.3 and 1.3/1.7 fold, respectively (Pappas and Bradley, unpublished results, see Appendix C). Finally, COX-2, another inflammatory molecule, is a potent mitogen that was recently shown to be correlated with poor prognosis in NSCLC (*47*), and was strikingly elevated in JP-2-treated A-549 cells. Interestingly, COX-2 overexpression was also found to be a marker of poor prognosis in stage I non-small cell lung cancer, as was RARβ2 (*47*).

The results obtained from the microarrays support the hypothesis that RARβ2 acts as a tumor promoter rather than a tumor suppressor in certain contexts, and elucidate the genes that may be responsible for duality of function. Similar duality of function has recently been shown for the first time in three genes: (1) the transcription factor E2F1, which regulates a group of genes involved in G1/S transition (*48*); (2) the transforming growth factor TGFβ, which is involved in multiple cellular processes including proliferation, differentiation, transformation and apoptosis (*49,50*); and (3) the oncogene Kras2, which is a GTP-binding protein involved in signal transduction (*51*). Akin to these genes, RARβ2 is involved in multiple cellular processes, including proliferation, differentiation, immunomodulation and apoptosis. In addition, the transcription factor E2F1 itself is down-regulated by 48% and up-regulated by 10% in A-549 cells treated

with JP-2 or ATRA, respectively (Table XXIV), it may therefore confer duality downstream of RARβ2.

We postulate that RARβ2 inactivation may be followed by reactivation as the cells transition from one context to another. Contexts may vary with regard to the accumulation of particular mutations or epigenetic events, or with regard to the transit from one tissue (e.g. the pulmonary epithelium) to another (e.g. the lymphatic vasculature) during metastatic spread, for example (Fig. 34). In particular, our findings suggest that RARβ2 may provide both a proliferative impetus as well as a heightened immunological visibility to cancer cells, and it is plausible that upon spread to a tissue with high immunological surveillance, such as lymph nodes, RARβ2 may be selected against in order to decrease immunomodulatory genes such as ICAM-1, IL-1β and MHC Class I, whereas it might be selected for in order to increase expression of proliferative genes such as TUBA3 upon spread to a tissue with relatively low immunological surveillance (Fig. 34). Evidently, cells subjected to various treatments, including RA, may respond with up-regulation of genes coding for catabolic enzymes or resistance to antimitotic agents, such as cyp24, ALDH1 or TUBA3, consequently reducing the efficiency of the given therapy.

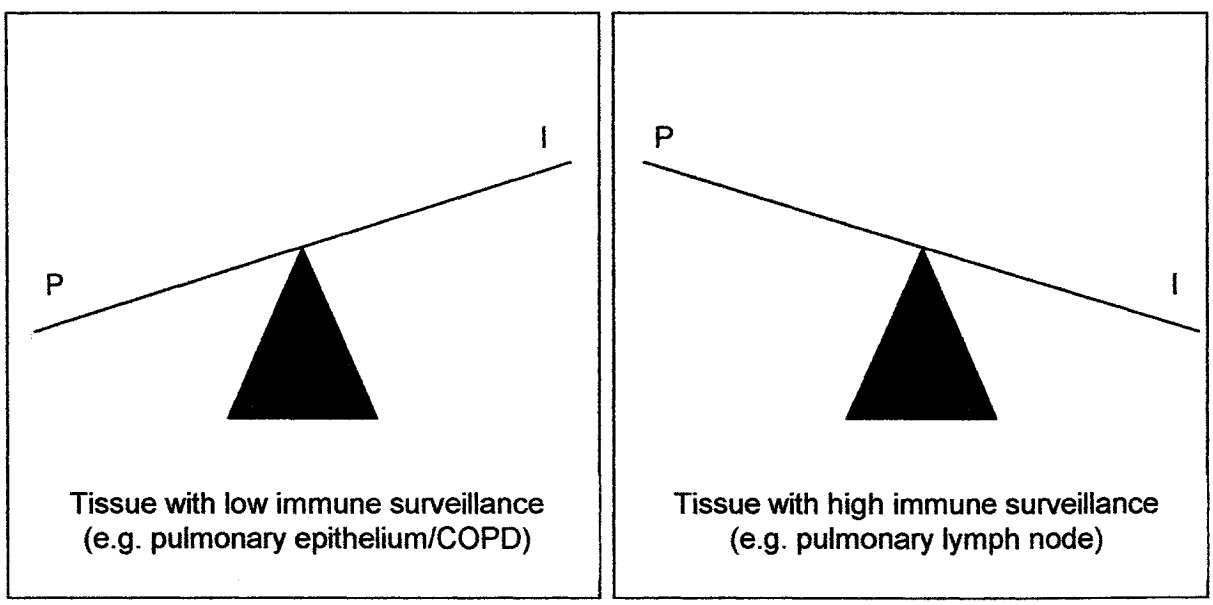

FIGURE 34: Diagram illustrating the hypothesized pivotal role that RARβ2 may play between proliferation and immunomodulation as a cancer cell migrates from a tissue with low immune surveillance, such as the pulmonary epithelium in a patient with COPD, to a tissue with high immune surveillance, such as a pulmonary lymph node.

Previous evidence that RARβ2 may play a dual role in cancer exists at three levels. First, the theory of field cancerization suggests that multiple cells within the epithelial lining are subject to similar genetic alterations predisposing to cancer (*52*). It has been shown that smokers at risk for lung cancer have large fields along the lining of their respiratory epithelium that contain cellular foci that have lost RARβ2 expression (*26*). If RARβ2 acted solely as a tumor suppressor, then it would be expected that most cancers that arise within these fields would be RARβ2-negative, when in fact, ~50% are negative and ~50% are positive (*25*). It would be interesting to perform a comparative analysis on the status of RARβ2 expression in primary versus local and distant metastatic cancers. It is plausible that RARβ2 is capable of such diametrically opposed roles because of its pleiotropic functions as a modulator of gene expression. Second, there is retrospective evidence that RARβ2 expression, at least in NSCLC, is correlated with worse prognosis than lack thereof (*24*). Third, the alleged protective effects of retinoids in cancer, which likely manifest themselves at least partially through RARβ2, are controversial: while some studies confirm them (*53,54*), others negate them (*27,28*).

In addition, RARβ2 is frequently inactivated via promoter methylation in multiple cancers, including head and neck, nasopharyngeal (*55*), lung (*56*), esophagus (*57*), gastric (*58,59*), breast (*60*), cervical (*61*), ovarian (*62*) and prostate (*63*) cancers, and methylation is a reversible process. It has been shown to be reversible in cancer cells lines using DNA methyltransferase inhibitors, such as 5-aza-2'-deoxycytidine (*15,64-66*). Also, several groups have shown the existence of cell lines where RARβ2 inactivation is not correlated with methylation (*15-23*), and we have recently shown that several cancer cell lines exhibit *divergent methylation*, the coexistence of differentially methylated alleles within the same cell (i.e. hypo- and hypermethylated alleles) (*23*), suggesting that another mechanism is responsible for RARβ2 inactivation on the hypomethylated allele. Though it is not yet known why certain genes are primarily inactivated via methylation (*67*), or why others are primarily inactivated via mutation, methylation and divergent methylation represent mechanisms that are potentially reversible and may allow for alternation between gene turn-off and reactivation. This may be the case for RARβ2.

The role of RARβ2 as a putative tumor suppressor remains controversial after more than 15 years of investigation: some experimental studies clearly demonstrate its tumor

suppressive effects in certain contexts (*38*), but there is yet to be a consensus within the scientific community as to whether it is a *bone fide* tumor suppressor. Until recently, this was mainly due to two reasons: (1) No genetic alteration affecting its remaining allelic copy has ever been found and such an alteration was a requirement for classification as a proper tumor suppressor gene (14); (2) The region affected by LOH is thought to contain putative tumor suppressor genes other than RARβ2. The findings shown here are therefore relevant both at the fundamental and the clinical level.

Only two previous experimental studies on RARβ2 have looked at the effects of the inhibition of RARβ2 expression (*13,68*), and both support the hypothesized tumor suppressor function of the gene. However, these studies looked at the effects of reduced effective level of RARβ2 in normal mouse cells, whereas this work aims to elucidate the potential plasticity of RARβ2 expression within the changing environment or genetic make-up of the cancer cell itself. Also, these studies did not confirm that the decreased tumor suppression observed was due to RARβ2 inactivation or increased overall RAR function. Early studies using RARβ-null or panRAR-null mice found no change in frequency of tumor formation (*69,70*). Many potentially compensatory mechanisms are present in the mouse model, such as expression of other retinoid receptors for instance, and these may obscure the specific role that RARβ2 plays in the cell's fate. Indeed, nearly all previous experimental studies on RARβ2 have focused on the tumor suppressive effects that exogenous RARβ2 transgene constructs have on cell lines that have lost RARβ2 expression, such as CALU-1 (*38,71*) or NCI-H157 (*38*). Reactivation of RARβ2 in such lines is expected to reactivate those molecular pathways that would have allowed tumor suppression, had RARβ2 not been selected against during the process of carcinogenesis. However, cancer cells that have retained the expression of the endogenous RARβ2 gene may have radically distinct expression profiles than those that have not. This may be explained by differences in the cancer cells' histological origins, genetic profiles, or microenvironmental histories, for example. In such circumstances, RARβ2 may have been selected for rather than against because of a potentially stimulatory effect it may have had on certain pathways, eventually allowing cancer cell survival or leading to promotion along the route of neoplastic progression. Thus, RARβ2 may not have uniquely tumor suppressive effects in all lung cancers, but may promote neoplastic progression in some.

In conclusion, we have shown that RARβ2 can play dual or opposing roles in the process of neoplastic progression, and this sheds light not only on previous experimental, epidemiological and clinical findings, but also on potential issues in cancer prevention and therapy. For example, cancers in which RARβ2 plays a beneficial role (as a tumor suppressor) will need to be distinguished from cancers in which RARβ2 plays a deleterious role (as a tumor promoter), especially when RA, a known stimulant of RARβ2 expression and function is being considered as a therapeutic modality for anti-cancer treatment. A simple assay of RARβ2 expression in conjunction with expression analysis of key RARβ2-downstream genes may prove to be prognostic in ascertaining whether or not retinoids should or should not be prescribed.

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References

(*1*) Chambon P. A decade of molecular biology of retinoic acid receptors. Faseb J 1996;10:940-54.

(*2*) Brand N, Petkovich M, Krust A, Chambon P, de The H, Marchio A, et al. Identification of a second human retinoic acid receptor. Nature 1988;332:850-3.

(*3*) Benbrook D, Lernhardt E, Pfahl M. A new retinoic acid receptor identified from a hepatocellular carcinoma. Nature 1988;333:669-72.

(*4*) Whang-Peng J, Kao-Shan CS, Lee EC, Bunn PA, Carney DN, Gazdar AF, et al. Specific chromosome defect associated with human small-cell lung cancer; deletion 3p(14-23). Science 1982;215:181-2.

(*5*) Houle B, Leduc F, Bradley WE. Implication of RARB in epidermoid (Squamous) lung cancer. Genes Chromosomes Cancer 1991;3:358-66.

(*6*) Gebert JF, Moghal N, Frangioni JV, Sugarbaker DJ, Neel BG. High frequency of retinoic acid receptor beta abnormalities in human lung cancer. Oncogene 1991;6:1859-68.

(*7*) Nervi C, Vollberg TM, George MD, Zelent A, Chambon P, Jetten AM. Expression of nuclear retinoic acid receptors in normal tracheobronchial cells and in lung carcinoma cells. Exp Cell Res 1991;195:163-70.

(*8*) Roman SD, Clarke CL, Hall RE, Alexander IE, Sutherland RL. Expression and regulation of retinoic acid receptors in human breast cancer cells. Cancer Res 1992;52:2236-42.

(*9*) Geradts J, Chen JY, Russell EK, Yankaskas JR, Nieves L, Minna JD. Human lung cancer cell lines exhibit resistance to retinoic acid treatment. Cell Growth Differ 1993;4:799-809.

(*10*) Swisshelm K, Ryan K, Lee X, Tsou HC, Peacocke M, Sager R. Down-regulation of retinoic acid receptor beta in mammary carcinoma cell lines and its up-regulation in senescing normal mammary epithelial cells. Cell Growth Differ 1994;5:133-41.

(*11*) Moghal N, Neel BG. Evidence for impaired retinoic acid receptor-thyroid hormone receptor AF-2 cofactor activity in human lung cancer. Mol Cell Biol 1995;15:3945-59.

(*12*) Seewaldt VL, Caldwell LE, Johnson BS, Swisshelm K, Collins SJ, Tsai S. Inhibition of retinoic acid receptor function in normal human mammary epithelial cells results in increased cellular proliferation and inhibits the formation of a polarized epithelium in vitro. Exp Cell Res 1997;236:16-28.

(*13*) Berard J, Laboune F, Mukuna M, Masse S, Kothary R, Bradley WE. Lung tumors in mice expressing an antisense RARbeta2 transgene. Faseb J 1996;10:1091-7.

(*14*) Haber D, Harlow E. Tumour-suppressor genes: evolving definitions in the genomic age. Nat Genet 1997;16:320-2.

(*15*) Cote S, Sinnett D, Momparler RL. Demethylation by 5-aza-2'-deoxycytidine of specific 5-methylcytosine sites in the promoter region of the retinoic acid receptor beta gene in human colon carcinoma cells. Anticancer Drugs 1998;9:743-50.

(*16*) Bovenzi V, Le NL, Cote S, Sinnett D, Momparler LF, Momparler RL. DNA methylation of retinoic acid receptor beta in breast cancer and possible therapeutic role of 5-aza-2'-deoxycytidine. Anticancer Drugs 1999;10:471-6.

(*17*) Arapshian A, Kuppumbatti YS, Mira-y-Lopez R. Methylation of conserved CpG sites neighboring the beta retinoic acid response element may mediate retinoic acid receptor beta gene silencing in MCF-7 breast cancer cells. Oncogene 2000;19:4066-70.

(*18*) Bovenzi V, Momparler RL. Quantitation of inhibition of DNA methylation of the retinoic acid receptor beta gene by 5-Aza-2'-deoxycytidine in tumor cells using a single-nucleotide primer extension assay. Anal Biochem 2000;281:55-61.

(*19*) Virmani AK, Rathi A, Zochbauer-Muller S, Sacchi N, Fukuyama Y, Bryant D, et al. Promoter methylation and silencing of the retinoic acid receptor-beta gene in lung carcinomas. J Natl Cancer Inst 2000;92:1303-7.

(*20*) Widschwendter M, Berger J, Hermann M, Muller HM, Amberger A, Zeschnigk M, et al. Methylation and silencing of the retinoic acid receptor-beta2 gene in breast cancer. J Natl Cancer Inst 2000;92:826-32.

(*21*) Gazdar AF, Zochbauer-Moller S, Virmani A, Kurie J, Minna JD, Lam S. RESPONSE: Re: Promoter Methylation and Silencing of the Retinoic Acid Receptor-beta Gene in Lung Carcinomas. J Natl Cancer Inst 2001;93:67-8.

(*22*) Lamy A, Metayer J, Thiberville L, Frebourg T, Sesboue R. Re: Promoter methylation and silencing of the retinoic acid receptor-beta gene in lung carcinomas. J Natl Cancer Inst 2001;93:66-8.

(*23*) Pappas JJ, Bradley WEC. The divergent methylation of the RARbeta2 promoter is correlated with an allelic bias for methylation. 2004.

(*24*) Khuri FR, Lotan R, Kemp BL, Lippman SM, Wu H, Feng L, et al. Retinoic acid receptor-beta as a prognostic indicator in stage I non-small-cell lung cancer. J Clin Oncol 2000;18:2798-804.

(*25*) Ayoub J, Jean-Francois R, Cormier Y, Meyer D, Ying Y, Major P, et al. Placebo-controlled trial of 13-cis-retinoic acid activity on retinoic acid receptor-beta expression in a population at high risk: implications for chemoprevention of lung cancer. J Clin Oncol 1999;17:3546-52.

(*26*) Xu XC, Sozzi G, Lee JS, Lee JJ, Pastorino U, Pilotti S, et al. Suppression of retinoic acid receptor beta in non-small-cell lung cancer in vivo: implications for lung cancer development. J Natl Cancer Inst 1997;89:624-9.

(*27*) Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, et al. Risk factors for lung cancer and for intervention effects in CARET, the Beta-Carotene and Retinol Efficacy Trial. J Natl Cancer Inst 1996;88:1550-9.

(*28*) Lippman SM, Lee JJ, Karp DD, Vokes EE, Benner SE, Goodman GE, et al. Randomized phase III intergroup trial of isotretinoin to prevent second primary tumors in stage I non-small-cell lung cancer. J Natl Cancer Inst 2001;93:605-18.

(*29*) Stein CA. Does antisense exist? Nat Med 1995;1:1119-21.

(*30*) Chen Y, Dougherty E, Bittner M. Ratio-based decisions and the quantitative analysis of cDNA microarray images. Biomedical Optics 1997;2:364-74.

(*31*) Chen Y, Kamat V, Dougherty ER, Bittner ML, Meltzer PS, Trent JM. Ratio statistics of gene expression levels and applications to microarray data analysis. Bioinformatics 2002;18:1207-15.

(*32*) Diehn M, Sherlock G, Binkley G, Jin H, Matese JC, Hernandez-Boussard T, et al. SOURCE: a unified genomic resource of functional annotations, ontologies, and gene expression data. Nucl. Acids. Res. 2003;31:219-23.

(*33*) Brown AJ. Mechanisms for the selective actions of vitamin D analogues. Curr Pharm Des 2000;6:701-16.

(*34*) Sladek NE. Aldehyde dehydrogenase-mediated cellular relative insensitivity to the oxazaphosphorines. Curr Pharm Des 1999;5:607-25.

(*35*) Olie RA, Simoes-Wust AP, Baumann B, Leech SH, Fabbro D, Stahel RA, et al. A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cancer cells to chemotherapy. Cancer Res 2000;60:2805-9.

(*36*) Balmer JE, Blomhoff R. Gene expression regulation by retinoic acid. J Lipid Res 2002;43:1773-808.

(*37*) Toulouse A, Loubeau M, Morin J, Pappas JJ, Wu J, Bradley WE. RARbeta involvement in enhancement of lung tumor cell immunogenicity revealed by array analysis. Faseb J 2000;14:1224-32.

(*38*) Houle B, Rochette-Egly C, Bradley WE. Tumor-suppressive effect of the retinoic acid receptor beta in human epidermoid lung cancer cells. Proc Natl Acad Sci U S A 1993;90:985-9.

(*39*) van der Leede BM, van den Brink CE, van der Saag PT. Retinoic acid receptor and retinoid X receptor expression in retinoic acid-resistant human tumor cell lines. Mol Carcinog 1993;8:112-22.

(*40*) Manna SK, Aggarwal BB. All-trans-retinoic acid upregulates TNF receptors and potentiates TNF-induced activation of nuclear factors-kappaB, activated protein-1 and apoptosis in human lung cancer cells. Oncogene 2000;19:2110-9.

(*41*) Fazely F, Ledinko N. Retinoic acid and epidermal growth factor binding in retinoid-mediated invasion suppressed human lung carcinoma cells. Anticancer Res 1990;10:667-70.

(*42*) Shen S, Kruyt FA, den Hertog J, van der Saag PT, Kruijer W. Mouse and human retinoic acid receptor beta 2 promoters: sequence comparison and localization of retinoic acid responsiveness. DNA Seq 1991;2:111-9.

(*43*) Kyu-Ho Han E, Gehrke L, Tahir SK, Credo RB, Cherian SP, Sham H, et al. Modulation of drug resistance by alpha-tubulin in paclitaxel-resistant human lung cancer cell lines. Eur J Cancer 2000;36:1565-71.

(*44*) Baxter RC. Signalling pathways involved in antiproliferative effects of IGFBP-3: a review. Mol Pathol 2001;54:145-8.

(*45*) Bhat PV, Samaha H. Kinetic properties of the human liver cytosolic aldehyde dehydrogenase for retinal isomers. Biochem Pharmacol 1999;57:195-7.

(*46*) Passlick B, Pantel K, Kubuschok B, Angstwurm M, Neher A, Thetter O, et al. Expression of MHC molecules and ICAM-1 on non-small cell lung carcinomas: association with early lymphatic spread of tumour cells. Eur J Cancer 1996;32A:141-5.

(*47*) Khuri FR, Wu H, Lee JJ, Kemp BL, Lotan R, Lippman SM, et al. Cyclooxygenase-2 overexpression is a marker of poor prognosis in stage I non-small cell lung cancer. Clin Cancer Res 2001;7:861-7.

(*48*) Johnson DG. The paradox of E2F1: oncogene and tumor suppressor gene. Mol Carcinog 2000;27:151-7.

(*49*) Akhurst RJ, Derynck R. TGF-beta signaling in cancer--a double-edged sword. Trends Cell Biol 2001;11:S44-51.

(*50*) Wakefield LM, Roberts AB. TGF-beta signaling: positive and negative effects on tumorigenesis. Curr Opin Genet Dev 2002;12:22-9.

(*51*) Zhang Z, Wang Y, Vikis HG, Johnson L, Liu G, Li J, et al. Wildtype Kras2 can inhibit lung carcinogenesis in mice. Nat Genet 2001;29:25-33.

(*52*) Slaughter DP, Southwick HW, Smejkal W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. Cancer 1953;6:963-8.

(*53*) Graham S. Epidemiology of retinoids and cancer. J Natl Cancer Inst 1984;73:1423-8.

(*54*) Sankaranarayanan R, Mathew B. Retinoids as cancer-preventive agents. IARC Sci Publ 1996:47-59.

(*55*) Kwong J, Lo KW, To KF, Teo PM, Johnson PJ, Huang DP. Promoter hypermethylation of multiple genes in nasopharyngeal carcinoma. Clin Cancer Res 2002;8:131-7.

(*56*) Toyooka S, Toyooka KO, Maruyama R, Virmani AK, Girard L, Miyajima K, et al. DNA methylation profiles of lung tumors. Mol Cancer Ther 2001;1:61-7.

(*57*) Kuroki T, Trapasso F, Yendamuri S, Matsuyama A, Alder H, Mori M, et al. Allele loss and promoter hypermethylation of VHL, RAR-beta, RASSF1A, and FHIT tumor suppressor genes on chromosome 3p in esophageal squamous cell carcinoma. Cancer Res 2003;63:3724-8.

(*58*) Oue N, Motoshita J, Yokozaki H, Hayashi K, Tahara E, Taniyama K, et al. Distinct promoter hypermethylation of p16INK4a, CDH1, and RAR-beta in intestinal, diffuse-adherent, and diffuse-scattered type gastric carcinomas. J Pathol 2002;198:55-9.

(*59*) Oue N, Oshimo Y, Nakayama H, Ito R, Yoshida K, Matsusaki K, et al. DNA methylation of multiple genes in gastric carcinoma: Association with histological type and CpG island methylator phenotype. Cancer Sci 2003;94:901-5.

(*60*) Fackler MJ, McVeigh M, Evron E, Garrett E, Mehrotra J, Polyak K, et al. DNA methylation of RASSF1A, HIN-1, RAR-beta, Cyclin D2 and Twist in in situ and invasive lobular breast carcinoma. Int J Cancer 2003;107:970-5.

(*61*) Narayan G, Arias-Pulido H, Koul S, Vargas H, Zhang FF, Villella J, et al. Frequent Promoter Methylation of CDH1, DAPK, RARB, and HIC1 Genes in Carcinoma of Cervix Uteri: Its Relationship to Clinical Outcome. Mol Cancer 2003;2:24.

(*62*) Evron E, Dooley WC, Umbricht CB, Rosenthal D, Sacchi N, Gabrielson E, et al. Detection of breast cancer cells in ductal lavage fluid by methylation-specific PCR. Lancet 2001;357:1335-6.

(*63*) Nakayama T, Watanabe M, Yamanaka M, Hirokawa Y, Suzuki H, Ito H, et al. The role of epigenetic modifications in retinoic acid receptor beta2 gene expression in human prostate cancers. Lab Invest 2001;81:1049-57.

(*64*) Cote S, Momparler RL. Activation of the retinoic acid receptor beta gene by 5-aza-2'-deoxycytidine in human DLD-1 colon carcinoma cells. Anticancer Drugs 1997;8:56-61.

(*65*) Sirchia SM, Ferguson AT, Sironi E, Subramanyan S, Orlandi R, Sukumar S, et al. Evidence of epigenetic changes affecting the chromatin state of the retinoic acid receptor beta2 promoter in breast cancer cells. Oncogene 2000;19:1556-63.

(*66*) Sirchia SM, Ren M, Pili R, Sironi E, Somenzi G, Ghidoni R, et al. Endogenous reactivation of the RARbeta2 tumor suppressor gene epigenetically silenced in breast cancer. Cancer Res 2002;62:2455-61.

(*67*) Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. Oncogene 2002;21:5427-40.

(*68*) Berard J, Luo H, Chen H, Mukuna M, Bradley WE, Wu J. Abnormal regulation of retinoic acid receptor beta2 expression and compromised allograft rejection in transgenic mice expressing antisense sequences to retinoic acid receptor beta1 and beta3. J Immunol 1997;159:2586-98.

(*69*) Mendelsohn C, Mark M, Dolle P, Dierich A, Gaub MP, Krust A, et al. Retinoic acid receptor beta 2 (RAR beta 2) null mutant mice appear normal. Dev Biol 1994;166:246-58.

(*70*) Lohnes D, Mark M, Mendelsohn C, Dolle P, Decimo D, LeMeur M, et al. Developmental roles of the retinoic acid receptors. J Steroid Biochem Mol Biol 1995;53:475-86.

(*71*) Toulouse A, Morin J, Dion PA, Houle B, Bradley WE. RARbeta2 specificity in mediating RA inhibition of growth of lung cancer-derived cells. Lung Cancer 2000;28:127-37.

CHAPTER 4

DISCUSSION

1 Summary

Regarding methylation studies, it was found that: hypo- and hypermethylated RARβ2 alleles frequently co-exist in lines in which RARβ2 is inactivated (5/11, 45%); such divergent methylation at RARβ2 is heritable in the majority of subclones analyzed (6/8, 75%); and copies are subject to methylation allelic bias at a ratio of ~2:1 (3/3 CpG sites, 100%). This work is therefore the first to report the co-existence and heritability of hypo- and hypermethylated allelic copies of a gene that is completely inactivated in cancer; that hypermethylation in cancer is subject to allelic bias reminiscent of *de novo* imprinting; and that a promoter methylation-independent *cis*-acting silencing mechanism is involved in RARβ2 inactivation. Regarding antisense studies, it was found that: cellular proliferation is correlated with RARβ2 expression levels ($p=0.0003$) in the A-549 cell line; the most effective oligo reduces cellular proliferation by up to 80% in cancer cell lines in which RARβ2 expression has been retained (3/3, 100%), but has no apparent effects in lines in which it has been lost (3/3, 100%); reduction in cell growth following oligo treatment is at least partially due to activation of programmed cell death; and over a dozen pro-carcinogenesis genes are downregulated following RARβ2 knockdown, whereas half are upregulated following ATRA stimulation. This work is therefore the first to report that RARβ2 expression may be necessary for the growth and oncogenic phenotype of certain RARβ2-expressing cancer cells; and methylation - since it is a reversible silencing mechanism, as well as the methylation-independent silencing mechanism referred to above – since it does not genetically alter the allele, may represent mechanisms allowing alternation between RARβ2-inactivation and re-expression. These findings help explain the paradoxical effects of retinoids and RARβ2 in cancer, and form excellent starting grounds for subsequent studies (detailed below).

For more ample details regarding these conclusions, as well as the relevance and implications of these findings, please refer to Chapter 2, section 4. *Discussion*, pp. 150-60, and Chapter 3, section 4. *Discussion*, pp. 192-198.

2 Divergent methylation of the RARβ P2 promoter; Methylation studies

It is appropriate to mention that though methylation allelic bias was only found in colon (LS-180) and breast (T2 and T8) cancer cells, it may also affect lung cancer cells, especially since divergent methylation was found in all three cancer cell types.

The following mechanistic and descriptive studies are suggested to deepen the understanding of divergent methylation in cancer.

2.1 Mechanistic studies

2.1.1 What is the nature of the promoter methylation-independent silencing event?

Evidently, since the cell lines found to be divergently methylated in this project (CALU-1, SK-MES, MDA-MB-231, ZR-75B and LS-180) contain alleles expected to be affected by a *cis*-acting event active at only one allele, they may be used in experiments geared toward discovering the nature of this mechanism. These cell lines may for example be used in pull-down assays in order to identify whether or not different proteins, factors or macromolecular structures, differentially bind the various alleles. Antibodies that bind methylating enzymes, such as DNMTs, or methyl-binding proteins, including MeCPs, could form the start of such a project, and alleles could be distinguished using the rs755661 SNP or other novel SNPs, which are being discovered with increasing frequency. Identified proteins could then be isolated and characterized.

2.1.2 What are the size and characteristics of the region affected by promoter-independent inactivation?

To delineate the region in/proximal to the RARβ2 locus on 3p24 which is sensitive to the *cis*-acting suppressive effect, RARβ itself as well as upstream and downstream regions could be targeted for reporter gene knock-in at multiple sites in tandem. For example, enhanced green fluorescent protein (EGFP) cDNA flanked by various sequences homologous to RARβ2 could be used in transfection experiments to exploit the naturally occurring processes of homologous recombination. DNA from clones expressing EGFP and consequently fluorescent could be subjected to Southern blot and sequencing for

precise identification of the site of insertion linked to expression (i.e. absence of the *cis*-acting effect).

2.1.3 Do the genes that are frequently methylated in cancer co-localize in nuclear sub-compartments where DNA methylation machinery has gone awry?

The nucleus contains various sub-compartments, including the nucleolus, chromosomes, nuclear foci, nuclear periphery and splicing speckles. Recent studies have shown that nuclear proteins are often mis-compartmentalized in genetic diseases (*1*) and this may be the case in cancer as well. In fact, it has been demonstrated that the RARα fusion partners PML and PLZF heterodimerize and co-localize to nuclear bodies in NB4 APL cells (*2*). It would thus be of interest to determine the subnuclear location of the proteins involved in DNA methylation as well as their target genes, which are known to be differentially methylated in disease such as carcinogenesis, in order to determine whether they co-localize in nuclear sub-compartments where they may be simultaneously hypermethylated by methylation machinery gone awry, for example. Such genes include RARβ2, RASSF1A, CDH1, cyclin D2, p15/Ink4a, APC and DAPK, etc. Fluorescence *in situ* hybridization (FISH) using probes hybridizable to the corresponding gene sequences to label the genes and reporter fusion gene constructs (such as the β–galactosidase-neomycin-phosphotransferase cassette) to stain/immunostain and thereby localize the proteins [described in (*3*)], in combination with live cell imaging, digital microscopy and confocal laser scanning microscopy could be used toward this aim. This same combination of techniques, but targeted toward the hypo- and the hypermethylated alleles of a same gene (e.g. RARβ) may also be employed to determine whether differentially methylated allelic copies are located in different subnuclear regions.

2.1.4 Are retinoid signaling and DNA methylation linked in cancers of epithelial origin?

DNA methylation is reminiscent of retinoid signaling in that it is both essential to the differential control of gene expression during ontogeny and development, and it is conserved in a wide range of species from *D. melanogaster* to *H. sapiens*, suggesting that there is a possible link. The only link found to date is the finding that the PML-RARα

fusion protein recruits DNA methyltransferases DNMT-1 and 3A, thereby silencing genes including RARβ2, via the targeted methylation of their promoters, and RA treatment was associated with demethylation and derepression of the RARβ2 gene. (*4*). Also, RARβ2 is one of the genes most frequently methylated in several cancers. Based on these findings, it would be relevant to investigate whether or not DNMTs involved in the methylation-associated gene inactivations (MAGIs) seen in cancer, including that of RARβ2 itself, or p15/Ink4a, β-catenin, etc., may be targeted through formation of "RARβ2:DNMT" complexes and subsequent binding and methylation of RARE-containing genes in non leukemic cells. As performed by Di Croce *et al.*, co-immunoprecipitation experiments using antibodies against the various DNMTs and against RARβ2 could be performed, but on protein extracts from lung, breast or colon cancer cells, for example, rather than PML cells.

2.1.5 Is RARβ2 involved in methylation?

To determine whether RARβ2 is involved in methylation, differential methylome profiling, using cells in which RARβ is reactivated versus inactivated, might provide clues as to which genes are important in this process.

2.1.6 Does the demethylation of the RARβ2 promoter result in earlier replication?

To determine whether hypermethylation of the RARβ2 promoter is correlated with late S phase replication as seen in FISH experiments (section 3.10 of Chapter 2), 5'-azadeoxycytidine treatment could be used to treat LS-180 cells prior to repeating the same labeling protocol. However, demethylating agents have genome-wide effects, which could potentially demethylate genes involved in cell cycling or DNA synthesis, and hence present confounding factors in such a study.

2.2 Descriptive studies

2.2.1 Are hypomethylated alleles in divergently methylated cell lines associated with differentially acetylated histones?

Little is know about the interactions and regulations of DNA methylation and histone deacetylation. Sirchia *et al.* showed, that as expected, RARβ2 is unmethylated and

acetylated in the RARβ2-expressing HS-578T and HCC 2185 breast cancer cell lines, and methylated and deacetylated in the non-expressing HCC712 and MCF-7 cell lines (5). Intriguingly, the breast cancer cell line that was found to lack expression yet be unmethylated at all alleles sequenced in this study, T47D, was found to be hypoacetylated by this group. It would therefore be of interest to determine whether hypomethylated alleles in divergently methylated cell lines are associated with differentially acetylated histones. Chromatin immunoprecipitation (ChIP), which consists of the labeling of cross-linked DNA:protein complexes with antibodies against various chromatin factors, such as HDAC1 or acetylated H4, for example, could be used. Also, to identify factors differentially associated with the hypomethylated allele, MeCP pull-downs, which are assays that allow the determination of protein:protein interactions, could be designed and performed in homo- and divergently methylated lines, and identified proteins could be isolated and characterized.

On a similar note, the knock-in of an unrelated sequence containing CpGs or the creation of radiation hybrids using divergently methylated cell lines may be used to determine whether divergent methylation persists, to estimate the length of the sequence affected, and to determine and identify whether certain sequences behave as barriers. This however would be a very time-consuming initiative.

2.2.2 Is lack of bioavailable RA correlated with RARβ2 methylation?

RA bioavailability is an issue in the development of lung cancer (section 2.7), and RARβ2 expression, which is RARE-dependent, requires RA for transcriptional activation since RXR:RAR heterodimers require RA for activation. Based on the assumption that genes which are infrequently or never transcribed may be methylated as a mechanism for global transcriptional optimization, it is plausible that RARβ methylation may result following a prolonged period during which RA is unavailable. Hence, it would be appealing to determine whether the regions or cells within cancerization fields where RA is not bioavailable are the same regions in which RARβ2 is inactivated via hypermethylation. A potentially feasible experimental design could include a mouse model, created from embryonic stem (ES) cells transfected with a $(RARE)_3$-LUC reporter plasmid, and exposed to tobacco distillates till the overt formation of lung tumors. Using this design, RARβ2 expression, methylation as well as 3p LOH statuses could be

analyzed in affected fields via laser micro-dissection. Both LOH and methylation affecting 3p24, the locus of RARβ2, are thought to be early events in lung cancer, but it is not yet clear whether they occur in the same preneoplastic cells.

3 RARβ2 duality of function; Antisense studies

It may be postulated that RARβ2 inactivation may be followed by reactivation as the cells transition from one context to another. Contexts may vary with regard to the accumulation of particular mutations or epigenetic events, or with regard to the transit from one tissue (e.g. the pulmonary epithelium) to another (e.g. the lymphatic vasculature) during metastatic spread, for example. In particular, our findings suggest that RARβ2 may provide both a proliferative impetus as well as a heightened immunological visibility to cancer cells, and it is plausible that upon spread to a tissue with high immunological surveillance, such as lymph nodes, RARβ2 may be selected against in order to decrease immunomodulatory genes such as ICAM-1, IL-1β and MHC Class I. On the opposite side of the coin, upon spread to a tissue with relatively low immunological surveillance, it might be selected for in order to increase expression of proliferative genes such as TUBA3. Evidently, cells subjected to various treatments, including RA, may respond with up-regulation of genes coding for catabolic enzymes or resistance to antimitotic agents, such as CYP24, ALDH1 or TUBA3, consequently reducing the efficiency of the given therapy. (Please refer to chapter 3 for the relevance and implications of these findings).

3.1 The use of antisense oligonucleotides as opposed to other current antagonists

Currently available RARβ2 antagonists have a panoply of non-specific effects. Most importantly, while these non-specific effects include the modulation of other RAR isotypes and isoforms, which can severely confound the results of this study, the non-specific effects of antisense oligos are normally restricted to protein complexes and are not expected to affect other RARs. In fact, the mRNA expression of RARα was found to be unaffected by antisense oligo treatment (results not shown).

3.2 Genes possibly involved in the pivotal response to RARβ2-dependent regulation

Five genes are of particular interest in the work presented here, because while they are strongly inhibited by JP-2, they are also up-regulated by ATRA, and may therefore represent important effectors of RARβ2- and/or ATRA-mediated tumor promotion, in light of their pivotal response to RARβ2-dependent regulation. They are: Cyp24, IGFBP3, ALDH1, ET-1, and TUBA3 (some mentioned above).

The findings concerning Cyp24, TUBA3 and ALDH1 up- and down-regulation following both ATRA and JP-2 treatment, respectively, in the A-549 lung adenocarcinoma cell line *in vitro*, highlight mechanisms potentially rendering lung cancer therapies less than optimal *in vivo*.

3.2.1 Cyp24

Cyp24 (cytochrome protein 24, a.k.a. vitamin D_3 24-hydroxylase) was found to be expressed at 4.41 and 0.38 in the ATRA and JP-2 microarrays, respectively. Cyp24 is a cytochrome-containing enzyme involved in the catabolism of certain chemotherapeutic agents, such as vitamin D_3 (calcitriol) and vitamin D analogs (*6*), which are potent agents of differentiation and proliferation blockade in cancer, especially breast cancer. Cyp24 is regulated by RA (*7*) as well as by vitamin D though vitamin D responsive elements (VDREs) (*8*). It has been shown to be amplified via comparative genomic hybridization (CGH) array in breast cancer (*9*) and over-expressed in several cancers, including esophageal cancer (*10*). This finding is particularly relevant to breast cancer research, since vitamin D and its derivatives are key agents used in the differentiation therapy of RA-sensitive breast cancers. Also, certain CYP enzymes are thought to play significant roles in the activation of tobacco smoke-derived procarcinogens. In fact, blocking CYP induction reduces the formation of benzo[a]pyrene-derived carcinogens (*11*), which is enhanced by β-carotene (*12*). To date, there is little data available regarding the majority of human CYP enzymes other then CYP1A1, which is involved in the metabolism of polycyclic aromatic hydrocarbons (PAHs) and thought to be partly responsible for the difference in individual susceptibility to lung carcinogenesis, and CYP3A5, the main CYP3A expressed in the pulmonary mucosa (*13*). It is important to

clarify that Cyp24 is not involved in ATRA metabolism, though CYP2B6, 2C8, 3A4/5, 2A6 and 26 are (*14*).

3.2.2 TUBA3

TUBA3 (tubulin α3) was found to be expressed at 2.04 and 0.51 in the ATRA and JP-2 microarrays, respectively. TUBA3 forms heterodimers with β-tubulin to form microtubules, which are involved in intracellular and extracellular transport, intracellular organization, motility, mitosis and cell shape. Both β- and α-tubulins are over-expressed in many cancers, including lung cancer. Tubulin α has recently been shown to confer strong resistance against several antimitotic agents in the NCI-H460 lung cancer cell line, including paclitaxel, colchicine, vinblastine and nocodazole (*15*).

3.2.3 ALDH1

ALDH1/ALDH1A1 (aldehyde dehydrogenase 1) was found to be expressed at 1.92 and 0.50 in the ATRA and JP-2 microarrays, respectively. There are at least 14 members of the ALDH family known to be expressed in human tissues. Little is known with regard to ALDH1 because of its wide spectrum of substrates, including acetaldehyde, retinal and aldophosphamide. ALDH1 is a key metabolic enzyme involved in the catabolism of certain chemotherapeutic compounds, such as nitrogen mustards, cyclophosphamides and RAs. Cyclophosphamides and other oxazaphosphorines are bifunctional cytotoxicants: these alkylating agents cause DNA cross-links and depurination. Cyclophosphamide is metabolized into the pivotal aldophosphamide prodrug, which is either converted into the anti-neoplastic agent phosphoramide mustard (and the acrolein bi-product) through exposure to alkaline substances (desirable), or it is enzymatically inactivated into carboxyphosphamide through ALDH1 catalysis (not desirable) (*16*). Little is known regarding human ALDH1 regulation, however it has been shown to be over-expressed in certain lung cancers, and was identified via differential display as one of four over-expressed genes in NSCLC-N6 cells treated with the NSCLC-chemotherapeutic compound VT1 [methyl-4-methoxy-3-(3-methyl-2-butanoyl)benzoate] (*17*). It is also thought to act as a retinaldehyde dehydrogenase in RA metabolism in the mouse. Retinaldehyde dehydrogenation is the second irreversible and rate-limiting step of the oxidative conversion of *all-trans*-retinal to ATRA (Fig. 6, p.29), and is crucial for

RAR signaling since ATRA selectively activates the RARs. Also, ALDH1 is known to be down-regulated via a negative feedback inhibitory mechanism in the presence of elevated hepatic RA levels (*18*). This finding therefore demonstrates one potential mechanism causing chemotherapy resistance in RA-treated cells.

3.2.4 IGFBP-3

IGFBP-3 (insulin-like growth factor binding protein-3) was found to be expressed at 2.56 and 0.56 in the ATRA and JP-2 microarrays, respectively. IGFBP-3, a member of a family of six structurally-related IGFBPs, is involved in the modulation (both positive and negative) of IGF signaling, mainly due to its high affinity binding of its ligand (10^{-10} M), IGF. IGF is known to stimulate the proliferation of multiple cancer cell types, including SCLC, melanoma, breast and prostate cancer cells, for example. IGFBP-3 is the major IGF carrier in the human circulation and acts as a mediator of both cell cycle blockade and apoptosis (*19*), and these effects comprise both IGF-dependent and independent mechanisms. Interestingly, it has previously been found to be specifically controlled by RARβ2 in CALU-1 cells by our group (*20*) as well as others (*21,22*). It was found to mediate both RA and TGF inhibition of proliferation of MDA-MB-231 breast cancer cells (*23*), and has equally been found to be up-regulated in MCF-7 breast cancer cells treated with ATRA via microarray (*24*). Also, paired comparisons of RNA expression in tumor versus normal tissues (via oligonucleotide microarray; 12,000 genes and ESTs) in three patients with bronchioalveolar carcinoma showed that IGFBP3 was one of the 12 genes consistently upregulated, increases ranging from 3.4 to 6.1 –fold (*25*). Deal *et al.* recently found a positive correlation between circulating retinol levels and circulating IGFBP-3 levels (*26*). Consistent with the hypothesis of duality of function, IGFBP3 was shown to be up-regulated by approximately 124% in RARβ2-negative CALU-1 cells via RARβ2 transfection (*20*), but was down-regulated by approximately 45% in RARβ2-positive A-549 cells treated with JP-2. IGFBP3 mediates TGF and RA inhibitory effects on cell growth in human breast cancer cells (*23,27*).

3.2.5 ET-1

ET-1 (endothelin 1/EDN-1) was found to be expressed at 2.10 and 0.49 in the ATRA and JP-2 microarrays, respectively. ET-1 is a mitogen (*28*) and highly potent inducer of

neoangiogenesis (*29*). It is not thought to be regulated directly by RA or through a RARE, but rather through transcription intermediary factors (TIFs) (*30*), and recent studies show that it may act as a paracrine growth factor in lung cancer (*31*).

3.3 Genes involved in the mitotic or apoptotic responses

Seven other genes are of particular importance because they are involved in the regulation of the cell cycle or apoptosis, they are known to be abnormally regulated in cancer, and they are significantly down-regulated by JP-2. They are: NTRK3 (0.36), survivin (0.46), cyclins A2 and B1 (0.49 and 0.51, respectively), PLK (0.48), FGFR4 (0.49), MYBL2 (0.51), E2F1 (0.52).

3.3.1 NTRK3

NTRK3/c-trk (neurotrophic tyrosine kinase 3) is one of two nerve growth factor (NGF) receptors (other than p75). NGF has been shown to increase the rate of cellular proliferation by as much as threefold in three lung cancer cell lines (HTB 119, HTB 120 and CCL 185), and the effect was shown to be specific through its reversal using antibodies against NGF or the tyrosine kinase inhibitor genistein (*32*). Interestingly NGF and IGF-1, mentioned above, are two of the main growth factors known to form autocrine growth loops in SCLC.

3.3.2 Survivin

Survivin/BIRC5 is an inhibitor of apoptosis (IAP) that regulates the G2/M phase of the cell cycle. It is expressed in many cancers, such as lung, pancreas, breast and prostate cancers, but not in their corresponding normal tissues. In fact, it is so commonly and significantly upregulated in tumor tissues as opposed to their normal corresponding counterparts as evidenced by transcriptome studies (*33*), that autoantibodies against it may serve as markers of cancer progression (*34*). Its expression in lung cancer has been correlated with poor prognosis and with vessel invasion (*35*). Interestingly, Olie *et al.* found that treatment of A-549 cells with antisense oligos against survivin down-regulated its mRNA expression by 70%, increased caspase-3-like protease activity, nuclear condensation and fragmentation, and trypan blue uptake, and sensitized tumor cells to the chemotherapeutic agent etoposide (*36*).

3.3.3 Cyclin A2

Cyclin A2 promotes G1/S and G2/M transitions (*37*) and may be used as a proliferation marker; cyclin B1 promotes G2/M.

3.3.4 FGF4

Studies have shown that FGF isotypes 1, 2, 7 and 10 may be involved in lung carcinogenesis. However, little is known about the function of fibroblast growth factor receptor 4 (FGFR4). Various studies indicate that it may be related to cancer progression, mainly in pituitary adenomas (*38*), but its mechanisms of action and regulation remain unknown. The transcription factor "Ikaros", a zinc-finger-containing protein mainly active in lymphoid cells and which may recruit HDACs, is known to bind the FGFR4 promoter (*39*). It is important to mention that polyanions, including phosphorothioate oligos such as those used in this study (Chapter 3, section 2.1), are known to interact with and bind various growth factors including FGF4 through their aptameric effects (*40*). Hence, it is plausible to assume that the down-regulation of FGF4R may result as a consequence to the artifactual reduction of its ligand, FGF4, following antisense oligo transfection of cells. Moreover, the treatment is preceded by a 4-hr incubation in serumless medium, further reducing - if not eliminating – FGF, since FGF is a component of serum.

3.3.5 MYBL2

MYBL2 (v-myb homology-like-2 protein) is the most potent and highly-expressed of the myb oncogene family members and promotes cell growth by activating CDC2 and cyclin D1.

3.3.6 E2F1

The transcription factor E2F1 regulates a group of genes involved in G1/S transition (*41*). Rb, which normally acts to inhibit G1/S, directly regulates E2F1.

3.3.7 Cox-2

Cyclooxygenase-2/phospholipase A_2 (COX-2/PLA_2), an inflammatory molecule, is a potent mitogen which is also implicated in angiogenesis and invasion. Its expression is correlated with pulmonary adenocarcinoma (*42*). Surprisingly, its expression was strikingly elevated in JP-2-treated A-549 cells (3.69). COX enzymes include COX-1 and COX-2. COX-2 is inducible whereas as COX-1 is constitutively expressed. Also, retinoids are known to inhibit COX-2 induction. Inhibitors of COX-2, such as non-steroidal anti-inflammatory drugs and celecoxib, are associated with reduced number and size of colon tumors, including Familial Adenopolyposis (FAP) polyps and colorectal adenocarcinoma. Interestingly, COX-2 overexpression was found to be a marker of poor prognosis in stage I NSCLC, as was RARβ2 (*43*). In fact, the selective COX-2 inhibitor nimesulide was shown to reduce the proliferation of lung cancer cells *in vitro* and to cause apoptosis (*44*). Gocyk *et al.* found that the rate of *Helicobacter pylori* bacterial infection, which is linked to the formation of gastric ulcers, is significantly higher in lung cancer patients than in normal healthy control subjects, and that the expression levels of gastrin, the main proliferating stimulus of gastric epithelial cells, its receptor CCKB-R, COX-1 and 2 were also significantly upregulated (*45*). Interestingly, COX-2 and RARβ2 have recently taken centre stage as targets of novel therapeutic strategies (*46*).

3.4 Genes involved in the anti-tumoral response

Three genes, ICAM-1, MHC Class I and COX-2 (above), are of interest with regard to projects having begun in our laboratory (section 4.2 and Appendices B and C). Intercellular adhesion molecule 1 (ICAM-1) and major histocompatibility complex class I (MHC Class I/MHC1) are up-regulated by as much as 2-3 times by RARβ2 transfection in CALU-1 and SK-MES RARβ2-negative lung cancer cells (*20*). These molecules play central roles in the immune surveillance of cancer cells, more specifically the presentation of tumor antigens on (or derived from) tumor cells to cytotoxic T-lymphocyte precursors (CD8 cells) and their consequent activation and cytotoxic response. Consequently, loss of RARβ2 function (and thereby down-regulation of ICAM-1 and MHC Class I) has been associated with immune evasion. In the paper mentioned above (Appendix B), it was demonstrated that increased ICAM-1 and MHC Class I expression specifically increases the immunogenicity of RARβ2-negative lung cancer cells. Also, it has been found that ICAM-1 and MHC Class I expression levels are inversely correlated

with metastasis and poor prognosis in NSCLC, and are associated with the early lymphatic spread of tumor cells (*47*). Consistent with the hypothesis of duality of roles conducted by RARβ2 in different contexts (one of which is the retained expression of RARβ2), we found that these molecules were up-regulated in JP-2-treated A-549 cells, at an average of 2.82 and 1.52, respectively, rather than down-regulated. A-549 cells treated with ATRA (A-549 ± ATRA microarray) did not show up-regulation of these genes, however, A-549 cells retrovirally transduced with the RARβ2 gene and treated or not with ATRA (-ATRA/+ATRA) up-regulated both ICAM-1 and MHC Class I by 2.0/3.3 and 1.3/1.7 fold, respectively (Appendix C).

3.5 Duality of function: RARβ2 versus E2F1, TGFβ and Kras2

The results obtained from the microarrays support the hypothesis that, in certain contexts, RARβ2 acts as a tumor promoter rather than a tumor suppressor, and elucidate the genes that may be responsible for duality of function.

Similar duality of function has recently been shown for the first time in three genes: E2F1, TGFβ and Kras2. E2F1, mentioned above, regulates a group of genes involved in G1/S transition (*41*). Transforming growth factor (TGFβ) is involved in multiple cellular processes including proliferation, differentiation, transformation and apoptosis (*48,49*). The oncogene Kras2 is a GTP-binding protein involved in signal transduction (*50*). These genes, akin to RARβ2, are all involved in multiple cellular processes, and E2F1 itself is down- and up-regulated (48% and 10%, respectively) in A-549 cells treated with JP-2 or ATRA, respectively. It may therefore confer duality downstream of RARβ2.

3.6 Future directions

3.6.1 Can results from A-549 microarray studies be generalized to other cancers?

It would be of major interest to determine which of the genes identified via paired microarray analyses to be both upregulated and downregulated via ATRA and JP-2 treatments, respectively, are similarly regulated in multiple cancer cell lines. Northern blot analysis of multiple cell lines treated with ATRA or JP-2 and probed with

radiolabeled cDNA constructs could be performed against the genes identified in Chapter 3. These experiments have already begun (Table XXVIII).

TABLE XXVIII: List of 17 genes suggested for Northern blot follow-up studies. A: mRNA expression in A-549 lung cancer cells following 1 uM ATRA treatment as per microarray results; or B; 400 nM JP-2 antisense oligo treatment as per microarray results; C: RA regulation of gene expression as classified in reference (*30*). Categories are: 3 = Directly by RA-RXR:RAR-RARE; 2 = Probably like category 1 but more proof required; 1 = Definitely RA-regulated; 0 = Probably through intermediary factors. ATRA = all trans-retinoic acid; N/A = not available; RA = retinoic acid. Gene loci found using reference (*51*).

17 Genes suggested for Northern blot follow-up studies					
Gene	ATRA[A]	JP2[B]	Category of RA regul'n[C]	Function(s)	Clone Image
cyp24	4.41	0.38	2/Up	Catabolism of chemotherapeutic agents	266146
NTRK3	0.99	0.36	1/Up	Cell signaling	971199
Survivin	1.02	0.46	1/Down	Inhibition of apoptosis	796694
ET-1	2.10	0.49	0/Down	Mitogenesis/Neoangiogenesis	47833
ALDH1	1.92	0.50	1/Up	Chemo. catabolism /RA metabolism	855624
FGFR4	0.94	0.49	1/Down	Cell signaling/Motility	2516430
cyclinA2	0.95	0.49	N/A	G2/M promotion	814270
cyclinB1	1.02	0.57	N/A	G2/M promotion	36374
E2F1	1.10	0.52	N/A	Rb binding/Apoptosis	768260
COX-2	N/A	3.69	1/Up&Down	Mitogenesis	845477
IGFBP3	2.56	0.56	1/Up&Down	IGF signaling	898218
TUBA3	2.04	0.51	N/A	Mitosis/Intra & extracellular transport	1470060
PLK	0.65	0.48	N/A	Tumorigenesis/Transformation	744047
CDKN1a	N/A	12.08	1/Up&Down	Induces G1/S arrest/pro-apoptotic	2549557
ICAM-1	N/A	2.99	1/Up	Intercellular adhesion/CD8+ costimul.	293413
MHC1	N/A	2.30	2/Up	Autologous antigen presentation	198453
MYBL2	1.04	0.51	1/Down	Regulates G1/S	815526

3.6.2 Can microarray results be generalized in the isogenic variants of a same cell line?

The microarray results compare the experimental effects of RA stimulation (via ATRA treatment) versus RARβ2 knock-down (via JP-2 antisense oligo treatment). They do not however compare the gene expression profiles of a same cell line having undergone a genetic or other type of event leading to the creation of isogenic RARβ2 variants, which is a more realistic phenomenon. Thus, two other major goals would be to determine whether inactivation or stimulation of exogenous RARβ2 expression in an isogenic cell line results in different gene expression profiles, and to determine whether these are the same dual profiles as those elucidated in the A-549 cancer cell line. A straightforward method would be to repeat the paired microarray analyses previously performed on RA or JP-2 treated A-549 lung cancer cells except using the existing C64 subclones of CALU-1 lung cancer cells that have been transfected with exogenous RARβ2.

3.6.3 Are parameters of transformation reduced in cancer cells in which RARβ2 expression is reduced?

To determine whether or not formally accepted characteristics of transformation, such as anchorage independence and motility, are reduced upon RARβ2-inhibition in cancer cells having retained RARβ2 expression, NCI-H125 lung cancer cells, for example, may be seeded, treated or not with JP-2, transferred to soft agar or matrigel dishes, and counted for colony formation and extent of membrane traverse, respectively.

3.6.4 Does the simultaneous inhibition of RARβ2 and COX-2 lead to synergistic effects?

COX-2, as mentioned above, is a potent mitogen known to be upregulated in lung cancer, especially adenocarcinoma. Since COX-2 expression was significantly up-regulated in A-549 cells treated with JP-2, it would be of clinical interest to determine if the combined treatment of RARβ2 and COX-2 inhibitors [i.e. JP-2 and Celecoxib (Pharmacia), respectively] has additive or synergistic effects on the growth, programmed cell death and gene expression profile of lung cancer cells. These experiments were begun in the laboratory and results appear promising.

4 The link between methylation studies and antisense studies

As detailed in section 6 of Chapter 1, previous evidence that RARβ2 may play a dual role in cancer exists at many levels, and RARβ2 is frequently inactivated via hypermethylation in multiple cancers. Moreover, methylation has been shown to be reversible in cancer cells lines using DNA methyltransferase inhibitors, such as 5-aza-2'-deoxycytidine (*5,52-54*), and several groups including ours have shown the existence of cell lines where RARβ2 inactivation is not correlated with methylation [(*53,55-62, Manuscript 1 in Chapter 2 (i.e. MM-1 and T47D)*]. We show that several cancer cell lines exhibit *divergent methylation*, the coexistence of differentially methylated alleles within the same cell (i.e. un/hypomethylated and hypermethylated alleles) (*62*), suggesting that another mechanism is responsible for RARβ2 inactivation on the un/hypomethylated allele. Though it is not yet known why certain genes are primarily inactivated via methylation (*63*), or why others are primarily inactivated via mutation, methylation and divergent methylation represent mechanisms that are potentially reversible and may allow for alternation between gene turn-off and reactivation, and hence the manifestation of duality of function with regard to tumor suppression versus promotion. And, this may be the case for RARβ2. After all, the role of RARβ2 as a putative tumor suppressor remains controversial after more than 15 years of investigation. Some experimental studies clearly demonstrate its tumor suppressive effects in certain contexts (*64*), but there is yet to be a consensus within the scientific community as to whether it is a *bone fide* tumor suppressor. Until recently, this was mainly due to two reasons: (1) no genetic alteration affecting its remaining allelic copy has ever been found, and such an alteration was a requirement for classification as a proper tumor suppressor gene (TSG) (*65*); (2) the region affected by LOH is thought to contain putative TSGs other than RARβ2. The findings shown here are therefore relevant both at the fundamental and the clinical level. At the fundamental level, together, the results suggest that the retention of one unaltered allele may allow the alternation between loss of expression and reactivation expected with duality of function. At the clinical level, cancers in which RARβ2 plays a beneficial role (as a tumor suppressor) will need to be distinguished from cancers in which RARβ2 plays a deleterious role (as a tumor promoter), especially when RA, a known stimulant of RARβ2 expression and function is being considered as a therapeutic modality for anti-cancer

treatment. A simple assay of RARβ2 expression in conjunction with expression analysis of key RARβ2-downstream genes may prove to be prognostic in ascertaining whether or not retinoids should or should not be prescribed.

5 Next steps

5.1 Does the status of methylation of the RARβ2 promoter change upon metastasis?

It would be pertinent to determine the statuses of expression and methylation of RARβ2 in primary versus corresponding metastatic lung tumors, with the prerequisite that their clonal relationship be confirmed (*66*), in order to determine whether or not RARβ2 expression is alternately active and inactive along the metastatic process and whether alternate methylation statuses play a role. Hence, the straightforward analysis/comparison of DNA methylation such as through BGS using DNA from primary and metastatic tumors would provide these data.

5.2 Does RARβ2 get reactivated during carcinogenesis or metastasis?

An intriguing objective is to determine whether the effects of RARβ2 inactivation and reactivation are different in different tissues or different points along the carcinogenic process, such as the lung, when and where primary lung cancers occur, or lymph nodes or other sites such as bone, when and where metastases tend to occur. Various conditional mouse models, including *cre/loxP*, *Tet*, and *Tamoxifen*, have been used to activate and inactivate oncogenes, including Myc, in a time- and tissue-specific manner, to determine if their effect in the gene in question induces or stops the progression of carcinogenesis. *Cre* is a DNA recombinase (from the P1 bacteriophage) that is functional only at *loxP* recognition sites, which do not exist in the mouse except for the exogenous sequences created in the transgene mice. Therefore, two RARβ2 functional domains, separated by a polyadenylation site to prevent expression and under the control of a tissue-specific promoter, could be floxed by *loxP* sites (i.e. ligated with *loxP* sequences at its 5' and 3'-most ends) and transfected into mouse ES cells. Meanwhile, the *cre* gene, under the control of the Tet-O promoter which requires doxocycline for Tet-O binding and activation, could also be transfected into these cells. The resulting transgenic mouse would express functional RARβ2 only in the tissue targeted by the

promoter and only upon treatment with doxocycline. cDNA microarray could be used to further elucidate the RARβ2 downstream genes responsible for this duality.

5.3 RARβ2 expression in cancer cell lines; three different scenarios

Regarding RARβ2, there appear to be three different scenarios in cancer cell lines, and these reveal a complexity till now unseen when tumors alone are analyzed; cells may be: (1) RARβ2-negative at baseline but RARβ2-inducible by RA, and RA-sensitive; (2) RARβ2-negative permanently, due to epigenetic silencing, and RA-resistant; or (3) RARβ2-positive constitutively, yet RA-resistant. We have shown that the absence of RARβ2 has detrimental biological consequences, and we have also shown that in some cases, the constitutive expression appears to have detrimental consequences as well.

6 Oncogenes and tumor suppressor genes; a philosophical reappraisal of the terms "growth stimulation" and "growth inhibition"

Theodor Boveri and his wife Marcella O'Grady attempted to determine the precise molecular and cellular origins of cancer nearly 100 years ago (*67*). They postulated that "growth stimulating" and "growth inhibitory" chromosomes cause the "out-of-balance" behavior of cancer cells and that "multi-polar" rather than bi-polar mitosis was the cause of the abnormal chromosomal constitution of cancer cells (*68,69*). Current postulates have not shifted significantly from this paradigm since cancer cell progression is frequently described as a dichotomy between growth promotion and growth suppression mainly controlled by "oncogenes" and "tumor suppressor genes" (TSG), respectively. Hence, the opposing forces on growth were attributed to multi-gene entities (chromosomes) by the Boveris, whereas they are now attributed to genetic-units (genes). It is interesting to note that the term "oncogene" was originally coined to describe the corresponding viral precursor gene ("v-onc") of a cellular oncogene ("c-onc") and thought to be causally associated with cancer development, but this term is now used to describe genes that contribute to cancer-related processes when activated (mainly by mutation), and TSGs ("anti-oncogenes") inhibit cancer-related processes unless inactivated. Nevertheless, there actually exist several hypotheses on the origin of cancer, some based on parameters other than this paradigm, and some of which are competing.

Indeed, Wunderlich stated that our current vision of cancer is nearly identical to that formed by the Boveris, except that we now have a bounty of intelligence concerning the genes within the chromosomes to which the Boveris were referring (*70*), suggesting that we haven't made enormous progress in the last 100 years. In addition, Solé and Deisboeck underline that we view and target cancer as a simple rather than a complex disease (*71*), signifying that our failures to control or eradicate cancer stem from a misguided vision of what cancer really is.

It is true that even though more than a dozen classes of cellular and molecular processes are studied for dysregulation in modern, post-Watson-Crick, cancer research, we ultimately view all abnormal processes as contributors to growth "promotion" or "suppression". These processes include DNA replication, repair, methylation and transcription, RNA processing, inhibition and translation, protein modification such as phosphorylation, and protein localization, DNA packaging, histone deacetylation, chromosomal segregation and cellular partitioning, etc. In fact, this remains the case even in light of several newly discovered cancer-related processes, including neo-angiogenesis (*72*), immune evasion (*73*) and programmed cell death avoidance (*73*). Thus, while highlighting the appreciation for Boverian dogmas, the use of only two concepts, oncogene and TSG, reveals an attempt to simplify the complex. This position is supported by the suggestion that our failures to reduce cancer incidence and mortality and to increase cancer survival rates (Chapter 1, section 1).

The act of classifying a gene in one or the other category also blurs the possibility that it may possess both growth stimulating *and* inhibiting functions, depending on various factors. Though it is evident that growth stimulating and growth inhibitory processes are balanced in normal cells and unbalanced in cancer cells, it is also evident that cancer is a multifactorial disease and so are the genes that play a part in it. It might thus behove us to pose questions such as "*Which abnormal molecular processes are causal and which are consequential?*", and "*How does the role of a given gene product change in different microenvironments?*" It may also be beneficial to summarize and fuse cohesive hypotheses on carcinogenesis and fit complex gene function within known theories.

Another area of concern regards is the nature of the phenomenon studied. Three types of carcinogenic events are thought to exist: (1) primary events, which are causally-related to carcinogenesis; (2) secondary events, which are phenomena generated as a consequence of primary events but do not play causal roles; and (3) tertiary events, which constitute epiphenomena – they do not play – nor are derived from - causal roles. Indeed, literature from the last five years shows that there is a new effort to distinguish causal events from epiphenomena (e.g. (*74-79*).

The development of certain technologies such as pathological X-ray crystallography in the 1970's (*80*), high-throughput DNA sequencing in the late 1980's (*81*), and microarrays technology in the 1990's, have confirmed – if not shown for the first time in some case - that cancer and other diseases such as diabetes, are *families of separate illnesses,* which have distinguishing molecular biological etiologies and thus potentially different responses to the "one-drug-fits-all" approach of recent and current cancer medical practices. In addition, other elements of molecular biological research from the last quarter century, such as the finding that certain sequences of molecular events, such as a particular set of DNA mutations occur in normal non-malignant cells well before the development of an overt cancer (*82*), evoke the idea that cancer is a *progressive* condition rather than a sudden or near-sudden onset disease. Moreover, elements though to be integral to the carcinogenic process, such as spontaneous DNA mutations, take place in normal human cells in a continuous and ubiquitous manner prior to the development of a covert tumor. (The spontaneous rate of DNA mutation is thought to be approximately 10^{-10} mutations per nucleotide, per cell generation (*83*). Thus, the overall mutation rate of an average healthy human diploid epithelial cell, containing the average 6×10^9 nucleotides per human genome, and replicating every 2 days, is approximately 1.2 mutations per cell division). Also, much time (years, decades) is required for cells to acquire cancer-related aberrations, such as mutational activation or inactivation of key genes, such as *ras* or *myc*, respectively, for example (*82*). Thus, the field of molecular biology brought depth and refinement to the definition of cancer, and has consequently allowed a similar refinement of the tools that allow the diagnosis and intervention of cancer progression.

More recent examples include the completion of the human genome project (*84*), and the bounty of medical applications emerging from it [for a review see reference (*85*)], which are spurring the creation and coming-of-age of new fields of medicine. Two cases in point are: (1) *pharmacogenetics,* which seeks to develop novel biological markers of disease and diminish individual undesired physiological responses to drugs by tailoring molecular biological formulas to the individual patient; and (2) *molecular epidemiology,* which aims to dissect and assess gene-environment and gene-risk behavior interactions. [For an in-depth overview of the pharmacogenetic view and suggested approaches to non-small cell lung cancers, see the review by Danesi (*86*)].

Thus, the term "cancer" may define a set of clinically similar diseases, but at the cellular and molecular levels, two related cancers might warrant different treatments based both on the tumor's molecular characteristics and the patients' genomic and proteomic backgrounds. Moreover, in light of these increasingly complex findings, it is more and more obvious that the terms oncogene and TSG need reappraisal. RARβ2 is a case in point.

References

(*1*) Wilson KL, Zastrow MS, Lee KK. Lamins and disease: insights into nuclear infrastructure. Cell 2001;104:647-50.

(*2*) Koken MH, Reid A, Quignon F, Chelbi-Alix MK, Davies JM, Kabarowski JH, et al. Leukemia-associated retinoic acid receptor alpha fusion partners, PML and PLZF, heterodimerize and colocalize to nuclear bodies. Proc Natl Acad Sci U S A 1997;94:10255-60.

(*3*) Sutherland HG, Mumford GK, Newton K, Ford LV, Farrall R, Dellaire G, et al. Large-scale identification of mammalian proteins localized to nuclear sub-compartments. Hum Mol Genet 2001;10:1995-2011.

(*4*) Di Croce L, Raker VA, Corsaro M, Fazi F, Fanelli M, Faretta M, et al. Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. Science 2002;295:1079-82.

(*5*) Sirchia SM, Ren M, Pili R, Sironi E, Somenzi G, Ghidoni R, et al. Endogenous reactivation of the RARbeta2 tumor suppressor gene epigenetically silenced in breast cancer. Cancer Res 2002;62:2455-61.

(*6*) Brown AJ. Mechanisms for the selective actions of vitamin D analogues. Curr Pharm Des 2000;6:701-16.

(*7*) Allegretto EA, Shevde N, Zou A, Howell SR, Boehm MF, Hollis BW, et al. Retinoid X receptor acts as a hormone receptor in vivo to induce a key metabolic enzyme for 1,25-dihydroxyvitamin D3. J Biol Chem 1995;270:23906-9.

(*8*) Darwish HM, DeLuca HF. Recent advances in the molecular biology of vitamin D action. Prog Nucleic Acid Res Mol Biol 1996;53:321-44.

(*9*) Albertson DG, Ylstra B, Segraves R, Collins C, Dairkee SH, Kowbel D, et al. Quantitative mapping of amplicon structure by array CGH identifies CYP24 as a candidate oncogene. Nat Genet 2000;25:144-6.

(*10*) Mimori K, Tanaka Y, Yoshinaga K, Masuda T, Yamashita K, Okamoto M, et al. Clinical significance of the overexpression of the candidate oncogene CYP24 in esophageal cancer. Ann Oncol 2004;15:236-41.

(*11*) Perocco P, Mazzullo M, Broccoli M, Rocchi P, Ferreri AM, Paolini M. Inhibitory activity of vitamin E and alpha-naphthoflavone on beta-carotene-enhanced transformation of BALB/c 3T3 cells by benzo(a)pyrene and cigarette-smoke condensate. Mutat Res 2000;465:151-8.

(*12*) Perocco P, Paolini M, Mazzullo M, Biagi GL, Cantelli-Forti G. beta-carotene as enhancer of cell transforming activity of powerful carcinogens and cigarette-smoke condensate on BALB/c 3T3 cells in vitro. Mutat Res 1999;440:83-90.

(*13*) Hukkanen J, Vaisanen T, Lassila A, Piipari R, Anttila S, Pelkonen O, et al. Regulation of CYP3A5 by glucocorticoids and cigarette smoke in human lung-derived cells. J Pharmacol Exp Ther 2003;304:745-52.

(*14*) Marill J, Cresteil T, Lanotte M, Chabot GG. Identification of human cytochrome P450s involved in the formation of all-trans-retinoic acid principal metabolites. Mol Pharmacol 2000;58:1341-8.

(*15*) Kyu-Ho Han E, Gehrke L, Tahir SK, Credo RB, Cherian SP, Sham H, et al. Modulation of drug resistance by alpha-tubulin in paclitaxel-resistant human lung cancer cell lines. Eur J Cancer 2000;36:1565-71.

(*16*) Sladek NE. Aldehyde dehydrogenase-mediated cellular relative insensitivity to the oxazaphosphorines. Curr Pharm Des 1999;5:607-25.

(*17*) Jacquot C, Lanco X, Carbonnelle D, Sevestre O, Tomasoni C, Briad G, et al. Effect of four genes (ALDH1, NRF1, JAM and KBL) on proliferation arrest in a non-small cell bronchopulmonary cancer line. Anticancer Res 2002;22:2229-35.

(*18*) Andreola F, Fernandez-Salguero PM, Chiantore MV, Petkovich MP, Gonzalez FJ, De Luca LM. Aryl hydrocarbon receptor knockout mice (AHR-/-) exhibit liver retinoid accumulation and reduced retinoic acid metabolism. Cancer Res 1997;57:2835-8.

(*19*) Baxter RC. Signalling pathways involved in antiproliferative effects of IGFBP-3: a review. Mol Pathol 2001;54:145-8.

(*20*) Toulouse A, Loubeau M, Morin J, Pappas JJ, Wu J, Bradley WE. RARbeta involvement in enhancement of lung tumor cell immunogenicity revealed by array analysis. Faseb J 2000;14:1224-32.

(*21*) Segars JH, Nagata T, Bours V, Medin JA, Franzoso G, Blanco JC, et al. Retinoic acid induction of major histocompatibility complex class I genes in NTera-2 embryonal carcinoma cells involves induction of NF-kappa B (p50-p65) and retinoic acid receptor beta-retinoid X receptor beta heterodimers. Mol Cell Biol 1993;13:6157-69.

(*22*) Shang Y, Baumrucker CR, Green MH. Signal relay by retinoic acid receptors alpha and beta in the retinoic acid-induced expression of insulin-like growth factor-binding protein-3 in breast cancer cells. J Biol Chem 1999;274:18005-10.

(*23*) Gucev ZS, Oh Y, Kelley KM, Rosenfeld RG. Insulin-like growth factor binding protein 3 mediates retinoic acid- and transforming growth factor beta2-induced growth inhibition in human breast cancer cells. Cancer Res 1996;56:1545-50.

(*24*) Dokmanovic M, Chang BD, Fang J, Roninson IB. Retinoid-induced growth arrest of breast carcinoma cells involves co-activation of multiple growth-inhibitory genes. Cancer Biol Ther 2002;1:24-7.

(*25*) Goodwin LO, Mason JM, Hajdu SI. Gene expression patterns of paired bronchioloalveolar carcinoma and benign lung tissue. Ann Clin Lab Sci 2001;31:369-75.

(*26*) Deal C, Ma J, Wilkin F, Paquette J, Rozen F, Ge B, et al. Novel promoter polymorphism in insulin-like growth factor-binding protein-3: correlation with serum levels and interaction with known regulators. J Clin Endocrinol Metab 2001;86:1274-80.

(*27*) Oh Y, Muller HL, Ng L, Rosenfeld RG. Transforming growth factor-beta-induced cell growth inhibition in human breast cancer cells is mediated through insulin-like growth factor-binding protein-3 action. J Biol Chem 1995;270:13589-92.

(*28*) Lahaye DH, Walboomers F, Peters PH, Theuvenet AP, Van Zoelen EJ. Phenotypic transformation of normal rat kidney fibroblasts by endothelin-1. Different mode of action from lysophosphatidic acid, bradykinin, and prostaglandin f2alpha. Biochim Biophys Acta 1999;1449:107-18.

(*29*) Fagan KA, McMurtry IF, Rodman DM. Role of endothelin-1 in lung disease. Respir Res 2001;2:90-101.

(*30*) Balmer JE, Blomhoff R. Gene expression regulation by retinoic acid. J Lipid Res 2002;43:1773-808.

(*31*) Ahmed SI, Thompson J, Coulson JM, Woll PJ. Studies on the expression of endothelin, its receptor subtypes, and converting enzymes in lung cancer and in human bronchial epithelium. Am J Respir Cell Mol Biol 2000;22:422-31.

(*32*) Oelmann E, Sreter L, Schuller I, Serve H, Koenigsmann M, Wiedenmann B, et al. Nerve growth factor stimulates clonal growth of human lung cancer cell lines and a human glioblastoma cell line expressing high-affinity nerve growth factor binding sites involving tyrosine kinase signaling. Cancer Res 1995;55:2212-9.

(*33*) Velculescu VE, Madden SL, Zhang L, Lash AE, Yu J, Rago C, et al. Analysis of human transcriptomes. Nat Genet 1999;23:387-8.

(*34*) Rohayem J, Diestelkoetter P, Weigle B, Oehmichen A, Schmitz M, Mehlhorn J, et al. Antibody response to the tumor-associated inhibitor of apoptosis protein survivin in cancer patients. Cancer Res 2000;60:1815-7.

(*35*) Ikehara M, Oshita F, Kameda Y, Ito H, Ohgane N, Suzuki R, et al. Expression of survivin correlated with vessel invasion is a marker of poor prognosis in small adenocarcinoma of the lung. Oncol Rep 2002;9:835-8.

(*36*) Olie RA, Simoes-Wust AP, Baumann B, Leech SH, Fabbro D, Stahel RA, et al. A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cancer cells to chemotherapy. Cancer Res 2000;60:2805-9.

(*37*) Pagano M, Pepperkok R, Verde F, Ansorge W, Draetta G. Cyclin A is required at two points in the human cell cycle. Embo J 1992;11:961-71.

(*38*) Qian ZR, Sano T, Asa SL, Yamada S, Horiguchi H, Tashiro T, et al. Cytoplasmic expression of fibroblast growth factor receptor-4 in human pituitary adenomas: relation to tumor type, size, proliferation, and invasiveness. J Clin Endocrinol Metab 2004;89:1904-11.

(*39*) Ezzat S, Yu S, Asa SL. Ikaros isoforms in human pituitary tumors: distinct localization, histone acetylation, and activation of the 5' fibroblast growth factor receptor-4 promoter. Am J Pathol 2003;163:1177-84.

(*40*) Stein CA. Does antisense exist? Nat Med 1995;1:1119-21.

(*41*) Johnson DG. The paradox of E2F1: oncogene and tumor suppressor gene. Mol Carcinog 2000;27:151-7.

(*42*) Chan DC. Anti-growth factor therapy for lung cancer. Drug Resist Updates 1998;1:377-88.

(*43*) Khuri FR, Wu H, Lee JJ, Kemp BL, Lotan R, Lippman SM, et al. Cyclooxygenase-2 overexpression is a marker of poor prognosis in stage I non-small cell lung cancer. Clin Cancer Res 2001;7:861-7.

(*44*) Hida T, Kozaki K, Muramatsu H, Masuda A, Shimizu S, Mitsudomi T, et al. Cyclooxygenase-2 inhibitor induces apoptosis and enhances cytotoxicity of various anticancer agents in non-small cell lung cancer cell lines. Clin Cancer Res 2000;6:2006-11.

(*45*) Gocyk W, Niklinski T, Olechnowicz H, Duda A, Bielanski W, Konturek PC, et al. Helicobacter pylori, gastrin and cyclooxygenase-2 in lung cancer. Med Sci Monit 2000;6:1085-92.

(*46*) Hirsch FR, Franklin WA, Bunn PA, Jr. Expression of target molecules in lung cancer: challenge for a new treatment paradigm. Semin Oncol 2002;29:2-8.

(*47*) Passlick B, Pantel K, Kubuschok B, Angstwurm M, Neher A, Thetter O, et al. Expression of MHC molecules and ICAM-1 on non-small cell lung carcinomas: association with early lymphatic spread of tumour cells. Eur J Cancer 1996;32A:141-5.

(*48*) Akhurst RJ, Derynck R. TGF-beta signaling in cancer--a double-edged sword. Trends Cell Biol 2001;11:S44-51.

(*49*) Wakefield LM, Roberts AB. TGF-beta signaling: positive and negative effects on tumorigenesis. Curr Opin Genet Dev 2002;12:22-9.

(*50*) Zhang Z, Wang Y, Vikis HG, Johnson L, Liu G, Li J, et al. Wildtype Kras2 can inhibit lung carcinogenesis in mice. Nat Genet 2001;29:25-33.

(*51*) Anonymous. Online Mendelian Inheritance in Man, OMIM (TM). Vol 2002: Center for Medical Genetics, Johns Hopkins University (Baltimore, MD) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD); 1996.

(*52*) Cote S, Momparler RL. Activation of the retinoic acid receptor beta gene by 5-aza-2'-deoxycytidine in human DLD-1 colon carcinoma cells. Anticancer Drugs 1997;8:56-61.

(*53*) Cote S, Sinnett D, Momparler RL. Demethylation by 5-aza-2'-deoxycytidine of specific 5-methylcytosine sites in the promoter region of the retinoic acid receptor beta gene in human colon carcinoma cells. Anticancer Drugs 1998;9:743-50.

(*54*) Sirchia SM, Ferguson AT, Sironi E, Subramanyan S, Orlandi R, Sukumar S, et al. Evidence of epigenetic changes affecting the chromatin state of the retinoic acid receptor beta2 promoter in breast cancer cells. Oncogene 2000;19:1556-63.

(*55*) Bovenzi V, Le NL, Cote S, Sinnett D, Momparler LF, Momparler RL. DNA methylation of retinoic acid receptor beta in breast cancer and possible therapeutic role of 5-aza-2'-deoxycytidine. Anticancer Drugs 1999;10:471-6.

(*56*) Arapshian A, Kuppumbatti YS, Mira-y-Lopez R. Methylation of conserved CpG sites neighboring the beta retinoic acid response element may mediate retinoic acid receptor beta gene silencing in MCF-7 breast cancer cells. Oncogene 2000;19:4066-70.

(*57*) Bovenzi V, Momparler RL. Quantitation of inhibition of DNA methylation of the retinoic acid receptor beta gene by 5-Aza-2'-deoxycytidine in tumor cells using a single-nucleotide primer extension assay. Anal Biochem 2000;281:55-61.

(58) Virmani AK, Rathi A, Zochbauer-Muller S, Sacchi N, Fukuyama Y, Bryant D, et al. Promoter methylation and silencing of the retinoic acid receptor-beta gene in lung carcinomas. J Natl Cancer Inst 2000;92:1303-7.

(59) Widschwendter M, Berger J, Hermann M, Muller HM, Amberger A, Zeschnigk M, et al. Methylation and silencing of the retinoic acid receptor-beta2 gene in breast cancer. J Natl Cancer Inst 2000;92:826-32.

(60) Gazdar AF, Zochbauer-Moller S, Virmani A, Kurie J, Minna JD, Lam S. RESPONSE: Re: Promoter Methylation and Silencing of the Retinoic Acid Receptor-beta Gene in Lung Carcinomas. J Natl Cancer Inst 2001;93:67-8.

(61) Lamy A, Metayer J, Thiberville L, Frebourg T, Sesboue R. Re: Promoter methylation and silencing of the retinoic acid receptor-beta gene in lung carcinomas. J Natl Cancer Inst 2001;93:66-8.

(62) Pappas JJ, Bradley WEC. The divergent methylation of the RARbeta2 promoter is correlated with an allelic bias for methylation. 2004.

(63) Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. Oncogene 2002;21:5427-40.

(64) Houle B, Rochette-Egly C, Bradley WE. Tumor-suppressive effect of the retinoic acid receptor beta in human epidermoid lung cancer cells. Proc Natl Acad Sci U S A 1993;90:985-9.

(65) Haber D, Harlow E. Tumour-suppressor genes: evolving definitions in the genomic age. Nat Genet 1997;16:320-2.

(66) Shattuck TM, Westra WH, Ladenson PW, Arnold A. Independent Clonal Origins of Distinct Tumor Foci in Multifocal Papillary Thyroid Carcinoma. N Engl J Med 2005;352:2406-12.

(67) Wright MR. Marcella O'Grady Boveri (1863-1950): her three careers in biology. Isis 1997;88:627-52.

(68) Boveri T. The Origin of Malignant Tumors. Baltimore: Williams and Wilkins; 1929.

(69) Boveri T. Zur Frage der Entstehung Maligner Tumoren. Jena: Fischer Verlag; 1914.

(70) Wunderlich V. JMM—past and present. Chromosomes and cancer: Theodor Boveri's predictions 100 years later. J Mol Med 2002;80:545-8.

(71) Solé RV, Deisboeck T. An error catastrophe in cancer? J Theor Biol. 2004:In press.

(72) Folkman J. Fundamental concepts of the angiogenic process. Curr Mol Med 2003;3:643-51.

(73) Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. Nat Immunol 2002;3:999-1005.

(74) Cahill DP, Kinzler KW, Vogelstein B, Lengauer C. Genetic instability and darwinian selection in tumours. Trends Cell Biol 1999;9:M57-60.

(75) Knudson AG. Two genetic hits (more or less) to cancer. Nat Rev Cancer 2001;1:157-62.

(*76*) Sonnenschein C, Soto AM. Somatic mutation theory of carcinogenesis: why it should be dropped and replaced. Mol Carcinog 2000;29:205-11.

(*77*) Bignold LP. The mutator phenotype theory of carcinogenesis and the complex histopathology of tumours: support for the theory from the independent occurrence of nuclear abnormality, loss of specialisation and invasiveness among occasional neoplastic lesions. Cell Mol Life Sci 2003;60:883-91.

(*78*) Duesberg P, Li R. Multistep carcinogenesis: a chain reaction of aneuploidizations. Cell Cycle 2003;2:202-10.

(*79*) Sieber OM, Heinimann K, Tomlinson IP. Genomic instability--the engine of tumorigenesis? Nat Rev Cancer 2003;3:701-8.

(*80*) Ghadially FN. Invited review. The technique and scope of electron-probe X-ray analysis in pathology. Pathology 1979;11:95-110.

(*81*) Hood LE, Hunkapiller MW, Smith LM. Automated DNA sequencing and analysis of the human genome. Genomics 1987;1:201-12.

(*82*) Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, et al. Genetic alterations during colorectal-tumor development. N Engl J Med 1988;319:525-32.

(*83*) Jackson AL, Loeb LA. The contribution of endogenous sources of DNA damage to the multiple mutations in cancer. Mutat Res 2001;477:7-21.

(*84*) Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, et al. Initial sequencing and analysis of the human genome. Nature 2001;409:860-921.

(*85*) Goldstein DB, Tate SK, Sisodiya SM. Pharmacogenetics goes genomic. Nat Rev Genet 2003;4:937-47.

(*86*) Danesi R, de Braud F, Fogli S, de Pas TM, Di Paolo A, Curigliano G, et al. Pharmacogenetics of anticancer drug sensitivity in non-small cell lung cancer. Pharmacol Rev 2003;55:57-103.

APPENDIX A

PULMONARY CELL TYPES

TABLE XXX. List of 20 cell types found in the lung. A. Epithelial cells; B. Immune cells; C. Structural and support cells; D. Endothelial cells. *Cell types known to have progenitor potential (see Section 4.4.1 Bronchial epithelium-specific progenitor stem cells). BM = basement membrane.

A. PULMONARY EPITHELIAL CELLS (OF LUNG ORIGIN)			
CELL	FUNCTION	LOCATION	*REFERENCES*
CILIATED EPITHELIAL CELLS	Primary cell type within the respiratory epithelium. Project cilia that beat and propel mucus anteriorly toward the pharynx.	Form the continuous lining of the respiratory tract within the bronchi and bronchioles.	*(1)*
GOBLET CELLS*	Secrete viscous mucus to protect against shear and chemical stress and entrap small particles. Rapidly proliferate leading to goblet cell hyperplasia upon various insults.	Found primarily in the trachea and bronchi (proximal RT), and in slightly diminishing numbers in distal and terminal bronchioles.	*(2)*
CLARA CELLS*	Synthesize and secrete lipoproteins, and metabolize xenobiotics via cytochrome p450 enzymes.	Found in progressively increasing numbers along R.T. Comprise totality of mucus-secreting cells in respiratory bronchioles (distal RT).	*(3)*
BASAL CELLS*	Function as the interface between the columnar epithelium and the basement membrane, and progress apically during differentiation.	Regularly scattered in epithelial lining, mainly in bronchi/bronchioles. Attached to BM via hemidesmosomal junctions.	*(4)*
SEROUS CELLS	Secrete thin and watery mucus (mucus of lower density than that secreted by goblet (mucus) cells). Increase number and function in certain diseases.	Within the continuous lining of the respiratory tract, but in increasingly lower numbers within distal and terminal bronchioles.	*(5)*
NEUROENDOCRINE CELLS*	Part of the diffuse neuroendocrine system. Secrete serotonin, bombesin, calcitonin, gastrin-releasing hormone, neuro-specific enolase and leucine-enkephalin.	Found as single cells or groups of approximately 100 cells called neuroendocrine bodies (NEBs), within the trachea, bronchi and bronchioles.	*(6)*
TYPE I PNEUMOCYTES	Form the protective lining of the alveolar sac. Protect against leakage and allow gas exchange between the air and the blood.	Lining/surface of alveoli. Relatively little known, but identification of new surface markers will allow progress.	*(7)*
TYPE II PNEUMOCYTES*	Secrete surfactant which reduces surface tension at the liquid/air interface and thus prevents alveolar collapse, surfactant also acts in non-specific immunity.	Form the alveolar septa (wall junctions).	*(8)*

B. PULMONARY IMMUNE CELLS (OF BONE MARROW ORIGIN)			
CELL	FUNCTION	LOCATION	*REFERENCES*
ALVEOLAR MACROPHAGES	Primary defense against invading microbes and inhaled particulates. Engulf and ingest them and may initiate a cellular-mediated immune type response. Secrete cytokines, eicosanoids and growth factors; respond with reactive oxygen and nitrogen species.	Reside mainly within the distal airspaces (in the lumen of the alveoli). Derive from circulating monocytes and other resident macrophages. Expelled via mucociliary elevator and cough (and thus may be present in the bronchioalveolar lavage fluid), or remain sequestered within the alveolar septum.	*(9,10)*
MAST CELLS	Normal function unknown, but release copious amounts of histamine in hypersensitivity type allergic responses and asthmatic diseases. Also known to secrete various other substances, such as tryptase, lysosomal enzymes, heparin, chondroitin, and various cytokines.	Reside mainly within the submucosal layer of the upper respiratory tract.	*(11)*
POLYMORPHO-NUCLEAR CELLS	One of several granulocytes that acts in non-specific immune responses by phagocytosing invading microorganisms, and releasing cytotoxic and proteolytic substances. Thought to take over when alveolar macrophage response is significantly increased (overwhelmed).	Mainly found within the pulmonary vasculature.	*(12)*
B and T LYMPHOCYTES	B cells secrete IgA within the U.R.T. and IgG within the L.R.T., which act as a sheath type barrier, and specifically (in binding antigen and eliciting a humoral response against inhaled microorganisms and antigens). T cells express specific surface T-cell receptors against cellular antigen and participate in anti-tumor responses.	B and T-cells are located within the lamina propria of the mucosa, compose the bronchial-associated lymphoid aggregates, and circulate in the lymph nodes.	*(13)*

C. PULMONARY STRUCTURE AND SUPPORT CELLS (OF MESENCHYMAL ORIGIN)			
CELL	FUNCTION	LOCATION	*REFERENCES*
SMOOTH MUSCLE CELLS	Contract to decrease passage diameter and thus increase resistance to air flow. Synthesize matrix proteins and muscle-specific alpha-actin, tropomyosin, desmin and myosin. Synthesize various other substances upon injury, such as tropoelastin, fibronectin, tenascin and cytokeratin 8. Vascular SMCs secrete VEGF, which mainly acts on endothelial cells*.	Highly heterogeneous cells found within the pulmonary vasculature and the mucosal layers of the upper and lower (but not distal) airways, and increase as the airway diameter decreases.	*(14,15)*
FIBROBLASTS	Secrete ECM components, such as ground substance, collagen and elastin. Respond to inflammatory cytokines by secreting collagen, and growth factors, and cytokines. Secrete excessive collagen during chronic inflammatory injury. Recently found to secrete keratinocyte growth factor (KGF), which regulates pulmonary epithelial cell proliferation.	Also found within alveolar walls.	*(16,17)*
MYOFIBRO-BLASTS	Have muscular and non-muscular characteristics, and play an important role in pulmonary homeostasis, and in development, repair and remodeling.	Also found within alveolar walls.	*(18)*
CHONDROCYTES	Secrete collagens (especially type I and III), elastins, microfibrils, proteoglycans, hyaluronic acid and chondronectin.	Within cartilagenous tissues and plates of serosal layers.	*N/A*

D. PULMONARY ENDOTHELIAL CELLS (HEMATOGENOUS AND LYMPHATIC ORIGIN)			
CELL	FUNCTION	LOCATION	*REFERENCES*
ENDO-THELIAL (Arterial, venous and capillary)	Accommodate 100% of cardiac output to vehicle the erythrocytes through lung allowing gas exchange, transport solutes, metabolize xenobiotics such as antibiotics, express angiotensin converting enzyme for regulating blood pressure, regulate hemostasis, inflammation, vasoregulation, angiogenesis, and vascular growth.	Heterogeneous cells found within the capillary network and the arterial, venous and capillary vasculature () and lymphatic vasculature of hematopoietic lineages.	*(19)*
ENDO-THELIAL (Lymph vessels)	Structural and functional components of the vascular and the lymphatic vessels. Regulated by several substances including VEGF, which is secreted by vascular SMCs.	Form the arterial, venous and capillary vessels, as well as the afferent and efferent lymphatic vessels. Involved in vascular development and angiogenesis.	(*20*)
ERY-THRO-CYTES	Contain hemoglobin for oxygen and carbon dioxide gas exchanges.	Found within capillaries where lung primary function of gas exchange occurs, as well as in transport (with blood flow) in other pulmonary vessels.	*(21)*

References

(*1*) Burkitt HG, Young B, Heath JW. Chapter 12. Respiratory system. In Burkitt HG, Young B, Heath JW, editors. Wheater's Functional Histology, A Text and Colour Atlas. 3 ed. Hong Kong: Churchill Livingstone; 1993. p. 220-34.

(*2*) Rogers DF. The airway goblet cell. Int J Biochem Cell Biol 2003;35:1-6.

(*3*) Komaromy L, Tigyi A. A unique cell type in the lung--the Clara cell (the non-ciliated bronchiolar epithelial cell). Acta Biol Hung 1988;39:17-29.

(*4*) Otto WR. Lung stem cells. Int J Exp Pathol 1997;78:291-310.

(*5*) Finkbeiner WE. Physiology and pathology of tracheobronchial glands. Respir Physiol 1999;118:77-83.

(*6*) Scheuermann DW. Comparative histology of pulmonary neuroendocrine cell system in mammalian lungs. Microsc Res Tech 1997;37:31-42.

(*7*) Williams MC. Alveolar type I cells: molecular phenotype and development. Annu Rev Physiol 2003;65:669-95.

(*8*) Fehrenbach H. Alveolar epithelial type II cell: defender of the alveolus revisited. Respir Res 2001;2:33-46.

(*9*) Guidi-Rontani C. The alveolar macrophage: the Trojan horse of Bacillus anthracis. Trends Microbiol 2002;10:405-9.

(*10*) Thomassen MJ, Kavuru MS. Human alveolar macrophages and monocytes as a source and target for nitric oxide. Int Immunopharmacol 2001;1:1479-90.

(*11*) Gurish MF, Boyce JA. Mast cell growth, differentiation, and death. Clin Rev Allergy Immunol 2002;22:107-18.

(*12*) Sibille Y, Marchandise FX. Pulmonary immune cells in health and disease: polymorphonuclear neutrophils. Eur Respir J 1993;6:1529-43.

(*13*) Pabst R, Tschernig T. Lymphocytes in the lung: an often neglected cell. Numbers, characterization and compartmentalization. Anat Embryol (Berl) 1995;192:293-9.

(*14*) Gittenberger-de Groot AC, DeRuiter MC, Bergwerff M, Poelmann RE. Smooth muscle cell origin and its relation to heterogeneity in development and disease. Arterioscler Thromb Vasc Biol 1999;19:1589-94.

(*15*) Low RB. Lung smooth muscle cell differentiation. Int. J. Biochem. Cell Biol. 1997:869-83.

(*16*) Shijubo N, Kojima H, Nagata M, Ohchi T, Suzuki A, Abe S, et al. Tumor angiogenesis of non-small cell lung cancer. Microsc Res Tech 2003;60:186-98.

(*17*) Carnevali S, Petruzzelli S, Longoni B, Vanacore R, Barale R, Cipollini M, et al. Cigarette smoke extract induces oxidative stress and apoptosis in human lung fibroblasts. Am J Physiol Lung Cell Mol Physiol 2003;284:L955-63.

(*18*) Walker GA, Guerrero IA, Leinwand LA. Myofibroblasts: molecular crossdressers. Curr Top Dev Biol 2001;51:91-107.

(*19*) Stevens T, Rosenberg R, Aird W, Quertermous T, Johnson FL, Garcia JG, et al. NHLBI workshop report: endothelial cell phenotypes in heart, lung, and blood diseases. Am J Physiol Cell Physiol 2001;281:C1422-33.

(*20*) Favre CJ, Mancuso M, Maas K, McLean JW, Baluk P, McDonald DM. Expression of genes involved in vascular development and angiogenesis in endothelial cells of adult lung. Am J Physiol Heart Circ Physiol 2003;285:H1917-38.

(*21*) Junqueira LC, Carneiro J, Kelley RO. Chapter 17: The Respiratory System. In Basic Histology. 9 ed: The McGraw-Hill Companies; 2002.

APPENDIX B

MANUSCRIPT 3

RARβ involvement in enhancement of lung tumor cell immunogenicity revealed by array analysis

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ABSTRACT **The retinoid receptors (RARs and RXRs) are mediators of the multiple effects of retinoic acid. Of these, the retinoic acid receptor β2 (RARβ2) has frequently been shown to be the principal mediator of the growth and tumor suppressive effects of retinoic acid; this gene is inactivated in many epithelial tumors and their derived cell lines. We have searched for genes that are regulated by this isoform and are potentially involved in tumor suppression. Using the Atlas human cDNA array I, we identified 27 genes (not counting RARβ itself) that are regulated, directly or indirectly, by RARβ2 when it is transfected into Calu-1, a lung tumor-derived line that does not normally express RARβ. Several of the affected genes code for proteins whose functions would augment the process of apoptosis and/or the host's immune response. The latter group included ICAM-1 and MHC class I heavy chain, whose protein products play particularly important roles in the mounting of an effective antitumor response. We then confirmed by flow cytometry that the observed increases in message levels were reflected in increased cell surface protein levels for ICAM-1 and MHC class I in RARβ2 transfectants of two RARβ-deficient lines, Calu-1 and the epidermoid lung cancer-derived line SK-MES. Finally, we showed that RARβ2 transfection of Calu-1 cells enhanced the heterologous CTL response in both the induction and the effector phases by up to threefold. These results support the hypothesis that down-regulation of these genes (and possibly others) in RARβ-deficient tumor cells contributes to immune system evasion, and suggest a novel therapeutic approach for this disease.—Toulouse, A., Loubeau, M., Morin, Pappas, J. J., Wu, J., Bradley W. E. C. RARβ involvement in enhancement of lung tumor cell immunogenicity revealed by array analysis. *FASEB J.* 14, 1224–1232 (2000)**

Key Words: lung cancer · retinoic acid receptor β · tumor suppression · cDNA array

RETINOIC ACID (RA) is essential for development and epithelial differentiation, and has been shown in many epidemiological (1) and animal (2) studies to have tumor suppressive effects. These effects are mediated by two families of nuclear RA receptors (RARs), each comprising three genes (RARα, β, and γ as well as RXR α, β, and γ; for review, see ref 3). These receptors are transcription factors that specifically bind RA-responsive elements (RAREs) in the promoters of genes whose expression they control. Each gene codes for at least two functional isoforms (3); of these receptors, it is RARβ, and more specifically the isoform β2, that has been implicated most frequently in suppression of epithelial cancers. For example, it has been shown that a majority of lung, breast, and other tumor-derived cell lines as well as the tumors themselves no longer express RARβ whereas expression is detected in the corresponding normal tissues (4–10). In addition, its forced re-expression in RARβ$^-$ lines reduces or eliminates various aspects of the tumor phenotype, including anchorage independence, focus formation and growth rate *in vitro*, and tumor formation in nude mice (reviewed in ref 11). Transgenic mice expressing antisense or other constructs that down-regulate RARβ2 develop lung and breast cancer (12, 13). RARα appears to mediate growth rate suppression in some breast tumor lines by modulating RARβ expression (14), but otherwise there is little indication that the other RA receptors contribute to epithelial tumor suppression.

To understand the mechanism of RARβ2-mediated tumor suppression, we have searched for genes regulated either directly or indirectly by RARβ2 that could be implicated in this suppression. We considered it important to compare lines that were as similar as possible, so we made use of a panel of RAR-expressing derivatives of the Calu-1 cell line, in which RARβ2 tumor suppression has been well characterized (15). We then compared patterns of gene

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expression using the Atlas human cDNA array I and confirmed the differences for six of the cDNAs by an independent assay. In addition to RARβ itself, the expression levels of 27 cDNAs were reproducibly affected and 3 other genes were up-regulated by treatment with RA in a non-RARβ-specific fashion. A striking characteristic of the results is the concerted nature of the effect the alterations are predicted to have on tumor behavior. Thus, for example, we found increased expression levels of several genes that regulate the immunogenicity of the tumor cell, most notably intercellular adhesion molecule 1 (ICAM-1), major histocompatibility complex (MHC) class I, and interleukin 1β (IL-1β). Since the consequences of down-regulation of these would be the well-documented crippling of the immune system's capacity to detect and kill the nascent tumor, we hypothesized a role for RARβ in this function. To test this, we first confirmed that cell surface expression of the proteins was increased, and then demonstrated that the consequence of these changes was increased alloantigen-specific CTL response in both the induction and effector phases. These results have implications for understanding the nature of tumor evasion of immune surveillance.

MATERIALS AND METHODS

Cell culture

Two RARβ-deficient epidermoid lung cancer cell lines, Calu-1 and SK-MES, and their transfected derivatives were maintained in alpha medium supplemented with 10% fetal bovine serum (Gibco BRL, Burlington, Canada) and 1% penicillin-streptomycin (Gibco BRL, Burlington, Canada) at 37°C in a humid atmosphere provided with 5% CO_2. All transfectants were maintained in 400 μg/ml of G418 (Gibco BRL). The Calu-1 derivatives C30 (RARβ$^-$, transfected with the neomycin resistance gene only), C24, and C64 (both transfected with RARβ2) have been described elsewhere (15). The RARβ1-transfected line (Cβ10) was obtained by following exactly the same protocol, using the pSVL vector carrying the RARβ1 cDNA sequences (A. Toulouse et al., unpublished results). The RARβ2$^+$ derivative of SK-MES, named C102, was obtained by transfection with the RARβ2 expression vector as described previously (15). Where indicated, cells were treated with pharmacological doses (100 nM) of *all-trans* retinoic acid (Sigma, St. Louis, Mo.) prior to collection.

RNA preparation

All cells were cultured to 70% confluency, pelleted, and stored at −80°C for subsequent RNA extraction using the LiCl-urea method (16). Poly A+ mRNA was prepared using Qiagen's Oligotex mRNA midi kit (Mississauga, Canada) and its quality was assessed following the manufacturer's instructions.

Atlas human cDNA expression array

The Atlas human cDNA array kit was purchased from Clontech Laboratories (Palo Alto, Calif.). All procedures for labeling and purifying the probes were accomplished by following manufacturer's recommendations. Complex α-^{32}P-dCTP-labeled cDNA probes were generated by reverse transcription of mRNA from untreated Calu-1 cells as well as C30 (neo^r), C24 (RARβ2$^+$), and C64 (RARβ2$^+$) treated with RA for 48 h. The probes were purified by column chromatography (ChromaSpin) and met or exceeded the manufacturer's recommendation for specific activity. The membranes were hybridized in ExpressHyb solution overnight at 68°C, washed twice (20 min with 0.1× SSC and 0.5% sodium dodecyl sulfate), and exposed for varying periods of time on Kodak XAR autoradiographic films.

RNA analysis

Northern blot analysis was performed according to standard protocols (17). The blot was probed using the insert of an IL-1β construct generated by amplification of a cDNA fragment (primers: 5'-GCTGCTCTGGGATTCTCTTC-3', 5'-AGCACAGGACTCTCTGGGTA-3'), which was digested with *Hin*dIII and *Acc*I prior to being cloned into pGEM-3Z. RNase protection assays were performed using Ambion's RPA II kit (Austin, Tex.) and the riboprobes were synthesized following standard protocols (17). The actin probe used as control was described elsewhere (15). The ID-3 probe was prepared by amplification of a cDNA fragment overlapping nucleotides 342 to 751 (primers: 5'-GCACCTCTGGACTCACTC-3', 5'-TGGAGGTGTCAGGACACG-3'). The polymerase chain reaction (PCR) product was digested with *Sma*I; a 409 bp fragment was isolated and cloned into the *Sma*I site of pGEM-3Z (Promega, Madison, Wis.). The plasmid was linearized using *Hin*dIII and the probe was synthesized using T7 RNA polymerase. The GADD-45 probe was obtained from Dr. P. A. Dion.

Immunological reagents

Lympholite H and phycoerythrin-conjugated goat anti-mouse IgG1 were purchased from Cedarlane (Hornby, Ontario, Canada). FITC-conjugated mAb (clone W6/32) against the conserved region of the human HLA class I antigen was obtained from Sigma (Oakville, Ontario, Canada), and mAb against ICAM-1 (clone 8.4A6) was from BioSource International (Camarillo, Calif.). Chromium-51 was purchased from ICN (Costa Mesa, Calif.).

Flow cytometry

One-color flow cytometry analysis was performed as follows. Where indicated, the cells were treated with RA at 100 nM for 5 or 7 days. To detect MHC class I proteins, 10^6 cells were incubated with 2 μg of FITC-conjugated monoclonal antibody W6/32 against human MHC class I antigen for 30 min on ice. The cells were then washed with phosphate-buffered saline (PBS) and fixed in 1% paraformaldehyde in PBS. To detect ICAM-1 protein expression, 10^6 cells were incubated with 1 μg of monoclonal antibody 8.4A6 for 30 min on ice and washed with PBS. These cells were then incubated with 600 ng of a goat anti-mouse IgG-1 antibody conjugated to R-phycoerythrin. The samples were read on a Profile I flow cytometer (Coulter, Burlington, Ontario, Canada). Two-color cytometry was performed in the same way except that labeling with the two antibodies conjugated with the respective fluorochromes was done simultaneously. All cytometric determinations were performed at least twice.

Cytotoxic lymphocytes (CTL) assay

Peripheral blood mononuclear cells (PBMC) were prepared as described previously (18). The Calu-1 derivatives C30 and C64 were cultured in either the presence or absence of RA (100 nM) for 5 days, then treated with mitomycin C. These mitomycin C-treated cells were used as stimulator cells for PBMC in a 5 day alloreactive culture in the absence of exogenous RA (18). The culture was performed in 24-well plates with 2×10^6 PBMC/ml and 2×10^6 stimulators/ml in a final volume of 2 ml/well. IL-2 (10 U/ml) was added at the beginning of the culture. After 5 days, the cells were washed and recounted. Fresh C30 and C64 cells that had been treated with 100 nM RA for 5 days prior to the assay were labeled with ^{51}Cr and used as targets in a standard 4 h ^{51}Cr release assay as described in a previous publication (19). The ratios of effector/target cells were set at 30:1, 10:1, 3:1, and 1:1. A fixed number of 15,000 target cells/well was used for all the determinations. Samples were in triplicate. The percentage of lysis was calculated as

$$\% = \frac{(\text{cpm of test sample} - \text{cpm of spontaneous release})}{(\text{cpm of maximal release} - \text{cpm of spontaneous release})}$$

RESULTS

To screen for differential expression of hundreds of genes, we used Clontech's Atlas human cDNA expression array I, which allows for direct screening of 588 cDNAs each spotted in duplicate and arranged in sextants, A to F (see **Fig. 1**). Nine additional genes that are thought to be constitutively expressed are displayed on the bottom row, G. Three negative controls include lambda, M13, and plasmid DNA, also in row G, and genomic DNA is arrayed along the right-hand and bottom borders to allow assessment of uniformity of hybridization. The cDNAs present on the membrane have been shown to be expressed in a regulated fashion and are involved in key cellular processes. Hybridizations were performed on four separate membranes, with probes generated from Calu-1 (a RARβ-deficient cell line) cells grown in the absence of added RA and three transfected derivatives grown in the presence of 100 nM RA for 48 h: C24, C64 (both RARβ2-expressing), and C30 (a neo^r-derivative), which we hereafter call C24-RA, C64-RA, and C30-RA. The latter was chosen as the RARβ-deficient line to be grown in the presence of RA because we have established that the parental Calu-1 line occasionally expresses trace levels of RARβ when grown in RA-supplemented medium, whereas C30 does not (data not shown). Previous work (11) had shown that the RNA fingerprints of these four lines, as well as two other lines derived from Calu-1 by transfection of RARβ1, varied from one line to another in less than 0.5% of their transcripts as determined by arbitrarily primed reverse transcription PCR (20), so the probes were comparable.

Uniformity of hybridization was demonstrable by

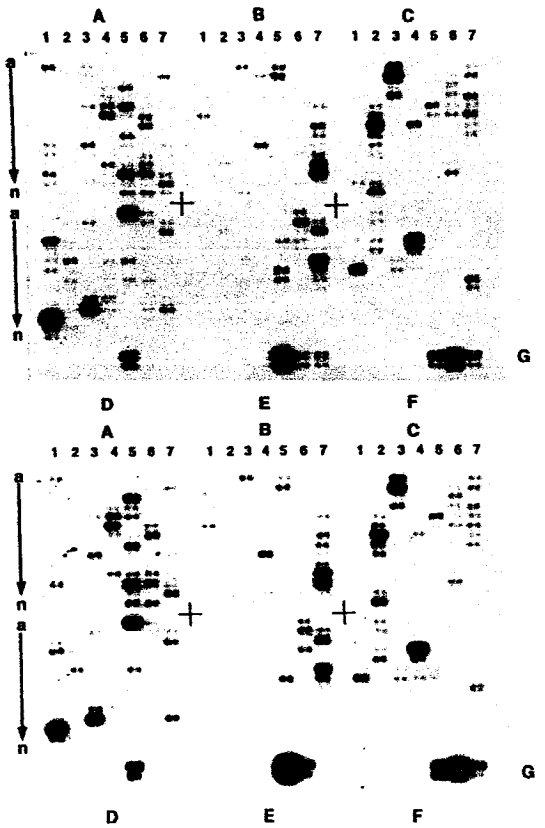

Figure 1. Atlas human cDNA array I. The probes were probed from RA-treated C64 cells (RARβ2$^+$) (top panel) or untreated Calu-1 cells (RARβ$^-$) (bottom panel). Capital letters designate sextants. Small letters indicate row positions and numbers refer to columns. Section G is the bottom row containing the 9 housekeeping controls. For cDNA identification, consult Clontech's Atlas array web page (atlas.clontech.com).

equality of intensity of hybridization of the genomic DNA spots on the extreme right border and the bottom row of section G on the original films. The negative controls built into the membrane in row G were all completely negative on all membranes (Fig. 1). To accommodate differences in specific activities of the probes used, the membranes were exposed for varying lengths of time so that meaningful comparisons could be made among all the membranes. A total of 71% of the cDNAs (428/597) were detectable after a 4 day exposure of the C64-RA-hybridized membrane (results available on request from W. E. C. Bradley), with somewhat lower numbers for the other membranes due to lower specific activity of the probes.

To evaluate the array results, spot densitometries were performed using the NIH Image software on the membranes probed with Calu-1, C30-RA, and C64-RA. Since the probe derived from C24-RA was weaker, densitometry of the corresponding array was

TABLE 1. *Evaluations of genes differentially regulated in RARβ-expressing cells*

Gene name	Position on array	C64-RA / Calu-1	C64-RA / C30-RA	Gene function
Up-regulated by RARβ				
C-yes	A3m	2.53	1.50	Protein tyrosine kinase
Inhibitor of DNA binding ID-1[a]	A5e	2.27	2.73	Inhibitor of differentiation, apoptosis
Cyclin A[a]	A6f	1.80	3.55	Cell cycle
p14 CDK-inhibitor (INK4B)	A7n	2.27	2.80	Cell cycle
RAB-5A RAS-related protein[a]	B4b	1.89	2.36	GTPase, endocytosis
IL-4 STAT[a]	B5aj	1.71	5.57	IL-4 signaling, inflammation
RARβ2[a]	C1k	[b]	[b]	Nuclear RA receptor
A1 protein (Bcl-2 related)	C4h	2.35	2.30	Apoptosis
Apoptosis inhibitor IAP1	C4i	3.68	2.69	Apoptosis
Cysteine protease ICE-LAP3	C5f	1.58	2.52	Apoptosis
GADD-45[a]	C7f	1.65	2.86	Growth arrest
Inhibitor of DNA-binding ID-3[a]	D1d	1.90	2.90	Inhibitor of differentiation, apoptosis
DNA binding protein SATB1[a]	D5h	1.77	1.68	MAR binding protein
Homolog of Disc Large (HDLG1)	D6k	1.69	2.11	Tumor suppressor in flies
Integrin alpha V[a]	E5d	2.83	2.18	Cell-matrix, cell-cell adhesion
Intercellular adhesion molecule-1[a]	E5h	8.99	3.91	Immune function, cell-cell adhesion
Macrophage inflammatory protein 2-α[a]	F5b	1.65	1.33	Inflammation, immune function
Interleukin-8	F5f	1.56	1.93	Inflammation, immune function
Interleukin-1β[a]	F5m	5.54	3.50	Inflammation, immune function
IGF-BP3[a]	F7i	2.24	3.48	IGF binding protein
MHC class I[a]	G14	3.23	6.78	Antigen presentation
Down-regulated by RARβ				
Ezrin	A4g	0.57	0.70	Cytoskeleton
Cyclin E	A6m	0.73	0.70	Cell cycle
Transducin-b1 subunit	B4f	0.60	0.56	GTPase
MKK3	B5g	0.49	0.82	Signal transduction
TFIIS	D4m	0.63	0.65	Transcription elongation factor
NF45	D6g	0.52	0.51	Subunit of NFAT (regulator of IL-2)
VEGF-related protein (VRP)	F4h	0.32	0.70	Growth factor
Non-RARβ RA regulated				
TNF receptor 1	C1m	2.16	0.93	Inflammation
FAS ligand[a]	C4m	3.17	1.17	Apoptosis
Inhibitor of DNA binding ID-2	D1g	2.04	1.02	Inhibitor of differentiation, apoptosis
Clonal variation	A4f, B7e, B7g, C7d, C7g, C7h, D4j, E7g, F1g, F2d, F3f			

[a] Regulation pattern was confirmed using the C24-RA-probed array.
[b] Absence of expression in Calu-1 and C30-RA cells.

less informative, and this array was used primarily to confirm by visual inspection results from the other arrays. Densitometric values for each spot were normalized to the mean value of the genomic DNA control spots on the membrane. Relative ratios were then calculated between the membranes (C64-RA/Calu-1 and C64-RA/C30-RA). Spots that were consistently changed in both ratios are reported in **Table 1**. Some of the changes are of the order of 1.5 to 2.5; these may be of marginal physiological significance, but are included for completeness (see Discussion).

A measure of the confidence one can place in these results is the comparison of their consistency between the two comparisons of RARβ$^+$ vs. RARβ-derived probes. A total of 28 cDNAs (including RARβ) were found to be altered in a consistent manner, with 21 being specifically up-regulated and 7 down-regulated in the presence of RARβ2 (Table

TABLE 2. *Densitometric evaluations of RNase protection (RPA) or Northern blot assays*[a]

	RARβ⁻		RARβ2+				RARβ1⁺	
	C30		C24		C64		Cβ10	
100 nM RA	−	+	−	+	−	+	−	+
IL-1β	1.1	1.3	0.3[b]	10	1.4	8.7	0.6[b]	0.9
ID-3	2.1	3.0	3.7	9.3	1.5	4.4	2.3	3.0
GADD-45	1.0	0.7	2.4	2.0	2.0	1.7	ND	ND

[a] Assays of IL-1β (RPA and Northern blot), ID-3 (RPA), and GADD-45 (Northern blot) were scanned using an Alpha Imager (Alpha Innotech Corp.). Ratios of band intensity to the band intensity of the internal control (actin) were calculated and these values were normalized to Calu-1 (without RA) = 1.0. Measurements were made using film exposure times to give band intensities in the linear range.
[b] These values are underestimates because the amount of material loaded was low.

1). Only three cDNAs varied in intensity in a way, suggesting regulation by RA rather than specifically by RARβ2 (most probably by other RARs present in the cells), namely, TNF receptor 1 (spot C1m), FAS ligand (spot C4m), and ID-2 (spot D1g) (Table 1). The ratios for these were close to 1.0 in C64-RA/C30-RA while being two- to threefold higher in the C64-RA/Calu-1 comparison, which suggests up-regulation in the RA-treated cells. A limited number of spots varied in what we interpret to be a clonal fashion, the intensities varying on the three membranes but not in a way suggestive of RA or RARβ2 regulation (Table 1). In addition, the results for 15 of the spots (in general, the more intense ones; identified in Table 1) were confirmed using the fourth membrane probed with RA-treated C24 cDNA. In no case was a discordance seen between this set of results and the others.

As expected, the expressed housekeeping controls in row G are also of similar relative intensity from one cell line to the other, with the striking exception of MHC class I (G14, see below), which is up-regulated by at least threefold in the RARβ2-positive line (confirmed with the C24-RA-probed array). To further assess the dependability of the array results, some genes listed in Table 1 have been tested by RNase protection or Northern blot for relative expression levels in a panel of RARβ1-positive (Cβ10), RARβ2-positive (C24 and C64), and RARβ-deficient (C30) derivatives of Calu-1 cells. RARβ itself was detected in the C64-RA and C24-RA probed arrays (albeit at a low intensity) but was not visible in the two arrays probed with cDNA from the RARβ-deficient cells (Table 1), and this is concordant with RNase protection and PCR assays (ref 15 and data not shown). Probes were also generated for several other genes that gave differences on both sets of arrays (GADD-45, ID-3, ICAM-1, and IL1-β, corresponding to C7f, D1d, E5h, and F5m, respectively). The results of RNase protection and Northern analyses of message levels of these genes (**Table 2** and results not shown) confirmed their differential expression. Only slight increases in the levels of expression were seen upon RA treatment of RARβ2-deficient cell lines (Calu-1, C30, and Cβ10). However, in the case of IL-1β and ID-3, transfection by RARβ2 resulted in a substantial RA response of about threefold or more. For GADD-45, an increase was seen in both RARβ2-transfected lines in comparison to Calu-1 and C30 in absence of retinoic acid, but no further increase was seen upon addition of exogenous RA. It may be that the physiological levels of RA present in the cells were sufficient to mediate the activation of this gene, but we cannot confirm this yet.

The modulation of the MHC class I and ICAM-1 genes may be of importance if we regard it in light of the theory of immune surveillance. The products of these genes are involved in the presentation of foreign or tumor antigens to cytotoxic cell precursors and in enhancing T cell and antigen-presenting cell interaction (21, 22). If down-regulation of these molecules occurs in tumor cells, they will have a better chance of evading the immune system since the signaling strength of any tumor-specific antigen at the cell surface would necessarily be compromised.

Since ICAM-1 and MHC class I can play these central roles in eliciting an immune response only if they are displayed on the cell surface, it was important to determine whether the RARβ2-induced increases in mRNAs were reflected at this level. We therefore assayed surface ICAM-1 and MHC class I by flow cytometry in two lung tumor lines and their RARβ⁺ transfectants. C30 (*neo*ʳ-transfectant, RARβ⁻) and C64 (RARβ2⁺) cells were labeled as described in Materials and Methods and analyzed by flow cytometry. The results in **Table 3** show that the mean fluorescence intensity (MFI) of ICAM-1 protein is ~threefold higher on the cell surface of the RARβ2-transfected C64 cells than on the C30 cells. Treating the cells with 100 nM RA for 7 days increased the expression in both cell lines but the increase was greater in C64 cells than in C30 cells, as demonstrated in Table 3 (2.7-fold vs. 1.7-fold). Table 3 also shows results obtained with antibody W6/32 against MHC class I proteins. As seen for ICAM-1 protein, the RARβ2-transfected C64 cells show slightly higher MHC class I MFI than C30 cells prior

TABLE 3. *Ratios of cell surface expression of ICAM-1 and MHC class I proteins*[a]

	Calu-1				SK-MES-1			
	C30 (RARβ⁻)		C64 (RARβ2⁺)		SK-MES-1 (RARβ⁻)		C102 (RARβ2⁺)	
100 nM RA	−	+	−	+	−	+	−	+
ICAM-1	1.0	1.7	3.1	8.3	1.0	1.4	2.3	3.9
MHC class I	1.0	1.0	1.5	3.1	1.0	1.3	3.1	3.5

[a] Mean fluorescence intensities from flow cytometry (see Materials and Methods) were normalized to the value of the RARβ-deficient cell line without RA treatment. Experiments were repeated twice with similar results.

to RA treatment. The 7 day incubation in the presence of retinoic acid resulted in the induction of MHC class I molecules in C64 cells, but no augmentation was seen with C30 cells after the treatment (Table 3).

To determine whether the RARβ-induced increase in expression of these proteins could be generalized to other lines, SK-MES, which is stably RARβ deficient (5), was transfected as described (15) and a derivative, C102, was characterized and shown to express RARβ2 (results not shown). SK-MES and C102 were grown in the presence or absence of RA (as above for Calu-1 derivatives) for 5 days and the proteins were assayed simultaneously by flow cytometry (Table 3). Again, an increase of ~twofold in ICAM-1 was seen in untreated C102 and the increase in MHC class I was more than threefold compared to that in SK-MES cells. RA treatment increased levels of both proteins to a similar extent (~30%) in both the parental and the transfected cell lines. This increase is less pronounced than seen in the Calu-1 RARβ2+ cells but, as mentioned above for GADD-45, it is possible that the basal level of bioavailable RA is sufficient in SK-MES to stimulate expression of these genes. In any event, the transfection of RARβ2 had a similar effect on cell surface expression of the two proteins in both cell lines. Furthermore, a similar effect has been observed on transfection of RARβ2 into breast and colon cancer cell lines (J. Pappas and W. E. C. Bradley, unpublished results), which supports the observations reported here and suggests that this RARβ2 effect applies to other cancers in addition to lung.

To show that this increase in potentially immunogenic proteins on the surface of the RARβ2⁺ cells actually had a physiological effect, we performed a CTL assay on these cells (23). We first isolated PBMC from heterologous donors and incubated them with either C30 or C64 stimulator cells that had been treated with RA for 5 days prior to incubation with the PBMC (see Materials and Methods). The PBMC were then incubated with ^{51}Cr-labeled target cells (RA-pretreated C64 cells or, for some combinations, RA-pretreated C30 cells) and the extent of lysis of these targets was determined. The C64 (RARβ2-expressing) line was 2.5- to 3-fold more efficient than the control line (C30) in inducing CTL effector cells (one experiment shown in **Fig. 2**) against a C64 target, as judged by chromium release. In addition, when used as target cells, the RARβ2⁺ line was 1.3- to 2-fold more susceptible to lysis by C64 RA-stimulated CTL cells than was the C30 RARβ⁻ line (**Fig. 3**).

DISCUSSION

In this paper we report the finding of genes that are regulated either directly or indirectly by RARβ. Most of the changes were in the direction of up-regulation, and this was particularly true for those genes where the change was substantial; for example, among the cDNAs listed in Table 1, 14 had intensities that differed by at least 2.5-fold in one of the comparisons; of these, however, only one, VRP, was

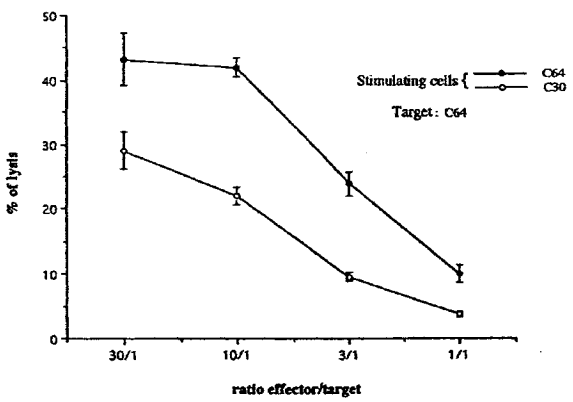

Figure 2. Overexpression of RARβ in Calu-1 cells enhances their antigenicity in the induction phase. A Calu-1 derivative (line C30) or Calu-1 transfected with RARβ (line C64) were cultured in the presence of RA (100 nM) for 5 days. These cells were then treated with mitomycin C and used as stimulating cells for PBMC in an alloreactive culture. After 5 days, the stimulated PBMC were used as effector cells and fresh RA-treated C64 cells (cultured in the presence of RA, 100 nM, for 5 days) were labeled with ^{51}Cr and used as targets in a standard 4 h ^{51}Cr release assay to measure cytotoxic T cell activity. Similar results were obtained from two experiments.

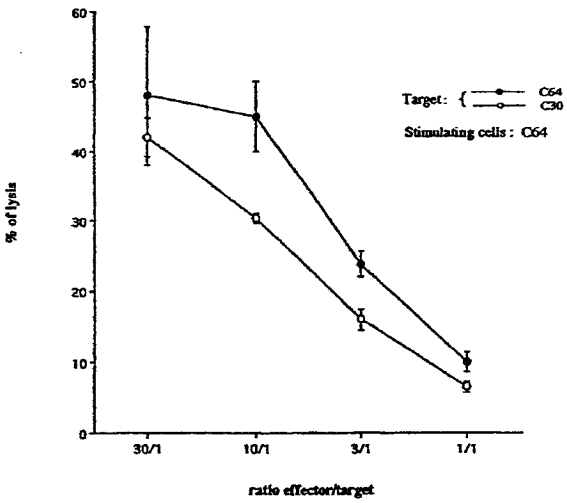

Figure 3. Overexpression of RARβ in Calu-1 cells enhances their antigenicity in the effector phase. The experimental design is similar to that described in Fig. 2. RARβ-transfected Calu-1 cells (C64) were used as stimulating cells for PBMC. The PBMC thus stimulated for 5 days were used as effector cells against ^{51}Cr-labeled C64 and C30 cells, which were used as target. Both the stimulating cells and target cells were cultured in the presence of 100 nM RA for 5 days before use. Similar results were obtained from two experiments.

down-regulated in the RARβ-expressing lines. We also found that three of the genes on the array were up-regulated by RA without specific dependence on the RARβ receptor, most probably through the other RA receptors expressed in these cells. We also tested six of the genes by an independent assay for RNA or protein level (Tables 2 and 3 and data not shown) and found up-regulation to levels similar to those shown by the array experiments. Based on this high degree of concordance, we are confident that the array results reflect genuine regulatory effects of RARβ2, at least in Calu-1 cells. The subsequent demonstration of up-regulation at the protein level of two of the genes in another cell line, SK-MES, increases our confidence that our results can be generalized.

Consistent with the high level of reliability of the array, several of the genes listed in Table 1 have previously been reported to be regulated by RA or other retinoids—for example, GADD-45 (24), ICAM-1 (25), IL-1β (26)—and the results presented here allow us to propose RARβ as the specific receptor mediating this regulation. Some other genes have been found by others to be controlled specifically by RARβ (MHC class I and recently IGF-BP3) and our data confirm the involvement of RARβ2 as a regulator of these genes (27, 28).

Of particular interest in the work presented here is the identification of genes that may be the downstream effectors of the RARβ2-mediated tumor suppression. Table 1 shows the functions of the regulated genes, and it is noteworthy that many of them are consistent with this role for RARβ2. These results highlight several potentially synergistic pathways regulated by RARβ2 that are involved in the control of cell behavior. In addition to the two major group discussed below, the expression of genes involved in repair (GADD-45), IGF signaling (IGF-BP3), as well as certain cell cycle regulators (p14) would be expected to contribute in a concerted fashion to suppress the development of tumor cells (29). The genes identified in this study as well as others yet to be identified will therefore provide a better understanding of the mechanisms by which RARβ2 promotes tumor suppression.

Of particular interest is that about a third (9/31) of the genes influence decision-making in the commitment to apoptosis, and another one-quarter are involved in provocation of an immune response. It is possible that the observed clustering of regulated genes into two groups reflects the selection of genes presented on the array; however, in light of the tumor suppressive properties of RARβ2 and the modification of the CTL response (discussed below), we believe they represent some of the mechanisms involved in RARβ2-mediated tumor suppression.

For the first of these major groups, genes that both promote and inhibit apoptosis are up-regulated, but close inspection of the arrays indicates that most of the latter are expressed at a very low level, so the predominant effect of RARβ2 may be to enhance the probability that the cell will commit to apoptosis if other requirements are fulfilled. Of particular interest within this group is the ID family of inhibitors of differentiation and DNA binding proteins. All three members on the array are up-regulated by RA, and in two of the cases—ID-1 and ID-3—specifically by RARβ2. In addition, the latter two are expressed at substantial levels. These genes promote passage from G1 to S-phase and have been shown to be potent inducers of apoptosis (30). Their functions and expression patterns overlap (31), suggestive of functional redundancy, so the conclusion that RARβ2 up-regulates them, if confirmed on analysis of other lines, may point to an important mechanism of defense against tumorigenesis.

The second group of genes that have similar function is that involved in immune response. As documented above, two genes essential for eliciting an effective cytotoxic immune response, MHC class I (G14) and ICAM-1 (E5h), are up-regulated by RARβ2. The results presented in Table 3 and those from Pappas and Bradley (unpublished results) also confirm that the effect of RARβ on levels of the two surface proteins is not restricted to one cell line. In addition, increased levels of several inflammatory cytokines that enhance this response (IL-1β, MIP2α,

and IL-8) were seen. These are potent attractants and activators of macrophages, granulocytes, and mast cells, and consequently of the immune system's ability to eliminate any lesions (reviewed in ref 32). In this context a reduced level of expression of RARβ would be hypothesized to weaken the overall immune response by compromising several of its components.

When this was tested in a heterologous CTL assay, the results (Figs. 2 and 3) showed that, at least as measured by this assay, the immune response would indeed be weakened against RARβ-deficient tumor cells. RARβ2-transfected cells were better stimulators and targets of the CTLs, establishing a role for RARβ2 in maintaining an effective immune response. The potential implications of these results are of major importance as they establish a physiological role for some of the genes whose expression is influenced by RARβ2. This physiological role may be exploitable for therapeutic benefits if RARβ2 expression can be up-regulated by gene therapy or by simple administration of retinoids, as has been shown in patients at risk (33, 34). FJ

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REFERENCES

1. Graham, S. J. (1984) Retinoids and lung cancer. *J. Natl. Cancer Inst.* **73,** 1423–1428
2. Rearick, J. I., and Jetten A. M. (1989) Effect of substratum and retinoids upon the mucosecretory differentiation of airway epithelial cells in vitro. *Environ. Health Perspect.* **80,** 229–237
3. Chambon, P. (1996) A decade of molecular biology of retinoic acid receptors. *FASEB J.* **10,** 940–954
4. Gebert, J. F., Moghal, N., Frangioni, J. V., Sugarbaker, D. J., and Neel, B. G. (1991) High frequency of retinoic acid receptor β abnormalities in human lung cancer. *Oncogene* **6,** 1859–1868
5. Houle, B., Leduc, F., and Bradley, W. E. C. (1991) Implication of RARβ in epidermoid (squamous) lung cancer. *Genes Chromosomes Cancer* **3,** 358–366
6. Roman, S. D., Clarke, C. L., Hall, R. E., Alexander, I. E., and Sutherland, R. L. (1992) Expression and regulation of retinoic acid receptors in human breast cancer cells. *Cancer Res.* **52,** 2236–2242
7. Swisshelm, K., Ryan, K., Lee, X., Tsou, H. C., Peacocke, M., and Sager, R. (1994) Down-regulation of retinoic acid receptor β in mammary carcinoma cell lines and its up-regulation in senescing normal mammary epithelial cells. *Cell Growth Differ.* **5,** 33–141
8. Xu, X.-C., Sneige, N., Liu, X., Nandagiri, R., Lee, J. J., Lukmanji, F., Hortobagyi, G., Lippman, S. M., Dhingra, K., and Lotan, R. (1997) Progressive decrease in nuclear retinoic acid receptor β messenger RNA level during breast carcinogenesis. *Cancer Res.* **57,** 4992–4996
9. Xu, X.-C., Sozzi, G., Lee, J. S., Lee, J. J., Pastorino, U., Pilotti, S., Kurie, J. M., Hong, W. K., and Lotan, R. (1997) Suppression of retinoic acid receptor β in non-small-cell lung cancer in vivo: implications for lung cancer development. *J. Natl. Cancer Inst.* **89,** 624–629
10. Widschwendter, M., Berger, J., Daxenbichler, G., Muller-Holzner, E., Widschwendter, A., Mayr, A., Marth, C., and Zeimet, A. G. (1997) Loss of retinoic acid receptor beta expression in breast cancer and morphologically normal adjacent tissue but not in the normal breast tissue distant from the cancer. *Cancer Res.* **57,** 4158–61
11. Toulouse, A., Morin, J., Ying, Y., Ayoub, J., and Bradley, W. E. C. (1998) Retinoic acid receptor β: an exploration of its role in lung cancer suppression and its potential in cancer prevention. In *Clinical and Biological Basis of Lung Cancer Prevention* (Martinet, Y., Hirsch, F. R., Martinet, N., Vignaud, J.-M., and Mulshine, J. L., eds) pp. 193–205, Birkhäuser-Verlag, Basel, Switzerland
12. Bérard, J., Gaboury, L., Landers, M., De Repentigny, Y., Houle, B., Kothary, R., and Bradley, W. E. C. (1994) Hyperplasia and tumours in lung, breast and other tissues in mice carrying a RARβ4-like transgene. *EMBO J.* **13,** 5570–5580
13. Bérard, J., Laboune, F., Mukuna, M., Massé, S., Kothary, R., and Bradley, W. E. C. (1996) Lung tumors in mice expressing an antisense RARβ2 transgene. *FASEB J.* **10,** 1091–1097
14. Liu, Y., Lee, M.-O., Wang, H.-G., Li, Y., Hashimoto, Y., Klaus, M., Reed, J. C., and Zhang, X. K. (1996) Retinoic acid receptor β mediates the growth inhibitory effect of retinoic acid by promoting apoptosis in human breast cancer cells. *Mol. Cell. Biol.* **16,** 1138–1149
15. Houle, B., Rochette-Egly, C., and Bradley, W. E. C. (1993) Tumor-suppressive effect of the retinoic acid receptor β in human epidermoid lung cancer cells. *Proc. Natl. Acad. Sci. USA* **90,** 985–989
16. Auffray, C., and Rougeon, F. (1980) Purification of mouse immunoglobulin heavy chain mRNAs from total myeloma tumor RNA. *Eur. J. Biochem.* **107,** 303–314
17. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual,* 2nd Ed, Cold Spring Harbor Laboratory Press, New York
18. Luo, H., Chen, H., Daloze, P., St. Louis, G., and Wu, J. (1993) Anti-CD28 antibody- and IL-4-induced human T-cell proliferation is sensitive to rapamycin. *Clin. Exp. Immunol.* **94,** 371–376
19. Chen, H., Luo, H., Daloze, P., Xu, D., and Wu, J. (1994) Rapamycin induced long-term allograft survival depends on persistence of alloantigen. *J. Immunol.* **152,** 3107–3118
20. Welsh, J., Chada, K., Dalal, S. S., Cheng, R., Ralph, D., and McClelland, M. (1992) Arbitrarily primed PCR fingerprinting of RNA. *Nucleic Acids Res.* **20,** 4965–4970
21. Garrido, F., Cabrera, T., Lopez-Nevot, M. A., and Ruiz-Cabello, F. (1995) HLA class I antigens in human tumors. *Adv. Cancer Res.* **67,** 155–195
22. van de Stolpe, A., and van der Saag, P. T. (1996) Intercellular adhesion molecule-1. *J. Mol. Med.* **74,** 13–33
23. Induction and measurement of cytotoxic T lymphocyte activity (1991) In *Current Protocols in Immunology* (Coligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M., and Strober, W., eds) John Wiley and Sons, New York
24. Rishi, A. K., Sun, R. J., Gao, Y., Hsu, C. K., Gerald, T. M., Sheikh, M. S., Dawson, M. I., Reichert, U., Shroot, B., Brewer, G., and Fontana, J. A. (1999) Post-transcriptional regulation of the DNA damage-inducible gadd45 gene in human breast carcinoma cells exposed to a novel retinoid CD437. *Nucleic Acids Res.* **27,** 3111–3119
25. Bouillon, M., Tessier, P., Boulianne, R., Destrempe, R., and Audette, M. (1991) Regulation by retinoic acid of ICAM-1 expression on human tumor cell lines. *Biochim. Biophys. Acta* **1097,** 95–102
26. Jarrous, N., and Kaempfer, R. (1994) Induction of human interleukin-1 gene expression by retinoic acid and its regulation at processing of precursor transcripts. *J. Biol. Chem.* **269,** 23141–23149
27. Segars, J. H., Nagata, T., Bours, V., Medin, J. A., Franzoso, G., Blanco, J. C. G., Drew, P. D., Becker, K. G., An, J., Tang, T., Stephany, D. A., Neel, B., Siebenlist, U., and Ozato, K. (1993) Retinoic acid induction of major histocompatibility complex class I gene in NTera-2 embryonal carcinoma cells involves induction of NF-κB (p50–p65) and retinoic acid receptor β-retinoid X receptor β heterodimers. *Mol. Cell. Biol.* **13,** 6157–6169
28. Shang, Y., Baumrucker, C. R., and Green, M. H. (1999) Signal relay by retinoic acid receptors alpha and beta in the retinoic acid-induced expression of insulin-like growth factor-binding protein-3 in breast cancer cells. *J. Biol. Chem.* **274,** 18005–18010

29. Herman, J. G., Jen, J., Merlo, A., and Baylin, S. B. (1996) Hypermethylation-associated inactivation indicates a tumor suppressor role for p15INK4B. *Cancer Res.* **56,** 722–727
30. Norton, J. D., and Atherton, G. T. (1998) Coupling of cell growth control and apoptosis functions of ID proteins. *Mol. Cell. Biol.* **18,** 2371–2381
31. Jen, Y., Manova, K., and Benezra, R. (1996) Expression patterns of ID1, ID2, and ID3 are highly related but distinct from that of ID4 during mouse embryogenesis. *Dev. Dynamics* **207,** 235–252
32. Bookman, M. A. (1998) Biological therapy of ovarian cancer: current directions. *Semin. Oncol.* **25,** 381–396
33. Xu, X. C., Lee, J. S., Lee, J. J., Morice, R. C., Liu, X., Lippman, S. M., Hong, W. K., and Lotan, R. (1999) Nuclear retinoic acid receptor β in bronchial epithelium of smokers before and during chemoprevention. *J. Natl. Cancer Inst.* **91,** 1317–1321
34. Ayoub, J., Jean-Francois, R., Cormier, R., Meyer, D., Ying, Y., Major, P., Desjardins, C., and Bradley, W. E. C. (1999) Placebo controlled trial of 13-cis-retinoic acid activity on retinoic acid receptor-β expression in a population at high risk: Implications for chemoprevention. *J. Clin. Oncol.* **17,** 3546–3552

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APPENDIX C

THE RARbeta2 TUMOR IMMUNOMODULATION PROJECT

THE ROLE OF RARβ2 EXPRESSION IN TUMOR IMMUNOGENICITY AS REFLECTED BY MHC CLASS I AND ICAM-1 EXPRESSION

Jane J. Pappas and W.E.C. Bradley

ABSTRACT

BACKGROUND: The retinoic acid receptor β2 (RARβ2) is a nuclear transcription factor and putative tumor suppressor that is frequently down-regulated in human cancers, including lung, breast and colon. The β2 isoform specifically mediates retinoic acid-induced growth inhibition of lung cancer cell lines (1). The β2 isoform also regulates the mRNA expression levels of the Major Histocompatibility Complex Class I β constant heavy chain (MHC Class I) and the Intercellular Adhesion Molecule-1 (ICAM-1), as well as several other genes also involved in the anti-tumor response (2). MHC Class I is essential in the targeted killing of cancer cells by cytotoxic T-lymphocytes (CTLs), forming a complex between the tumor cell and the CTL precursor, which consists of the Class I molecule, the antigenic peptide, and the T-cell receptor (TCR). ICAM-1 allows the tumor cell to adhere to the CTL, allowing the TCR to bind the MHC complex, and comprising one of the potential co-stimulatory signals that is necessary in CTL activation. This is of particular importance since absence of costimulation causes the clonal expansion of anergic T-cells, thereby enhancing tumor cell evasion. In addition, MHC Class I and ICAM-1 expression levels are inversely correlated with metastasis and poorer prognosis. Therefore, loss of RARβ2 expression, a common aberration in lung cancer (~50%), may be involved in the evasion of lung cancer cells from immune surveillance mechanisms. ***HYPOTHESIS:*** RARβ2 expression is correlated with MHC Class I and ICAM-1 expression in multiple cancer types, including lung, breast and colon. ***OBJECTIVE:*** To determine whether RARβ2 expression is correlated with MHC Class I and ICAM-1 in 14 cancer cell lines, including 7 lung, 4 breast and 3 colon lines. ***METHODS:*** Retroviral transduction of an RARβ2 gene construct to over-express RARβ2 in 14 cancer cell lines having lost *or retained* RARβ2 mRNA expression. The construct comprised a bicistronic message, where RARβ comprised the first cistron and enhanced green fluorescent protein (GFP) comprised the second, and these were interlinked via an inter-ribosomal entry site (IRES). Fluorescently-labeled monoclonal antibodies against MHC Class I or ICAM-1 were used to measure cell surface expression levels of

corresponding proteins via fluorimetry. ***Results:*** RARβ2 transduction of certain cancer cell lines, such as T47D, increases MHC Class I and ICAM-1 up to 300% and 700%, respectively. Though results were usually significant, they were also highly variable. ***Conclusions:*** RARβ2 plays a role in the immunomodulation of certain cancer cell lines. However, based on recent findings regarding the correlation between ICAM-1 expression and metastasis or poor prognosis, RARβ2-mediated up-regulation of ICAM-1 may act as a tumor-related process and hence comprises an undesirable event in cancer treatment.

1. Materials & Methods

We constructed a retroviral bicistronic vector construct (Fig. 35), rvRARβ2-IRES-GFP, in which RARβ comprised the first cistron, and enhanced green fluorescent protein (GFP) comprised the second, and these were interlinked via an inter-ribosomal entry site (IRES). Fluorescently-labeled monoclonal antibodies against MHC Class I or ICAM-1 were used to measure cell surface expression levels of corresponding proteins via fluorimetry.

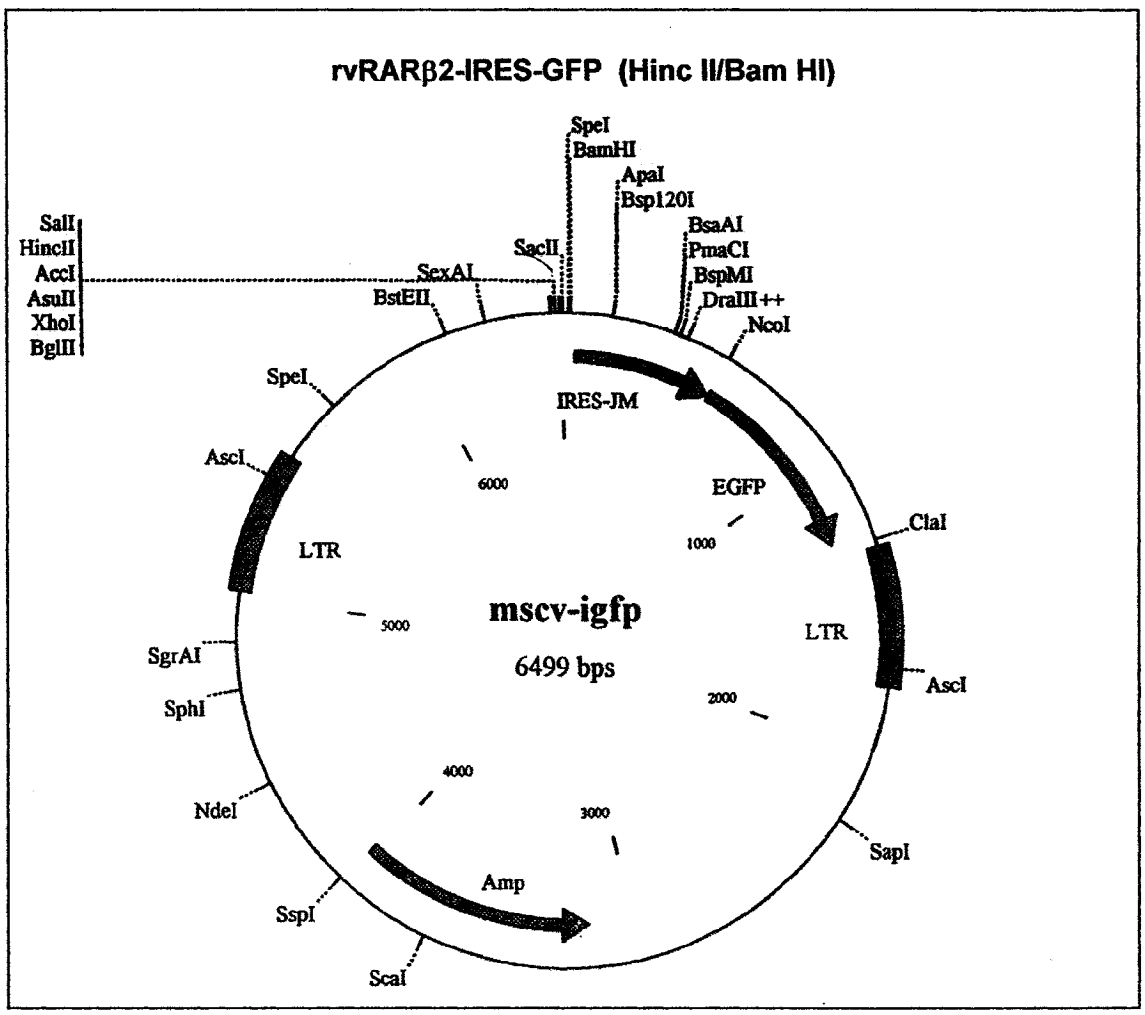

FIGURE 35. Diagram representing the retroviral vector *rvRARβ-IRES-EGFP* used in the transfection of 14 cancer cell lines. Reagents were kindly provided by Dr. Gallipeau (Lady Davis Institute, Jewish General Hospital).

2. Results

RARβ2 transduction generally increased MHC Class I and ICAM-1, and the effect was generally exacerbated in the presence of ATRA. RARβ2 transduction of certain cancer cell lines, such as T47D, increased MHC Class I and ICAM-1 up to 300% and 700%, respectively.

Table XXVII. List of effects of retroviral transduction of rvRARβ2-IRES-GFP on the levels of ICAM-1 and MHC Class I cell surface protein expression (FACS) in 13 cancer cell lines. The methylation status of the endogenous RARβ2 P2 promoter is included. ∅ = Ethanol alone. ATRA = *all-trans*-retinoic acid (dissolved in ethanol); DM = divergently methylated; EGFP = enhanced green fluorescent protein; IRES = internal ribosomal entry site; M = methylated; N/A = not available; RARβ2 = retinoic acid receptor isoform β2; U = unmethylated.

Effect of rvRARβ2-IRES-GFP on 14 cancer cell lines						
Cell line	RARβ2	ICAM-1		MHC I		Methylation
		∅	ATRA	∅	ATRA	
HCT-15	NEG	0.9	0.7	0.9	0.7	M
NCI-H596	NEG	1.0	1.3	1.2	1.3	U
HS-578T	POS	1.1	1.6	0.4	0.5	U
COLO-201	NEG	1.3	1.2	1.6	2.0	M
QUDB	POS	1.8	2.0	N/A	N/A	U
A-549	POS	2.0	3.3	1.3	1.7	U
NCI-H125	POS	2.4	3.7	1.7	1.7	U
CALU-1	NEG/IND	2.8	3.3	0.6	1.4	DM
MDA-MB-231	NEG	3.2	3.9	1.3	1.4	DM
NCI-H23	POS	3.5	7.8	1.3	1.6	U
NCI-H157	POS/IND	3.6	5.6	1.7	1.7	U
LS-180	NEG	4.3	5.5	3.9	3.8	DM
T-47D	NEG	4.7	11.9	2.4	4.8	U

References

(*1*) Toulouse A, Morin J, Dion PA, Houle B, Bradley WE. RARbeta2 specificity in mediating RA inhibition of growth of lung cancer-derived cells. Lung Cancer 2000;28:127-37.

(*2*) Toulouse A, Loubeau M, Morin J, Pappas JJ, Wu J, Bradley WE. RARbeta involvement in enhancement of lung tumor cell immunogenicity revealed by array analysis. Faseb J 2000;14:1224-32.

APPENDIX D

MOLECULAR CHARACTERIZATION OF 28 CELL LINES

TABLE XXIII List of the molecular characteristics of 28 cell lines studied, including: RARβ2 mRNA expression status; RARβ2 P2 promoter methylation status; ROH at THRβ2; ROH at D3S1583; and ROH D3S2335 statuses. Molecular characteristics include RARβ2 mRNA expression, methylation status of the RARβ2 P2 promoter, LOH at the THRβ2 locus, and LOH at the D3S1583 and D3S2335 loci. HET = heterozygous; HM = heteromethylated; IND = inducible; LOH = loss of heterozygosity; NEG = negative; N/A = not applicable; N/I = non-informative; N/T = not tested; pos = positive; M = methylated; RARβ2 = retinoic acid receptor isoform β2; SCLC = small cell lung carcinoma; U = unmethylated. Patterns refer to electrophoretic migration observed, they were used to inter-compare cell lines.

Molecular characterization of 28 cell lines											
TISSUE	**HISTOLOGY**	**NO.**	**CELL LINE**	**RARβ2**	**METHYLATION**	**THRβ2**	**Pattern**	**D3S1583**	**Pattern**	**D3S2335**	**Pattern**
LUNG	EPIDERMOID	1	CALU-1	NEG/IND	DM	HET	H/L=1.3	HET	1A	N/I	1A
		2	CALU-1 C19	POS	DM	HET	HL	HET	1A	N/I	1A
		3	CALU-1 C59	POS	DM	HET	HL	HET	1A	N/I	1A
		4	NCI-H157	POS/IND	U	N/I	L	HET	2	HET	2
		5	SKMES	NEG	DM	N/I	L	N/I	1B	N/I	1B
		6	SKMESC102	POS	all borderline	N/I	L	N/I	N/A	N/I	N/A
		7	NCI-H520	POS	U	N/I	H	N/A	N/A	N/A	N/A
	EPITHELIAL	8	NBE-E6E7	POS/IND	U	HET	HL	N/I	3A	N/I	1B
	ADENOSQUAMOUS	9	NCI-H596	NEG	M	N/I	L	N/I	1A	N/I	1A
		10	NCI-H125	POS	U	N/I	L	HET	2	HET	2
	ADENOCARCINOMA	11	NCI-H23	POS	U	N/A	N/A	N/I	4	N/I	3
		12	A-549	POS	Taq1alpha U	N/I	H	N/I	3A	N/I	4
		13	NCI-H69	POS	N/T	N/I	L	N/I	3A	N/A	5
	LARGE CELL	14	QUDB	POS	U	N/I	H	N/I	1C	N/I	5
	SCLC	15	H-82	POS/IND	U	N/I	H	N/I	3A	N/I	5
		16	MM-1	POS	U	N/I	L	N/I	3A	N/I	6
		17	RG-1	NEG	N/A	N/I	L	N/I	3A	N/T	6
BREAST	ADENOCARCINOMA	18	HS-578T	POS/IND	U	HET	H/L=2.0	HET	2	N/I	5
		19	T-47D	POS	U	N/I	H	N/I	3A1C	N/I	5
		20	MB-231	NEG	DM	N/I	H	N/I	3A	N/I	5
		21	ZR-75B	NEG	DM	N/I	H	N/I	3A	N/I	5
		22	MCF-7	NEG/IND	N/T	N/I	H	N/A	N/A	N/A	N/A
COLON	ADENOCARCINOMA	23	CACO-2	POS	U	HET	N/A	N/I	3B	N/I	5
		24	LS-180	NEG	DM	HET	H/L=0.2	HET	3D	N/I	6
		25	HCT-15	NEG	M	N/I	H	N/I	3A	N/I	5
		26	COLO-201	NEG	M	N/I	H	N/I	3A	N/I	5
		27	COLO-205	NEG	M	N/I	H	N/I	3A	N/I	5
		28	SW-1222	POS	U	HET	N/A	N/I	3D	N/I	5

APPENDIX E

THE RARbeta2 AND MHC CLASS I INVERSE CORRELATION IN THE HS-578T BREAST CANCER CELL LINE

Inverse correlation between MHC class I & RARβ2 expression AND ATRA dose in HS-578T breast cancer cells.

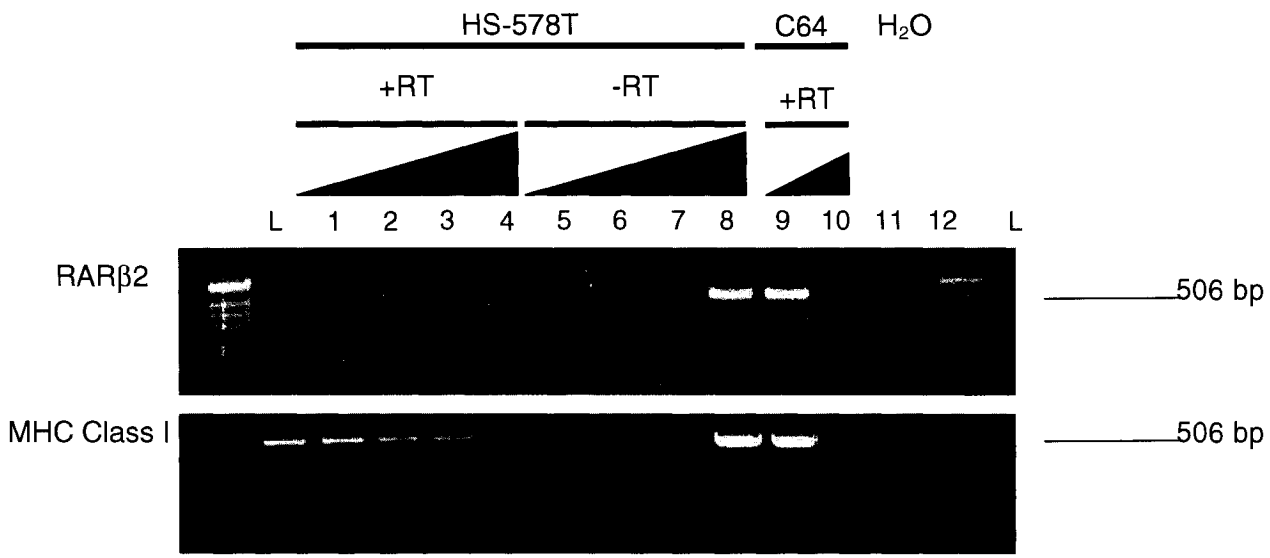

FIGURE 36. 2% agarose gel electrophoresis of RARβ2 versus MHC class I mRNA expression analyses (RT-PCR) in HS-578T breast cancer cells following a 24-hour treatment with ATRA. **Top**: RARβ2; **Bottom**: MHC class I . Lanes are: 1 = 0 nM; 2 = 10 nM; 3 = 100 nM; 4 = 1,000 nM; 5 = 0 nM; 6 = 10 nM; 7 = 100 nM; 8 = 1,000 nM; 9 = 0 nM; 10 = 100 nM; 11 = water; 12 = water. L = 1 kb Plus mol. Weight ladder; +RT = test sample; -RT = RT-negative controls; H2O = template replaced with water. CALU-1 clone C64 was used as a positive control.

APPENDIX F

CERTIFICATES OF AUTHORIZATION FOR USE OF BIOHAZARDOUS MATERIALS

CHUM

Montréal, le 21 juillet 2004

À qui de droit,

Objet: Avis de conformité – Laboratoire du Dr Ted Bradley
Institut du Cancer de Montréal, Centre de recherche du CHUM

Madame,
Monsieur,

Je confirme par la présente que les travaux de recherche de madame Jane J. Pappas effectués au sein du laboratoire du Dr Ted Bradley ont été réalisés en pleine conformité avec les normes de biosécurité requises pour ce genre de travail. En particulier, les expériences utilisant des rétrovirus ont toutes été réalisées dans le laboratoire de virologie du Centre de recherche de l'Hôpital Notre-Dame, lequel laboratoire fonctionne selon des règles très strictes de sécurité.

Espérant le tout conforme. N'hésitez pas à communiquer avec moi pour toute information supplémentaire.

Marc Bilodeau, M.D.
Président
Comité des biorisques
Centre de recherche du CHUM

CHUM

Bureau de la radioprotection
Direction des ressources technologiques
Centre hospitalier de l'Université de Montréal

Je certifie par la présente que Jane Pappas a suivi un cours en radioprotection donné au Centre de recherche de l'Hôpital Général de Montréal. Ce cours d'une durée de trois heures est destiné aux utilisateurs de radio-isotopes en laboratoires de recherche.
Les sujets suivants y sont entre autres abordés: physique des radiations, effets biologiques et risques, limites de doses, mesures, détection et dosimétrie, caractéristiques des différents radio-isotopes, utilisation sécuritaire des produits radioactifs en laboratoire, règlements d'utilisation, protection contre le rayonnement, contrôle de la contamination, gestion des déchets, mesures d'urgence et décontamination.
Étant donné cette formation, madame Pappas a été autorisée à utiliser des radio-isotopes dans le laboratoire du Dr. Bradley détenant le permis interne CHUM # 2148.

Lysanne Normandeau
Physicienne
Responsable de la radioprotection

APPENDIX G

CONSENT FORM for the "GENOMIC INSTABILITY IN BREAST CANCER STUDY"

FOR USE OF BREAST CANCER BIOPSY SAMPLES IN CHAPTER 2

Hôtel-Dieu de Montréal
3840, rue Saint-Urbain
Montréal (Québec)
H2W 1T8

FORMULE DE CONSENTEMENT ÉCLAIRÉ

TITRE DE L'ÉTUDE: INSTABILITÉ GÉNOMIQUE DANS LE CANCER DU SEIN

CHERCHEUR PRINCIPAL: DR. MARC BASIK

Financé par le Centre de Recherche de l'Hôtel-Dieu de Montréal, le Fonds pour la Recherche en Chirurgie de Montréal et la Fondation Linda Saab.

---

1. Cette étude a pour but de chercher à mieux comprendre les changements génétiques impliqués dans votre maladie du sein. Nous voulons étudier le nombre et le type de changements qui ont lieu au niveau des cellules de votre maladie du sein. Pour se faire, nous aimerions faire une prise de sang (de 2 tubes de sang) avant votre chirurgie et aussi prélever des échantillons de votre tumeur après qu'elle a été enlevée. Il s'agira donc de prélever une petite partie de la masse qui aura déjà été enlevée lors de votre chirurgie. Ceci ne modifiera en rien l'opération pratiquée par votre chirurgien, pas plus que la durée de l'intervention. Il n'y aura donc pour vous aucun risque supplémentaire.

De plus il sera nécessaire pour notre étude de pouvoir vérifier dans votre dossier quelques données, telles votre âge, la présence d'une histoire familiale de cancer ou encore la présence de facteurs de risque pour le cancer du sein (hormonothérapie, délai avant la première grossesse, etc...). Pendant votre suivi après l'opération, on contactera votre médecin ou vous-mêmes pour d'autres données cliniques.

2. Les résultats seront par la suite analysés mais ne vous seront pas transmis. Ils seront utilisés pour des fins strictement scientifiques. Votre confidentialité sera protégée en tout temps. Les échantillons seront numérotés sans que votre nom paraisse. De plus, les échantillons seront utilisés seulement pour ce projet de recherche ou de la recherche qui en découle ou qui est liée à celle-ci.

3.Il n'a pour vous aucun avantage médical relié à votre participation à l'étude et le seul avantage réside dans le fait que votre contribution aidera à l'approfondissement de la connaissance scientifique des maladies du sein, surtout le cancer du sein.

4.Je reconnais avoir été bien informé et avoir eu suffisamment de temps pour considérer ces information et pour demander conseil.

5.Je reconnais que le language médical et technique utilisé m'a été expliqué à ma satisfaction et que j'ai reçu les réponses satisfaisantes à mes questions.

6.Je consens à la publication des résultats de cette étude en autant que les informations demeurent anonymes et/ou déguisées et qu'aucune identification ne puisse être faite.

7.J'ai été informé que ma participation à l'étude est volontaire et que je suis entièrement libre de refuser d'y participer ou de me retirer de l'étude en tout temps sans que ceci ne modifie en rien la qualité des soins que je recevrai.

8.J'ai également été informé que le directeur de la recherche et le Comité d'éthique de la recherche de l'Hôpital ont approuvé le protocole de l'étude.

9.J'ai lu la présente formule et je consens volontairement à participer à cette étude.

10.Toute nouvelle information qui pourrait influencer ma décision de participer à l'étude me sera communiquée par mon médecin verbalement et par le biais d'une FORMULE DE CONSENTEMENT ÉCLAIRÉ RÉVISÉE sur lesquels les nouvelles mentions ou changements ou ajouts seront soulignés.

11.Pour de plus amples informations concernant l'étude, je peux communiquer avec le Dr. Mark Basik au numéro suivant: 843-2611, poste 4950.

12.Pour tout renseignement concernant mes droits en tant que participant à l'étude, je peux m'addresser à Mme. Yolande Audette, porte-parole de malades 843-2761.

Nom du patient: _____

Signature: _____ Date: _____

Nom du témoin: _____

Signature: _____ Date: _____

Nom du chercheur: _____

Signature: _____ Date: _____

Copies conformes avec signatures originales:
1)patient
2)service des archives médicales
3)chercheur

le 5 novembre 1996
révisé le 3 décembre 1996

CLAIMS TO ORIGINALITY

1. The present study is the first to report the *co-existence* and *heritability* of hypo- and hypermethylated alleles of a gene that is completely inactivated in cancer. It is also first to report that hypermethylation in cancer is subject to allelic bias, akin to mechanisms of imprinting. The phenomenon of co-existence was directly demonstrated through the use of independently bisulfite-treated DNA samples followed by molecular sub-cloning and sequencing. The phenomenon of heritability was directly demonstrated through the generation and use of cellular subclones derived from single cells and the processing of DNA in a manner identical to that described above. The phenomenon of allelic bias was directly demonstrated through the use of SNP analysis. These results suggest that: (1) a novel promoter methylation-independent *cis*-acting silencing event at the hypomethylated allele is frequently implicated in RARβ2-inactivation; (2) the potential exists for expression from the unmethylated allele; (3) methylation patterns are heritable; (4) methylation in cancer is subject to allelic preference. These findings provide a basis from which to study promoter methylation-independent mechanisms of gene silencing, and may be applicable to other genes, including tumor suppressor genes, candidate tumor suppressor genes, oncogenes, and other aberrantly repressed genes.

2. The present study is also the first to report that the potential for RARβ2 expression is *necessary for growth* and *maintenance of the oncogenic phenotype* in certain RARβ2-expressing cancer cell lines. The former was directly demonstrated through the use of antisense oligonucleotides to knockdown RARβ2 expression in cancer cells. The latter was described through the use of paired microarray experiments in which a molecular signaling system, in this case retinoid signaling, was stimulated in one experiment and inhibited in the other. This is a novel finding since exogenous RARβ2 expression has been shown to have tumor suppressive effects in multiple cancer cell lines having *lost* endogenous RARβ2 expression, and since the effects of RARβ2 knock-down have never been tested in the context of cancer and gene expression profiles. These results suggest that the efficacy of retinoids in lung cancer chemoprevention may depend on the particular role played by RARβ2 in the patients' cancer cells. Optimally, microarray technology would be used to assess the gene expression profile of patient biopsies. These findings may also be applicable to other genes, including tumor suppressor genes, candidate tumor suppressor genes, or oncogenes.

3. Together, these findings suggest that DNA methylation, a reversible process, is a mechanism that may allow RARβ2 reactivation at alternate points along the carcinogenic pathway. The phenomenon of alternation has not been reported before. This may depend, for example, on the cellular environment of the cancer cell along the carcinogenic pathway and its transit from one compartment to another during metastasis. The elucidation of duality of function may have wide implications in cancer research since it helps explain one potential source from which the inconsistent findings regarding the effects of retinoids/carotenoids and RARβ2 in cancer may come. Thus, the potential for expression from the unmethylated allele represents one line of evidence that certain mechanisms are in place to support RARβ2-reactivation.

4. The use of antisense oligonucleotides specific for - and hybridizable to - the putative tumor suppressor RARβ2.