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Retinoic acid receptors and mouse epidermal tumorigenesis and development

By

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Abstract

Retinoic acid (RA), the major biologically active form of vitamin A, plays important roles in regulating a broad range of biological processes. Pharmacological doses of RA affect epidermal homeostasis, and RA has been successfully used to treat some epidermal diseases. The biological functions of RA are mediated by two groups of retinoid receptors, the RA receptors (RARα, RARβ, RARγ, and their isoforms), and the retinoid X receptors (RXRα, RXRβ, RXRγ, and their isoforms). The epidermis expresses RARα, RARγ, RXRα, and RXRβ, with RARγ and RXRα being the predominant receptor types.

Progressive loss of RARs is associated with skin carcinogenesis both in human and animals. Despite such observations, the biological significance of RAR loss in skin carcinogenesis has not yet been clarified. To this end, we established keratinocyte cell lines deficient in RARα, RARγ, or both and employed a well-established tumorigenesis model to investigate whether loss of RARs is causally related to skin tumorigenesis. We found that RARγ is the major RAR subtype mediating the growth and AP-1 inhibitory effects of RA on keratinocytes in vitro. Consistent with this observation, loss of RARγ, but not RARα, predisposed keratinocytes to tumor formation, suggesting that RARγ may act as a tumor suppressor. Reconstitution of RARs in the RARαγ$^{-/-}$ keratinocytes inhibited their tumorigenic potential, further proving that RARs have tumor suppressive effects.

Transgenic mice expressing a dominant negative (dn) RARα in the basal epidermis exhibit severe defects of the epidermis, suggesting that RARs are essential for epidermal development. By contrast, targeted deletion of both RARα and RARγ, the total RARs expressed in the epidermis, does not cause any significant epidermal abnormalities, arguing against any major role of RARs in epidermal organogenesis. To explore a molecular basis for the discrepancy between these two models, I made a direct phenotypic comparison of RARαγ null mice and transgenic mice expressing dnRARα or dnRARα with a defective DNA-binding domain (DBD) (designated dnRARα^{DBD}) in basal keratinocytes.

As expected, expression of dnRARα resulted in profound epidermal defects. Intriguingly, dnRARα^{DBD} caused a virtually identical skin phenotype, suggesting that dnRARα acts to affect epidermal development via a DNA-binding-independent mechanism. The epidermal phenotype of these transgenic mice is reminiscent of that seen in the p63$^{-/-}$ mice, and p63 expression was indeed significantly reduced in the epidermis expressing dnRARα or dnRARα^{DBD}, suggesting that downregulation of p63 by dnRARα may be attributable to the epidermal phenotypes associated with the transgenic mice. These observations also suggest that DNA-binding is not required for dnRARα to attenuate p63 expression in the epidermis. Consistent with these observations, I also found that p63 is indeed not a RAR-target, as no overt changes in p63 expression were observed in the RARαγ$^{-/-}$ epidermis, which appeared normal.

Résume

L'acide rétinoique (AR), la principale forme biologiquement active de la vitamine A, joue des rôles importants dans plusieurs processus biologique. L'homéostasie épidermale est influencée par des doses pharmacologiques d'AR. De plus, l'AR est utilisé pour traiter certaines maladies épidermales. Les fonctions biologiques de l'AR sont médiées par deux groupes de récepteurs, les récepteurs à l'AR (RARα, RARβ, RARγ et leurs isoformes) et les récepteurs à l'AR de type X (RXRα, RXRβ, RXRγ et leurs isoformes). L'épiderme exprime RARα, RARγ, RXRα et RXRβ, avec RARγ et RXRα étant les récepteurs prédominants.

La perte progressive des RARs est associée avec le développement de la tumorigénèse de la peau dans les vertébrés. Malgré ces observations, le rôle des RARs dans la tumorigénèse de la peau n'est pas clair. Donc, nous avons généré des lignées cellulaires déficientes en RARα, RARγ ou les deux; nous avons employé un modèle bien définie de la tumorigénèse de la peau pour déterminer si la perte des RARs est reliée à la formation de tumeurs de la peau. Nous avons trouvé que RARγ est le sous-type majeur qui est impliquée dans la croissance et le contrôle des effets inhibiteurs de AP-1 engendré par l'AR dans des kératinocytes en culture. De plus, la perte de RARγ, et non celle de RARα, prédispose à la formation de tumeurs des kératinocytes, indiquant que RARγ pourrait agir comme suppresseur de tumeurs. Le potentiel tumorigénique de ces kératinocytes peut être

compensé par la reconstitution des RARs dans la lignée RARαγ$^{-/-}$, renforçant l'hypothèse que les RARs agissent comme suppresseur de tumeurs.

Des souris transgéniques exprimant un dominant négatif (dn) de RARα dans la couche basal de l'épiderme souffre de défauts épidermaux à la naissance, ce qui suggère que les RARs sont essentiels dans le développement de l'épiderme. Au contraire, la délètion constante de RARα et de RARγ ne cause pas d'anomalies dans l'épiderme, indiquant que les RARs ne jouent pas de rôles majeurs dans l'organogénèse de l'épiderme. J'ai fait des comparaisons phénotypiques entre les souris déficientes en RARα et RARγ et des souris transgéniques qui exprime un dnRARα ou un dnRARγ et qui ont un domaine de liaison à l'ADN défectif (désigné dnRARα^{DBD}) dans les kératinocytes basal afin de pouvoir discriminer les deux situations énumérées ci haut.

Tel que prévu, les kératinocytes basal exprimant un dnRARα développent des défauts dans l'épiderme. Par contre, le dnRARα^{DBD} développe un phénotype virtuellement identique dans la peau, ce qui suggère que le dnRARα affecte le développement épidermal en utilisant une voie de signalisation des RARs non-canonique. Le phénotype observé dans ces souris transgéniques est remeniscent du phenotype des souris déficiente en p63. De plus, l'expression de p63 était très fortement réduite dans l'épiderme des souris transgéniques dnRARα ainsi que dnRARα^{DBD}. Ces observations suggèrent que le phénotype associé aux souris transgéniques peut être attribué à la réduction de l'expression de p63 par le dnRARα, un mécanisme

indépendant de la présence du domaine de liaison à l'ADN. Pour mieux supporté cette notion, nous savons que p63 n'est pas une cible de l'AR, car les niveaux d'expression de p63 dans l'épiderme RAR$\alpha\gamma^{-/-}$ apparassent normals.

Preface

This thesis is submitted to the McGill University Faculty of Graduate Studies and Research. This thesis is presented according to a manuscript-based format:

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceding and following each manuscript are mandatory.

The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.

The thesis must include the following: a table of contents; a brief abstract in both English and French; an introduction which clearly states the rational and objectives of the research; a comprehensive review of the literature (in addition to that covered in the introduction to each paper); a final conclusion and summary; a thorough bibliography; Appendix containing an ethics certificate in the case of research involving human or animal subjects, microorganisms, living cells, other biohazards and/or radioactive material.

As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

When previously published copyright material is presented in a thesis, the candidate must include signed waivers from the publishers and submit these to the Graduate and Postdoctoral Studies Office with the final deposition, if not submitted

previously. The candidate must also include signed waivers from any co-authors of unpublished manuscripts.

Irrespective of the internal and external examiners reports, if the oral defense committee feels that the thesis has major omissions with regard to the above guidelines, the candidate may be required to resubmit an amended version of the thesis.

In no case can a co-author of any component of such a thesis serve as an external examiner for that thesis.

Chapter I is a general introduction and literature review of the relevant work of the research project. The experimental results are included in Chapter II, -III, and -IV. Chapter II is a published paper, and Chapter III and –IV are manuscripts which have been submitted for publication. Connecting texts are included in each preface of the chapter. Chapter V is a general discussion. The manuscripts included are the following:

1) Characterization of retinoic acid receptor-deficient keratinocytes. By Phillipe Goyette, **Changfeng Chen**, Wei Wang, Francois Seguin and David Lohnes. In Journal of Biological Chemistry 2000, 275, 16497-16505.

2) RARγ acts as a tumor suppressor in mouse keratinocytes. By **Changfeng Chen**, Phillipe Goyette, and David Lohnes. Submitted to Oncogene.

3) Dominant negative RARs elicit epidermal defects through a non-canonical pathway. By **Changfeng Chen** and David Lohnes. Submitted to Journal of Biological Chemistry.

The contributions of the co-authors to these publications and manuscripts are greatly acknowledged: Dr. David Lohnes, Director of the Molecular and Cellular Biology Research Laboratory at the Clinical Research

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List of figures and tables

Abbreviations

ACTR:	activator for thyroid hormone and retinoid receptors
ADH:	alcohol dehydrogenase
AEC:	ankyloblepharon-ectodermal dysplasia-clefting syndrome
AF-2:	activation function-2
AK:	actinic keratosis
AP-1:	activating protein-1
APL:	acute promyelocytic leukemia
BCC:	basal cell carcinoma
CBP:	CREB-binding protein
cdk:	cyclin dependent kinase
CoA:	transcription coactivator
CoR:	transcription corepressor
CRABP:	cellular retinoic acid-binding protein
CRBP:	cellular retinol binding protein
CRE:	cAMP-response element
DBD:	DNA-binding domain
DMBA:	7, 12-dimethylbenz[a]anthracene
DMSO:	dimethyl sulfoxide
dnRAR:	dominant negative RAR
DR:	direct repeat
DRIP:	vitamin D receptor-interacting protein
EBS:	epidermolysis Bullosa Simplex
ECM:	extracellular matrix
EEC:	ectodermal dysplasia, ectrodactyly, and cleft plate syndrome
EGFR:	epidermal growth factor receptor
EHK	epidermolytic hyperkeratosis
ER:	estrogen receptor
ERK:	extracellular-signal-regulated kinase
Fgfr:	fibroblast growth factor receptors
GR:	glucocorticoid receptor
HAT:	histone acetyltransferase
HDAC:	histone deacetyltransferase
4-HPR:	N-(4-hydroxyphenyl) retinamide
IFE:	interfollicular epidermis
IκB:	inhibitor of κB
IKK:	IκB kinase
IRES:	internal ribosomal entry site
IRS:	inner root sheath
IV:	ichthyosis vulgaris
JEB:	junctional epidermolysis bullosa
JNK:	c-jun N-terminal kinase
LBD:	ligand-binding domain
LI:	lamellar ichthyosis
LOH:	loss of heterozygosity

LRC:	label-retaining cell
MAPK:	mitogen activated protein kinase
NCoR:	nuclear receptor corepressor
NF-κB:	nuclear factor-κB
OD:	oligomerization domain
ORS:	outer root sheath
PBS:	phosphate-buffered saline
p/CAF:	p300/CBP-associated factor
PCR:	polymerase chain reaction
PKC:	protein kinase C
PML:	promeolocytic leukemia gene
PPAR:	peroxisome proliferator-activated receptor
PSEK:	progressive symmetric erythrokeratoderma
RA:	retinoic acid
RALDH:	retinaldehyde dehydrogenase
RAR:	retinoic acid receptor
RARE:	retinoic acid response element
RBP:	retinol-binding protein
RD:	repression domain
RID:	nuclear receptor-interacting domain
RXR:	retinoid X receptor
SAM:	sterile α-motif
SCC:	squamous cell carcinoma
SMRT:	silencing mediator for retinoid and thyroid hormone receptor
SOS:	son of sevenless
SRC-1:	steroid receptor coactivator-1
TA cell:	transit amplifying cell
TBP:	TATA-binding protein
TCF:	ternary complex factor
TG:	transglutaminase
TR:	thyroid hormone receptor
TRAP:	thyroid hormone receptor-associated protein
TRE:	TPA-response element
TPA:	12-O-tetradecanoylphorbol-13-acetate
UV:	ultraviolet radiation
VAD:	vitamin A deficiency
VDR:	vitamin D receptor
VS:	Vohwinkel's syndrome
XP:	xeroderma pigmentosum

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Chapter I

Literature review

Section I. Epidermal physiology and tumorigenesis

The skin is one of the largest organs in mammals with a primary biological function as a protective barrier to the environment, keeping harmful substances out, preventing loss of essential body fluids, and resisting physical trauma.

1. Structure of the mature skin

The mature skin of mouse and man is composed of two major tissue layers, the epidermis and the dermis, which are separated by a basement membrane (Figure 1-1a). Hair, sweat and sebaceous glands, and nails form the skin appendages, which are specialized derivative structures (Odland, 1991; Byrne and Hardman, 2002; Haake et al., 2001).

1.1 The epidermis

The outermost compartment of the skin is the epidermis, a stratified keratinizing epithelium (Figure 1-1a). As a continuous sheet that is only disrupted by glandular pores and hair follicles, the epidermis provides the body with a barrier isolating it from the outer environment (Byrne and Hardman, 2002; Haake et al., 2001; Odland, 1991).

About 90-95% of the epidermal cells are keratinocytes (Byrne and Hardman, 2002; Haake et al., 2001; Odland, 1991), so named because they

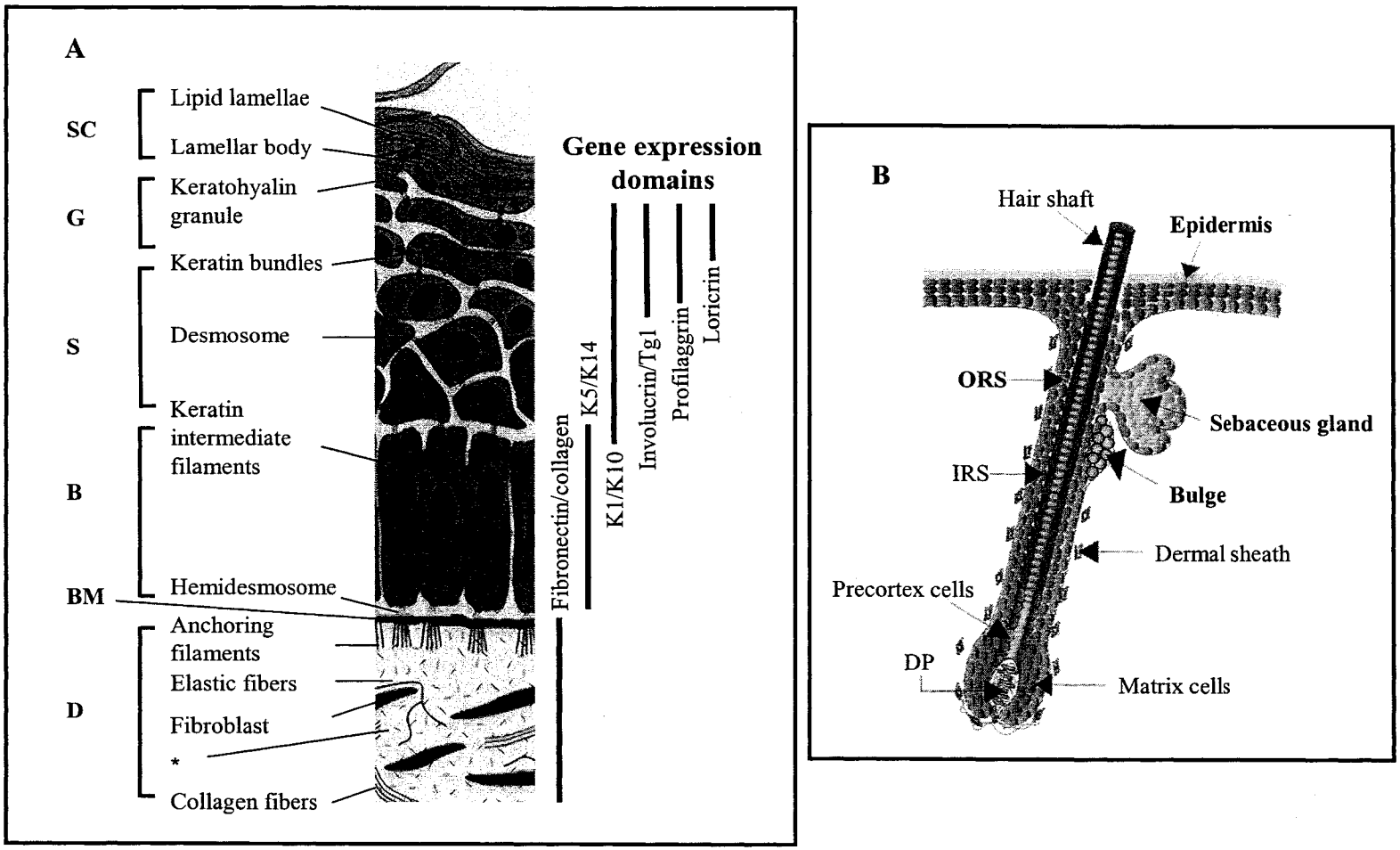


Figure 1-1. Diagrammatic representation of adult skin. A) The skin (Modified from Byrne and Hardman, 2002). *, GAG's, proteoglycans and glycoproteins; SC, stratum corneum; G, granular layer; S, spinous layer; B, basal layer; BM, basement membrane; D, dermis. B) Hair follicle (Modified from Alonso and Fuchs, 2003). ORS, outer root sheath; IRS, inner root sheath; DP, dermal papilla.

produce intermediate filament proteins, keratins, which are the major structural proteins of the epidermis (Fuchs, 1995; Fuchs, 1996).

Keratins (K) are expressed as a combination of type I (K9-K20) and type II (K1-K8) keratin pairs in a tissue- and differentiation-specific manner, and account for up to 85% of the total cellular proteins in the terminally differentiated keratinocyte (Fuchs, 1995; Fuchs, 1996). These proteins provide the epidermis with protection from mechanical stress and also play roles in cell signaling, stress response and apoptosis (Coulombe and Omary, 2002; Oshima, 2002).

Other cell types, such as melanocytes, Langerhans cells, and Merkel cells, can also be found in the epidermis. Melanocytes are responsible for epidermal pigment production, Langerhans cells for antigen-presentation, and Merkel cells are the epidermal neuroendocrine cells (Haake et al., 2001; Odland, 1991).

As a stratified epithelium, the epidermis is composed of several sub-compartments, including the basal layer, the spinous layer, the granular layer and the stratum corneum (Byrne and Hardman, 2002; Haake et al., 2001; Odland, 1991).

The basal layer (the stratum germinativum, Figure 1-1a) is composed of a single sheet of cuboidal keratinocytes that is attached to the basement membrane via hemidesmosomes, the specialized structures mediating cell/matrix interactions (Garrod, 1993; Garrod et al., 2002; Green and Jones, 1996; Nievers et al., 1999).

In adult epidermis, only the basal keratinocytes have proliferative abilities, and they serve as the source for epidermal renewal (Alonso and Fuchs, 2003).Transcription of K5 and K14 is characteristic of, and restricted to, the basal keratinocytes, although their protein products can persist in the upper cell layer (Fuchs, 1995; Fuchs, 1996).

Above the basal layer lie several layers of polyhedral keratinocytes, which form the so-called spinous layer (stratum spinosum or the prickle cell layer; Figure 1-1a) (Byrne and Hardman, 2002; Haake et al., 2001; Odland, 1991). It is so named because of the histological appearance of "spines" in the keratinocytes, a shrinkage artifact formed during tissue processing because of the abundance of cell-cell junctions (desmosomes) between keratinocytes. In the mature epidermis, keratinocytes lose their proliferative ability and begin to undergo the terminal differentiation program upon entering into the spinous layer. This process is accompanied with changes in gene expression, switching from expression of K5 and K14 to K1 and K10. Spinous keratinocytes become more flattened as they move outward, and new organelles, the lamellar granules, appear in the cytoplasm of the upper spinous keratinocytes (Byrne and Hardman, 2002; Haake et al., 2001; Odland, 1991).

The granular layer lies above the spinous layer, and the flattened granular keratinocytes can be recognized by their distinctive cytoplasmic structures, the keratohyalin granules (Figure 1-1a) (Byrne and Hardman, 2002; Haake et al., 2001; Odland, 1991). The keratohyalin granules are

insoluble aggregates, which appear as dense, irregular, or stellate-shaped globules composed of granular deposits along the keratin filament bundles (Odland, 1991; Resing and Dale, 1991).

There are two types of keratohyalin granules, one containing a histidine-rich protein, profilaggrin, and the other containing a cysteine-rich protein, loricrin. Proteolysis of profilaggrin gives rise to filaggrin, which functions as a scaffold to aggregate keratin filaments when cornification begins. In addition, free amino acids yielded by filaggrin breakdown are important for normal hydration of the stratum corneum (Resing and Dale, 1991). Loricrin is one of many proteins forming the cornified envelope of the terminally differentiated keratinocytes (Resing and Dale, 1991).

Lamellar granules, appearing first in the upper spinous layer, function primarily at the interface between the granular and cornified layer to deliver and process the precursors of stratum corneum lipids (Haake et al., 2001; Madison, 2003).

In the outermost layer of granular keratinocytes, morphological alterations, such as degradation of mitochondria and ribosomes, disappearance of the nuclear envelope, and ultimate degradation of the nucleus, occur in association with many biochemical changes, such as activation of caspases (Haake et al., 2001; Tsuruta et al., 2002). These changes lead to the formation of the stratum corneum, which acts as the major barrier to water loss and to permeation of environmental substances (Madison, 2003; Steinert, 2000; Tsuruta et al., 2002).

The stratum corneum (Figure 1-1a) is composed of multiple layers of non-viable, flattened, anucleated, terminally differentiated keratinocytes often referred to as corneocytes, which are filed with keratin bundles and covered with a very densely cross-linked protein structure, the cornified envelope (Madison, 2003; Steinert, 2000; Tsuruta et al., 2002; Haake et al., 2001).

The cornified envelope is formed by more than 20 proteins, including involucrin and loricrin, which are cross-linked by sulfhydryl oxidases and transglutaminases (TGs) (Kalinin et al., 2002). The interior surface of the cornified envelope is attached to keratin bundles, while the exterior surface is covered with a lipid envelope formed by very long chain ω-hydroxyceramides that are ester-linked to involucrin by TGs (Lorand and Graham, 2003; Nemes and Steinert, 1999; Kalinin et al., 2002). The major reinforcement protein in the cornified envelope is loricrin which is cross-linked to small amounts of small proline-rich proteins (SPRPs) (Steinert, 2000; Tsuruta et al., 2002). The extracellular spaces between corneocytes are filled with lipid lamellae, composed mainly of ceramides, cholesterol, and free fatty acids (Madison, 2003; Steinert, 2000).

1.2 The dermis

The dermis, derived from diverse mesodermal sources (primarily somites and lateral plate mesoderm), is the connective tissue compartment of the skin and lies below the epidermis (Figure 1-1a). It supplies the epidermis

with its entire nutrient requirements, and acts to cushion against mechanical injuries (Byrne and Hardman, 2002; Haake et al., 2001; Odland, 1991).

The dermis is much less cellular than the epidermis, being composed mainly of a fibrous, amorphous, extracellular matrix (ECM) that surrounds the dermal cells. The major dermal cell type is the fibroblast, which is responsible for synthesis and degradation of ECM constituents (Byrne and Hardman, 2002; Haake et al., 2001; Odland, 1991).

Among the ECM molecules, collagen is most abundant, accounting for roughly 75% of the dry weight of mature skin. Collagen proteins form fibers or bundles to provide skin with tensile strength. In adults, type I collagen is the predominant dermal collagen, which forms fibrils with type III and V collagens (Uitto and Bernstein, 1998).

Elastin fibers, composed of elastin and fibrillin, form a network throughout the dermis and function to restore the skin to its normal configuration following deformation (Pasquali-Ronchetti and Baccarani-Contri, 1997; Uitto and Bernstein, 1998).

The fibrous dermal components are embedded by a continuous gel, which is formed by glycosaminoglycans and proteoglycans. Such a gel-like "ground substance" supports cell migration and holds water, enzymes, and signaling molecules (Byrne and Hardman, 2002; Haake et al., 2001; Odland, 1991).

There are some other glycoproteins in the dermis including fibronectin, thrombospondin, and vitronectin. These proteins interact with other ECM

components and also with cells (Haake et al., 2001; Uitto and Bernstein, 1998).

1.3 The basement membrane

The cutaneous basement membrane located between the epidermis and the dermis, also known as the dermal-epidermal junction (DEJ), is a complex network of proteins, produced both by basal keratinocytes and by dermal fibroblasts (Figure 1-1a). It separates the dermal and epidermal compartments, and provides a dynamic interface between them. Interaction of the basement membrane with the basal epidermis occurs through hemidesmosomes, and with the dermis through anchoring fibrils (Bruckner-Tuderman, 1999; Burgeson and Christiano, 1997; Byrne and Hardman, 2002).

In a hemidesmosome, integrin $\alpha 6\beta 4$ located on the basal surface of a basal keratinocyte associates with a basement membrane protein, laminin 5, which also interacts with another basement membrane protein, type VII collagen. Other proteins in a hemidesmosome, such as plectin and Bullous pemphigoid antigens (BPAG1 and BPAG2), function to link integrin $\alpha 6\beta 4$ to the keratin intermediate filaments in the basal keratinocytes (Burgeson and Christiano, 1997).

Anchoring fibrils are centrosymmetrically cross-banded, fibrillar structures that originate from the dermis-proximal side of the basement membrane and extend into the dermis. The major, if not the exclusive,

component of the anchoring fibrils is type VII collagen, which associates with type IV collagen and laminin 5 in the basement membrane (Burgeson, 1993).

1.4 Hair follicles

Hairs are important epidermal appendages, which are produced by the hair follicle, a specialized structure consisting mainly of an outer root sheath (ORS) that is contiguous with the basal epidermal layer, an inner root sheath (IRS), and the hair shaft (Figure 1-1b) (Byrne and Hardman, 2002; Hardy, 1992; Stenn and Paus, 2001).

Mouse fur is derived from three types of hair follicles including vibrissal (whisker), tylotrich (guard), and non-tylotrich (awl, auchene, and zigzag) follicles. Vibrissa follicles are initiated at E12.5, tylotrich follicles at E14.5, and non-tylotrich follicles at E16.5 (Philpott and Paus, 1998).

Hair follicles are derived from primitive epidermis (Byrne and Hardman, 2002; Hardy, 1992; Stenn and Paus, 2001). In response of a still unknown epidermal cue, keratinocytes in the primitive epidermis accumulate and first form a placode and then a small downgrowth (germ) into the dermis. Subsequently, the mesenchymal cells underlying the hair germ condense, possibly due to a signal originating from the hair germ cells, eventually forming the dermal component of a hair follicle, the dermal papilla (Byrne and Hardman, 2002; Hardy, 1992; Stenn and Paus, 2001). A second dermal message from the dermal papilla is then transmitted back to the epithelial cells, instructing them to proliferate downwards. As development proceeds,

the epithelial cells differentiate, giving rise to first an ORS and IRS, and then near or at birth, a hair shaft at the center of the follicles (Byrne and Hardman, 2002; Hardy, 1992; Stenn and Paus, 2001).

The epithelial cells forming a cloak surrounding the dermal papilla are called matrix cells, which differentiate into upwardly moving cells. At the center, matrix cells give rise to precortical cells, which subsequently yield the cortex, medulla, and cuticle of the hair shaft. A surrounding concentric ring of matrix cells gives rise to the IRS, which degenerates at the skin surface to free the hair shaft (Byrne and Hardman, 2002; Hardy, 1992; Stenn and Paus, 2001).

Postnatal hair follicles proceed through cycles of active periods of hair growth (anagen), regression and shortening (catagen) and rest (telogen) (Byrne and Hardman, 2002; Hardy, 1992; Stenn and Paus, 2001). In adults, the lower epithelial portion of the hair follicle constantly cycles, while the upper portion remains throughout life. In rodents, the first two hair cycles are largely synchronous. Progressive asynchrony develops with subsequent cycles, possibly due to environment impact (Fuchs et al., 2001).

Below the sebaceous glands and at the base of the permanent segment of a hair follicle, there is a region known as the bulge, an important site hosting skin epithelial stem cells (Fuchs et al., 2001; Gambardella and Barrandon, 2003; Lavker and Sun, 2000; Niemann and Watt, 2002; Watt, 2001).

During the development of the hair follicle, a sebaceous gland emerges as an appendage to the upper segment of each hair follicle (Figure 1-1b) (Paus et al., 1999). The center of the sebaceous gland is open to and secretes lipid (sebum) into the hair canal at the skin surface (Downing and Strauss, 1982).

2. Murine epidermal development

The epidermis derives from non-neural embryonic ectoderm, a single layer of histologically undifferentiated epithelial cells (Figure 1-2) (Byrne and Hardman, 2002).

Between embryonic day 8.5-10 (E8.5-10), cell mitoses occur parallel to the ectodermal cell layer, resulting in ectodermal expansion (Byrne and Hardman, 2002). Between E9-12, regional stratification of the non-neural ectoderm gives rise to an outer surface layer of flattened cells, the periderm, which is shed off into the amniotic fluid starting at about E16.5 (M'Boneko and Merker, 1988). Although the periderm has been suggested to act as a transitory protective interface between the developing embryo and the amniotic fluid during early epidermal development in utero (M'Boneko and Merker, 1988) and a lubricant during birth in human, its precise biological function remains elusive (Agorastos et al., 1988).

Weak expression of K5 is first detected at E9.5 in both the basal and peridermal cells, and is likely induced by a mesenchymal cue, and a marked

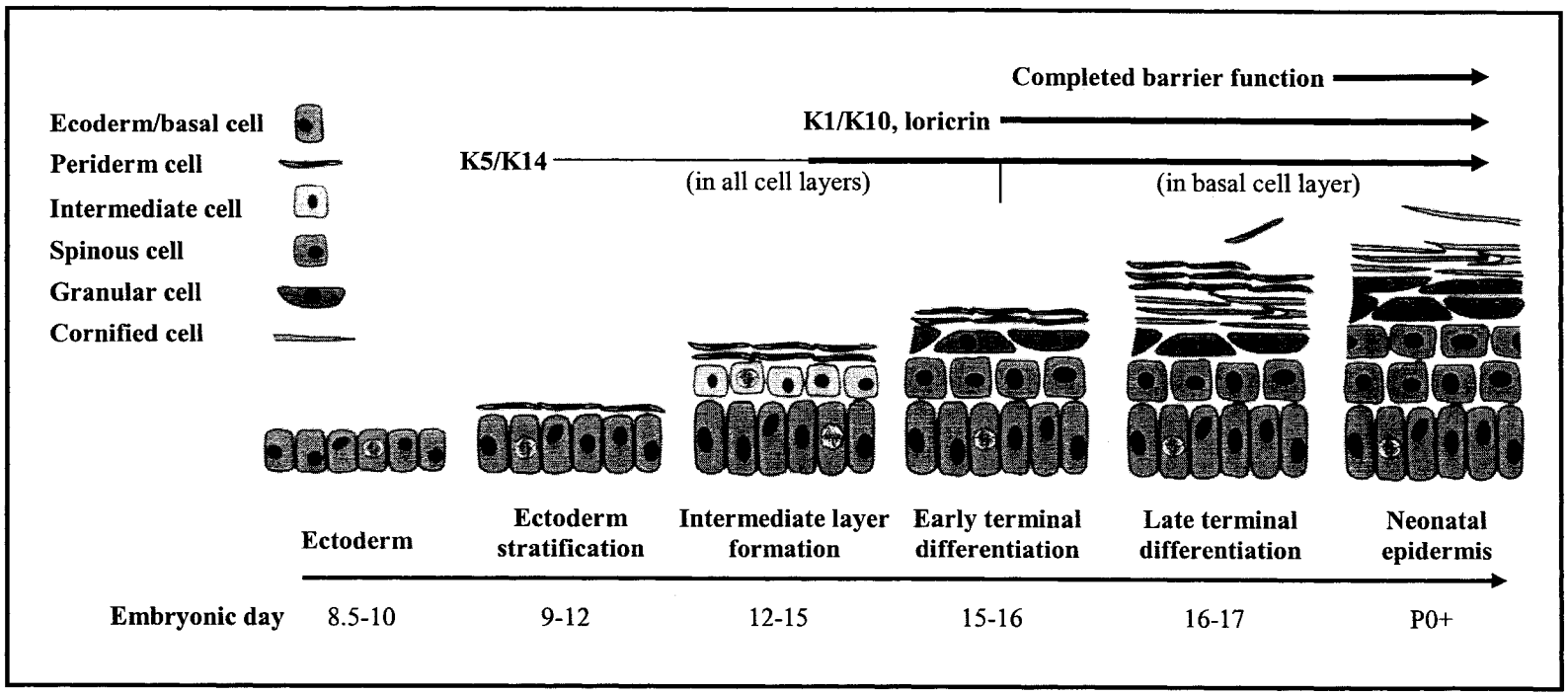


Figure 1-2. Diagrammatic representation of mouse epidermal development (Modified from Byrne and Hardman, 2002).

increase in K5 expression occurs between E13.5 and E14.5 (Byrne et al., 1994). The expression pattern of K14 is the same as that of K5 during mouse epidermal development (Byrne et al., 1994), although K5 expression is reported to slightly precede that of K14 during human epidermal development (Moll et al., 1982).

Starting from E12, cell division occurs in an orientation perpendicular to the initial ectodermal layer, leading to further stratification and the formation of the intermediate layer (stratum intermedium) (Byrne and Hardman, 2002; Sengel, 1990). At E13.5, differentiation-specific gene expression begins to occur in the developing epidermis. By E15.5, cells in the intermediate layer display expression of K1, K10 and loricrin, correlating with the appearance of the spinous and granular layers (Byrne et al., 1994; Sengel, 1990), while expression of K5 and K14 becomes restricted to the basal cells (Byrne et al., 1994).

By E16.5, the epidermis has fully stratified with the appearance of the stratum corneum (Byrne and Hardman, 2002; Sengel, 1990). The stratum corneum at this stage, however, does not exhibit its typical appearance seen in postnatal skin (Hardman et al., 1998). Concomitantly, the barrier function of mouse epidermis begins to form at E16, and is completed in a defined order, initiating first in dorsal epidermis and followed by the epidermal areas covering the head and tail, then limbs (Hardman et al., 1998). After E16.5, whole-mount in situ hybridization can no longer be used to study gene expression, because the skin has become impermeable to cRNA probes

(Byrne et al., 1994). The barrier function of E17.5 skin is essentially the same as that of the adult skin (Hardman et al., 1998).

3. Skin epithelial stem cells

As reviewed above, the epidermis and its appendages such as hair follicles and sebaceous glands are the differentiating epithelial compartments of the adult mammalian skin, which undergo constant self-renewal (Gambardella and Barrandon, 2003; Niemann and Watt, 2002; Watt, 2001).

The renewal program of the epidermis and sebaceous glands are continuous. In the epidermis, proliferation takes place in the basal layer, and the differentiating cells move through the suprabasal layers to the tissue surface where they form the stratum corneum (Byrne and Hardman, 2002; Fuchs and Raghavan, 2002).

Likewise, in the sebaceous glands, cell division occurs in the periphery of the gland (the basal layer), and the differentiating progeny accumulate sebum as they move toward the sebaceous duct and eventually rupture to release their contents into the pilosebaceous canal (Downing and Strauss, 1982).

Unlike the epidermis and sebaceous glands, the growth of adult hair follicles is cyclic. During active growth (the anagen phase), proliferation takes place in matrix cells in the hair bulb, with upward movement and differentiation to form the IRS and hair shaft at the center of the follicle (Hardy, 1992; Stenn and Paus, 2001). Like other constantly renewing tissues,

stem cells are responsible for the maintenance of these cutaneous epithelial tissues (Gambardella and Barrandon, 2003; Niemann and Watt, 2002; Watt, 2001; Raff, 2003).

3.1 Skin epithelial stem cells

The fundamental feature of a stem cell is that it has the capacity for self-renewal, i.e. the ability to generate additional stem cells and thus be long lived, and the capacity to generate progeny that are destined to differentiate into at least one type of differentiated descendants (Lajtha, 1979; Morrison et al., 1997; Raff, 2003; Slack, 2000; Watt and Hogan, 2000). Usually, between the stem cell and its terminally differentiated progeny there is an intermediate population of committed progenitors called transit amplifying (TA) cells that have limited proliferative capacity and restricted differentiation potential (Morrison et al., 1997; Slack, 2000; Watt and Hogan, 2000). Besides the difference between their dividing potentials, the cell kinetics of the skin epithelial stem cells and TA cells are distinct. In undamaged, steady-state postnatal skin, the stem cells are slow-cycling or infrequently dividing, while the TA cells divide relatively frequently (Gambardella and Barrandon, 2003; Niemann and Watt, 2002; Watt, 2001). The slow-cycling feature of skin epithelial stem cells is of particular importance, since it preserves the proliferative potential of the stem cells and minimizes errors associated with DNA replication (Lavker and Sun, 2000). Quiescent stem cells can be activated by wounding, treatment with phorbol ester (TPA) and certain in vitro

culture conditions (Braun et al., 2003; Lavker and Sun, 2000; Niemann and Watt, 2002; Watt, 2001).

Experimentally, the epithelial stem cells in the skin can be detected as the "label-retaining cells" (LRCs) based on their slow-cycling properties. This is done by labeling all skin cells in neonatal animals at a time of rapid tissue expansion via repeated injection of ^{3}H-thymidine or 5-bromo-2'-deoxyuridine (BrdU), and then identifying those cells retaining the label into adulthood. The LRCs are cells that do not divide frequently and are thus putative stem cells (Bickenbach, 1981; Bickenbach and Chism, 1998).

Label-retention assays are not applicable to human material. In this case, in vitro clonogenicity assays are useful to distinguish stem cells from TA cells. Under certain conditions, the growth of stem cells is activated, giving rise to large, actively growing clones in human keratinocyte cultures because of their great proliferative potential. By contrast, TA cells only form small, abortive clones due to their limited mitotic ability (Barrandon and Green, 1987; Jones and Watt, 1993; Rheinwald and Green, 1975). It is worth noting that such an approach can not be used to screen stem cells in mouse keratinocyte culture, since mouse keratinocytes do not perform well in clonal culture (Watt, 2001).

In recent years, extensive efforts have focused on identifying markers for skin epithelial stem cells (Gambardella and Barrandon, 2003; Lavker and Sun, 2000; Niemann and Watt, 2002; Watt, 2001), and several markers have been reported. For instance, integrin β1 enriches for stem cells in human

interfollicular epidermis (IFE) and in human keratinocyte culture (Jensen et al., 1999; Jones and Watt, 1993; Jones et al., 1995). In both mouse and human skin, the stem cells are located in areas expressing high levels of integrin $\alpha 6\beta 4$. Mouse skin epithelial stem cells can be enriched by sorting out keratinocytes that have strong expression of integrin $\alpha 6$ and weak expression of CD71 (transferrin receptor) (Tani et al., 2000). Likewise, the combination of high levels of integrin $\alpha 6$ and low expression of a proliferation-associated cell surface marker, 10G7ag, has also been used to screen stem cells from human epidermis (Kaur and Li, 2000; Li et al., 1998a).

Other putative markers for stem cells have also been suggested. These include K15 (Braun et al., 2003; Lyle et al., 1998), high levels of non-cadherin-bound β-catenin (Zhu and Watt, 1999), an elevated level of the proteoglycan NG2/MCSP (Legg et al., 2003), and high expression of Delta1, a Notch ligand (Lowell et al., 2000).

In the hair-bearing mouse skin, epithelial stem cell "clusters" are found in the bulge areas of the hair follicles (Gambardella and Barrandon, 2003; Lavker and Sun, 2000; Niemann and Watt, 2002; Watt, 2001). The IFE and sebaceous glands may also be minor stem cell niches (Braun et al., 2003; Cotsarelis et al., 1990; Morris and Potten, 1999; Ghazizadeh and Taichman, 2001; Taylor et al., 2000; Tumbar et al., 2004). The IFE stem cells located in the basal epidermis in the hairy mouse skin are not organized as clusters, but instead are distributed in a scattered manner (Braun et al., 2003).

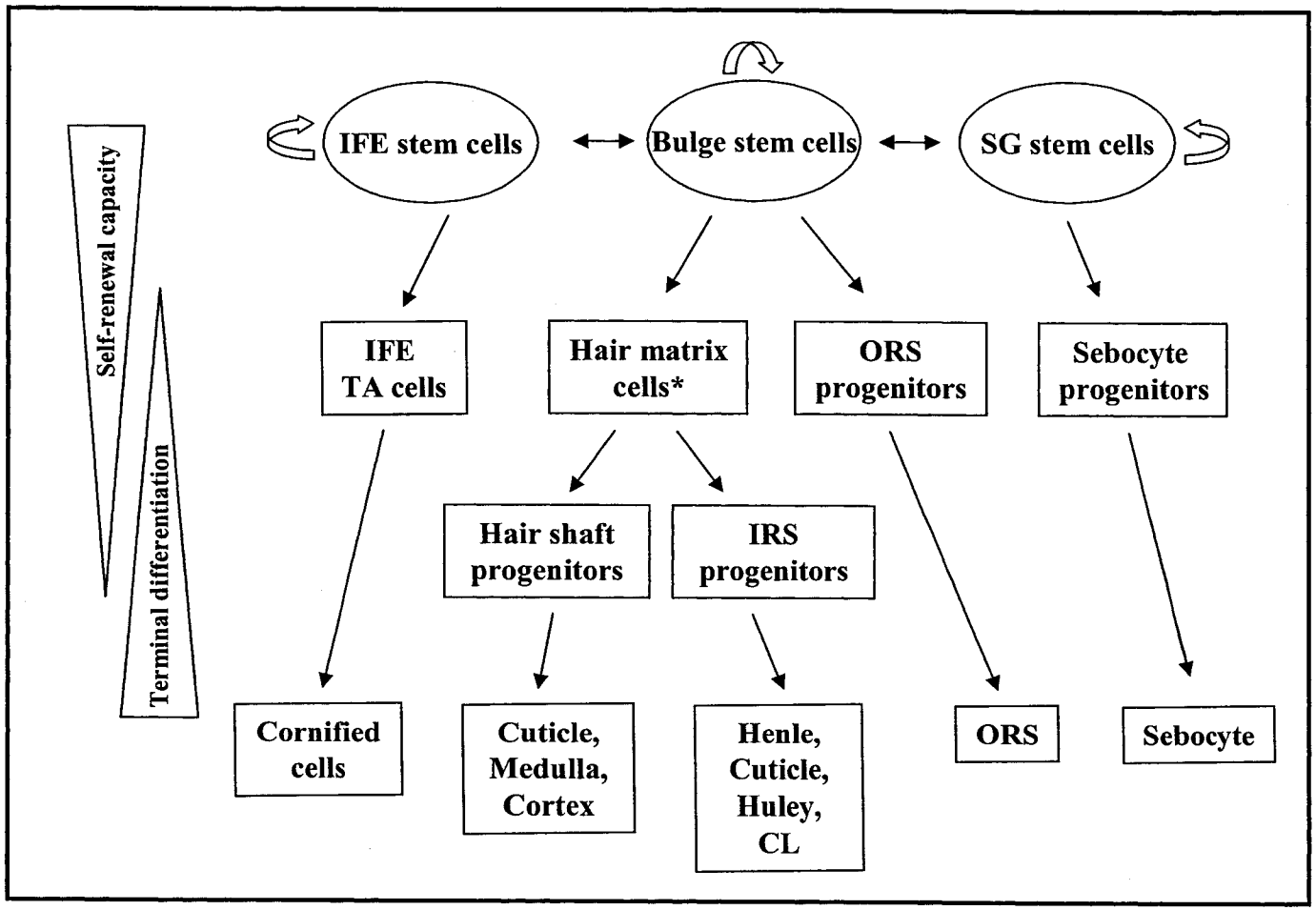


Figure 1-3. A model of lineage relationships within the epidermis (Modified from Niemann and Watt, 2002). IFE, interfollicular epidermis; SG, sebaceous gland; ORS, outer root sheath; IRS, inner root sheath; CL, companion layer. *, Stem cells are also suggested to be located in the hair bulb where the matrix cells are based. This, however, is still in debate (Niemann and Watt, 2002).

Human skin is much less hairy than mouse skin, and the basal IFE layer is the major skin epithelial stem cell-hosting location. Close to 10% of the basal keratinocytes in human epidermis are estimated to be stem cells, and are distributed as clusters (Jensen et al., 1999; Jones et al., 1995; Li et al., 1998a; Potten and Morris, 1988).

The bulge stem cells are multipotent, able to give rise not only to the hair lineages but also to IFE and sebocytes (Figure 1-3) (Oshima et al., 2001; Taylor et al., 2000). Although it is still under debate (Braun et al., 2003; Ghazizadeh and Taichman, 2001), the bulge stem cells are also thought to be the ultimate source of IFE keratinocytes in the hairy mouse skin (Lavker and Sun, 2000; Taylor et al., 2000).

As reviewed above, during embryonic development, the epidermis is derived from non-neural embryonic ectoderm, and its appendages, such as hair follicles and sebaceous glands, from the primitive epidermis (Byrne and Hardman, 2002). Due to the limitation of the current approaches used to identify stem cells, little is known about the properties of epithelial stem cells in the developing skin.

With the aid of gene-targeting and transgenesis technology, many factors which are involved in the maintenance and cell-fate determination of skin epithelial stem cells have been revealed (Brouard and Barrandon, 2003; Fuchs and Raghavan, 2002; Niemann and Watt, 2002; Watt, 2001). Among these factors, p63 may be essential for the maintenance of skin epithelial

stem cells (Fuchs and Raghavan, 2002; Yang and McKeon, 2000; Yang et al., 2002).

3.2 p63 and its role in epidermal development

p63 is a group of proteins homologous to p53 with highly conserved domains analogous to those seen in p53 including a central DBD and a carboxy-terminal oligomerization domain (OD) (Figure 1-4a). p63 proteins elicit both p53-related and -unrelated functions (Yang and McKeon, 2000; Yang et al., 2002).

3.2a p63 isoforms

Unlike the p53 gene, which encodes only one product, the p63 gene gives rise to two p63 subtypes, TA-p63 and ΔN-p63, due to differential usage of two promoters. In addition, both TA-p63 and ΔN-p63 are expressed as α, β and γ isoforms as a result of alternative splicing (Figure 1-4a) (Yang and McKeon, 2000; Yang et al., 2002).

The P1 promoter located upstream of exon 1 produces TA-p63 proteins containing a conserved acidic amino terminus with homology to the transactivation domain of p53 (Yang and McKeon, 2000; Yang et al., 2002). Indeed, the β and γ isoforms of TA-p63 proteins can mimic p53 function in vitro, bind to canonical p53 response elements and transactivate p53 target genes such as $p21^{cip1}$, mdm2, BAX, and GADD45 (Levrero et al., 2000; Osada et al., 1998; Shimada et al., 1999; Yang et al., 1998). Moreover, these

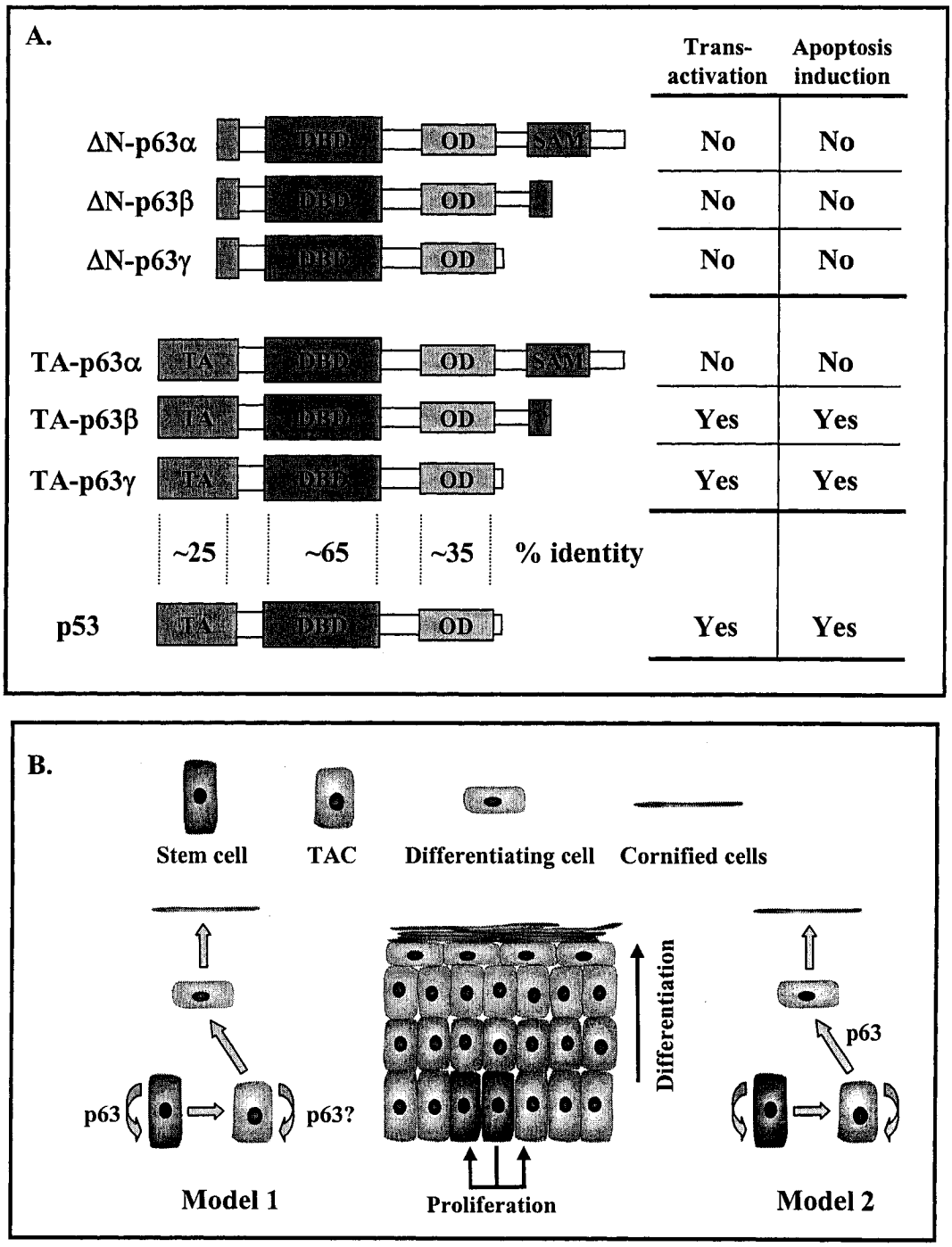


Figure 1-4. The role of p63 in the epidermis (Modified from Yang and Mckeon, 2000). DBD, DNA-binding domain; OD, oligomerization domain; TA, transactivation domain; SAM, sterile α-motif, TAC, transit amplifying cell.

isoforms can induce apoptosis after introduction into the cell (Osada et al., 1998; Yang et al., 1998). The ability of TA-p63β and TA-p63γ to mediate these p53-related activities is comparable to that of wild type p53 (Levrero et al., 2000; Osada et al., 1998; Shimada et al., 1999; Yang et al., 1998).

At the carboxy-terminus, the α isoform of TA-p63 (and ΔN-p63) contains an extra conserved sterile α-motif (SAM) that is thought to mediate protein-protein interactions and exert autoinhibitory effects on p53-like transactivation activity (Yang and McKeon, 2000). As a result, TA-p63α only weakly activates p53-target genes and is well tolerated by cells after transfection (Yang et al., 1998).

The P2 promoter, located in intron 3, produces ΔN-p63 proteins that lack the TA domains (Yang and McKeon, 2000; Yang et al., 2002). Unlike TA-p63, ΔN-p63 proteins do not transactivate p53-target genes and do not induce apoptosis when expressed in the cell. Instead, they show dominant negative effects on p53 as well TA-p63 both in vitro and in vivo (Yang and McKeon, 2000; Yang et al., 2002). Of note, virtually all p63 proteins expressed in the epithelial tissues examined belong to the ΔN-p63 subfamily (Yang et al., 1998).

In transcription assays, ΔN-p63 inhibits p53 as well as TA-p63-mediated gene transactivation (Yang et al., 1998). In vivo, ultraviolet (UV) radiation causes p53-dependent apoptosis in the epidermis, accompanied by a rapid loss of ΔN-p63 (but an increase in TA-p63 expression), and targeted expression of ΔN-p63 in the suprabasal epidermis results in a reduction in

UV-induced apoptosis, indicating that ΔN-p63 can antagonize p53 action in the epidermis (Liefer et al., 2000).

In contrast to the role of p53 as a tumor suppressor gene which is frequently lost in many human cancers, p63 may act as an oncogene (Yang and McKeon, 2000; Yang et al., 2002). 3q27-ter locus (denoted AIS for amplified in squamous cell carcinoma), where p63 is located, is frequently amplified in several types of squamous cell carcinomas. In these tumors, ΔN-p63 is expressed as the predominant form of p63. Moreover, overexpression of ΔN-p63 enhances the tumorigenicity of Rat 1a cells (Hibi et al., 2000).

3.2b Roles of p63 in epidermal development

p63 is constitutively expressed in many epithelial tissues such as the epidermis, cervix, tongue, esophagus, mammary glands, prostate, and urogenital tract (Yang et al., 1998). Specifically, strong nuclear expression is detected continuously in the basal epidermis and the ORS of the hair follicles (Mills et al., 1999; Yang et al., 1999). In the developing mouse embryo, p63 expression is first evident at E9.5 in the ectodermal cell layer which eventually gives rise to the epidermis and related derivatives (Mills et al., 1999). p63 is also expressed in the basal cells of many other stratified epithelia (Mills et al., 1999). In keeping with this expression pattern, targeted deletion of p63 causes severe defects in epithelial tissues including the epidermis, hair follicle, mammary tissue, prostate, lachrymal glands, salivary glands, urogenital tract, tongue and stomach (Mills et al., 1999; Yang et al., 1999).

p63 null mice also exhibit limb truncation and craniofacial malformation and die shortly after birth possibly due to dehydration (Mills et al., 1999; Yang et al., 1999).

The marked defects exhibited by p63 null mice are in striking contrast to the normal appearance of p53 null animals, suggesting p63 as a p53 homolog plays distinct roles in development (Celli et al., 1999; Van Bokhoven et al., 2001; McGrath et al., 2001; Mills et al., 1999; Yang et al., 1999; Donehower et al., 1992).

Perinatal p63 null mice reported by Yang et al. (1999) lack intact epidermis, with only patchy epidermal keratinocytes covering the dermis. These keratinocytes do, however, undergo normal terminal differentiation as evidenced by expression of differentiation-related markers, such as loricrin, filaggrin and involucrin. The residual epidermal cells do not express K5, a marker associated with the proliferative compartment of the epidermis, indicating defective keratinocyte growth. p63 is, therefore, thought to be essential for the maintenance of epidermal stem cells and/or TA cells (Model 1, Figure 1-4b) (Yang et al., 1999; Yang and McKeon, 2000; Yang et al., 2002).

In line with this notion, it has recently been shown that ΔN-p63, the major p63 subtype expressed in the epidermis, is essential for epidermal development by affecting cell proliferation rather than differentiation (Bakkers et al., 2002; Lee and Kimelman, 2002). Elevated expression of $p21^{cip1}$ and 14-3-3σ correlates with keratinocyte differentiation (Topley et al., 1999;

Dellambra et al., 2000; Macleod et al., 1995; Missero et al., 1995). Phosphorylated ΔN-p63α can bind to the p53 consensus sites in the $p21^{cip1}$ and 14-3-3σ promoters and transrepresses their expression in primary human keratinocyte cultures. Transrepression of $p21^{cip1}$ and 14-3-3σ is thus thought to be at least in part attributable for the effect of p63 on stem cell maintenance (Westfall et al., 2003).

Mice deficient in p63 generated by Mills et al. (1999) display a similar skin phenotype, but have an intact basal epidermal layer and an absence of suprabasal cells. Expression of differentiation-related markers, such as K1, filaggrin and loricrin, is undetectable, indicating a block in terminal differentiation. However, keratinocyte growth is still retained, as K14, a marker associated with the proliferative compartment of the epidermis, is expressed in the epidermis albeit at a relatively low level. These observations suggest that p63 may be involved in regulating keratinocyte differentiation rather than growth (Model 2, Figure 1-4b) (Koster et al., 2002; Mills et al., 1999). This mechanism is supported by gene expression studies showing that several genes implicated in epidermal development are p63 targets. Notably, TA-p63γ positively regulates the Notch ligands, JAG1 and JAG2 (Sasaki et al., 2002), and Notch1 has been known to be important for epidermal differentiation (Rangarajan et al., 2001).

The clinical signs of several human disorders such as ectodermal dysplasia, ectrodactyly, and cleft plate syndrome (EEC), an autosomal dominant disorder characterized by skin, limb, and craniofacial abnormalities,

and the ankyloblepharon-ectodermal dysplasia-clefting syndrome (AEC or Hay-Wells syndrome), an autosomal dominant disorder characterized by fused eyelids and severe scalp dermatitis (but normal limb formation), are reminiscent of the phenotypes of p63 null mice, although less severe. In agreement with this, p63 mutations have been found to be causally related both EEC and AEC (Celli et al., 1999; Van Bokhoven et al., 2001; McGrath et al., 2001).

4. Epidermal integrity

The epidermis forms a protective barrier which must remain uncompromised for health. Structural proteins such as keratins and cell-cell, cell-matrix interacting molecules play a very important role in maintaining epidermal integrity (Fuchs, 1995; Fuchs, 1996; Watt, 2002).

4.1 Roles of keratins and their associated proteins

Keratins are the major structural proteins in the epidermis and its appendages (Fuchs, 1995; Fuchs, 1996). The major basal keratins are K5 and K14, which are also expressed in the ORS of the hair follicle, while K1 and K10 are expressed in the suprabasal cells. Expression of filaggrin, loricrin, and involucrin are associated with late epidermal differentiation, and these proteins, together with keratins, are the major protein components forming the stratum corneum (Madison, 2003).

Epidermolysis bullosa simplex (EBS) is an autosomal dominant human skin disorder typified by skin blistering as a consequence of cell cytolysis within the basal epidermal layer (Fuchs, 1996; Takahashi et al., 1999; Coulombe and Fuchs, 1993; Coulombe and Omary, 2002). Dominant mutations in K5 and K14 and disrupted assembly of keratin intermediate filaments are associated with all human EBS variants (Fuchs, 1996; Takahashi et al., 1999). Transgenic mice expressing K14 with these various mutations recapitulate the skin phenotypes displayed in human EBS (Coulombe et al., 1991; Vassar et al., 1991). Ablation of K14 causes a generalized EBS phenotype that is accompanied by an increased mortality (Lloyd et al., 1995), while disruption of K5 also leads to EBS and death within hours of birth (Peters et al., 2001).

Epidermolytic hyperkeratosis (EHK), also known as bullous congenital ichthyosiform erythroderm (BCIE), is an autosomal dominant skin disorder characterized by erythematous bullous skin lesions during infancy and dry, scaly, fragile, and hyperkeratotic skin in adult patients (Fuchs, 1996; Takahashi et al., 1999). A link between K1 or K10 mutation and EHK has been found in many affected families (Cheng et al., 1992; Chipev et al., 1992; Rothnagel et al., 1992; Rothnagel et al., 1993), and transgenic mice expressing mutated forms of K10 and K1 exhibit EHK phenotypes (Bickenbach et al., 1996; Fuchs et al., 1992; Porter et al., 1998).

Mice expressing a truncated K10 protein with dominant negative effects present with a more severe EHK phenotype (Porter et al., 1996;

Reichelt et al., 1997). By contrast, K10 null mice display only mild EHK phenotypes with hyperkeratosis in adults. Enhanced stability of K5 and K14 in K10 null mice is thought to compensate for K10 loss and is thus able to partially "rescue" the EHK defects. Induction of c-Myc, cyclin D1, and 14-3-3σ correlates with epidermal hyperproliferation, and may be causally related to the hyperkeratosis phenotype of the K10 null epidermis (Reichelt et al., 2001; Reichelt and Magin, 2002).

Ichthyosis vulgaris (IV) is an autosomal dominant skin disorder characterized by mild to moderate scaling involving primarily the extensor surface of the extremities (Ishida-Yamamoto et al., 1998). In IV patients, profilaggrin mRNA is unstable, leading to a decrease in protein levels in the epidermis (Nirunsuksiri et al., 1998; Sybert et al., 1985).

Both progressive symmetric erythrokeratoderma (PSEK) and Vohwinkel's syndrome (VS) are autosomal dominant skin disorders. PSEK is characterized by symmetric red scaling plaques usually on the extremities, while VS leads to marked hyperkeratosis with a honeycomb appearance (Ishida-Yamamoto et al., 1998). In some PSEK and VS patients, loricrin mutations have been documented (Ishida-Yamamoto et al., 1997; Korge et al., 1997; Maestrini et al., 1996), and transgenic mice expressing a mutant form of loricrin display phenotypes characteristic of PSEK and VS (Suga et al., 2000). However, despite the fact that loricrin is abundant in the cornified envelope of the stratum corneum, loricrin null mice exhibit only a transient, mild, epidermal erythema after birth. Other protein components in the

cornified envelope are thought to have overlapping functions with loricrin and may functionally compensate for the loss of loricrin (Koch et al., 2000).

Overexpression of involucrin in mice leads to scaly skin phenotypes (Crish et al., 1993). By contrast, involucrin knockouts are unexpectedly normal. Again, compensatory mechanisms overcoming the absence of involucrin have been suggested (Djian et al., 2000).

Lamellar ichthyosis (LI) is a severe congenital generalized skin disorder characterized by variable erythema and scaling throughout the entire body surface. It is inherited as an autosomal recessive trait in most cases (Ishida-Yamamoto et al., 1998). Mutations in the TG1 gene are found in LI families (Hennies et al., 1998; Huber et al., 1995; Laiho et al., 1997; Russell et al., 1995), and ablation of TG1 in mouse causes erythrodermic skin and abnormal keratinization that resembles severe LI. $TG1^{-/-}$ mice die early after birth (Matsuki et al., 1998).

4.2 Roles of integrins and their ligands

Integrins are heterodimeric transmembrane receptors consisting of one α and one β subunit. Extracellular matrix proteins or counter-receptors of the Ig superfamily serve as their ligands (Giancotti, 2003).

In normal epidermis, $\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins are expressed constitutively, and are confined to the basal layer and the ORS of the hair follicles. $\alpha 6\beta 4$ integrin localizes primarily at the basal surface of the basal cells as a component of hemidesmosomes, while other integrins are

distributed over the basal, lateral, and apical surfaces of the basal cells. Integrins act to regulate epidermal cell adhesion, migration, growth and differentiation (Watt, 2002).

Mutations in α6 or β4 integrins have been found in some families with junctional epidermolysis bullosa (JEB), an autosomal recessive disorder with epidermal blistering (Ashton et al., 2001). Ablation of α6 or β4 integrin subunit in mice also causes severe blistering of the skin. The skin in these mice lacks hemidesmosomes and shows signs of tissue degeneration and disorganization, and mutant offspring die shortly after birth (Dowling et al., 1996; Georges-Labouesse et al., 1996; van der et al., 1996).

Integrin β1 deficiency causes embryonic death at a very early stage (Fassler and Meyer, 1995; Stephens et al., 1995), while conditional knockout in the epidermis leads to skin blistering and hair defects that are accompanied by hemidesmosome instability and disorganization of the epidermis (Brakebusch et al., 2000; Raghavan et al., 2000). In these mice, keratinocyte migration is impaired, thereby leading to severe wound healing defects (Grose et al., 2002). High levels of β1 integrin expression are associated with the regions which host epidermal stem cells, making β1 integrin a valuable marker for these stem cells (Watt, 2001).

Targeted deletion of integrin α3 causes disorganization of the basement membrane and occasional skin blistering (DiPersio et al., 1997). By contrast, there is no skin phenotype reported in integrin α2 knockout mice (Chen et al., 2002; Holtkotter et al., 2002).

Laminin 5, a heterotrimer composed of laminin α3, β3 and γ2 chains, serves as a ligand for both α6β4 and α3β1 integrins (Watt, 2002). Mutations in the genes encoding laminin 5, and, in particular, in LAMB3 (the β3 chain), have been found in many cases of JEB (Fine et al., 2000). Expression of wild type LAMB3 in affected human keratinocytes is able to correct the JEB symptoms in grafting experiments (Ortiz-Urda et al., 2003). Targeted disruption of LAMA3, the α3 chain coding gene, also causes JEB skin phenotypes (Ryan et al., 1999).

5. Epidermal keratinocyte growth and differentiation

Epidermal homeostasis is achieved by balancing cell growth and differentiation, which are regulated by many factors (Fuchs and Raghavan, 2002).

5.1 Regulation of epidermal growth

As reviewed above, basal keratinocytes, composed of stem cells and transit amplifying cells, have proliferative ability. Some growth factors have been shown to play important roles in regulating keratinocyte growth *in vivo*. The epidermal growth factor receptor (EGFR) belongs to a group of structurally related tyrosine kinase receptors, which are activated by polypeptide growth factors. In the adult epidermis, EGFR is mainly expressed in the basal layer. Mice lacking EGFR exhibit strain-dependent phenotypes affecting multiple organs, including the skin. In general, perinatal EGFR null

skin displays a thinner epidermis due to hypoproliferation, and short, waved, hairs due to disorganization of the hair follicles (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995).

Fibroblast growth factor receptors (Fgfr) are cell surface receptors with tyrosine kinase activity, which are activated by FGFs, a large group of polypeptide growth factors. Among Fgfrs, Fgfr2-IIIb is highly expressed in the basal layer of the epidermis. Disruption of Fgfr2-IIIb causes defects in multiple organs, including the skin. Fgfr2-IIIb null skin has a very thin epidermis caused by hypoproliferation of the basal keratinocytes, and reduced hair follicles (Li et al., 2001a; Petiot et al., 2003; Revest et al., 2001).

5.2 Epidermal differentiation

Starting from the spinous layer, keratinocytes lose their mitotic capacity, undergo terminal differentiation, and eventually form the stratum corneum of the epidermis.

In vitro, calcium is a potent differentiation inducer of keratinocytes. Many lines of evidence also suggest a major role of calcium in regulating keratinocyte differentiation in the epidermis, including the finding of an extracellular calcium gradient that peaks at the granular layer (Byrne and Hardman, 2002). Further evidence supporting the role of calcium in epidermal differentiation comes from the finding that CaR (calcium sensing receptor) null mice show epidermal defects, including abnormal differentiation (Oda et al., 2000; Komuves et al., 2002).

Many other factors are also involved in regulating epidermal differentiation. For instance, Notch1, a transmembrane receptor, acts as a direct determinant of keratinocyte growth arrest and differentiation. Conditional deletion of Notch1 in the epidermis results in hyperproliferation, thereby causing a thickened epidermis (Rangarajan et al., 2001). These conditional Notch1 null mice eventually develop basal cell carcinomas due to defective differentiation (Nicolas et al., 2003).

Nuclear factor-κB (NF-κB)/Rel family of proteins are dimeric transcription factors, with p50/p65 (RelA) the most frequent pair. In unstimulated cells, inhibitors of κB (IκBs) sequester NF-κB dimers in the cytosol. Activation of IκB kinases (IKK) by stimuli leads to phosphorylation, ubiquitination and degradation of IκB, resulting in translocation of the NF-κB dimers into the nucleus and activation of gene expression (Baldwin, Jr., 2001; Lin and Karin, 2003).

When exiting from the basal layer, p50 and p65 translocate from the cytosol to the nucleus of keratinocytes, suggesting that NF-κB activation may be important for promoting keratinocyte differentiation (Seitz et al., 1998; Takeda et al., 1999). However, this role is presently controversial due to the fact that there may be functional redundancy between NF-κB members, and that abnormal NF-κB activation may cause an inflammatory response in the epidermis, resulting in a host of secondary effects (Kaufman and Fuchs, 2000) .

Interestingly, IKKα, one of the catalytic subunits of the IKK complex, regulates epidermal homeostasis independent of the canonical NF-κB pathway. Conditional deletion of IKKα in the epidermis causes hyperproliferation of the basal keratinocytes and abortive differentiation of spinous cells (Hu et al., 1999; Li et al., 1999; Takeda et al., 1999). Loss of IKKα does not affect NF-κB activation, but inhibits the expression of a secreted factor, keratinocyte differentiation-inducing factor (kDIF). Loss of kDIF expression may underlie the epidermal abnormalities caused by loss of IKKα (Hu et al., 2001).

Several transcription factors have been found to specifically regulate the late stages of terminal differentiation. For instance, the Distal-less-related homeodomain gene Dlx3 is expressed in the granular layer of the epidermis. Overexpression of Dlx3 in basal epidermal cells inhibits keratinocyte growth and induces premature terminal differentiation in the basal epidermis, resulting in the absence of suprabasal layers (Morasso et al., 1996).

The transcription factor kruppel-like factor 4 (Klf4) is expressed in the upper spinous and granular layers of the epidermis. K1f4 null mice appear normal at term, but die of dehydration shortly after birth due to impaired barrier function (Segre et al., 1999). Ectopic expression of Klf4 in the basal keratinocytes from K5 promoter causes accelerated formation of the epidermal outer barrier (Jaubert et al., 2003).

5.3 Roles of AP-1 in epidermal development

Activating protein-1 (AP-1) transcription factors are homo- or heterodimers composed of basic region-leucine zipper (bZIP) proteins including Jun (c-Jun, JunB and JunD), Fos (c-Fos, FosB, Fra-1 and Fra-2), and ATF (ATF2, ATF3 and B-ATF) (Angel and Karin, 1991; Karin et al., 1997; Shaulian and Karin, 2002). Jun members can form homodimers and heterodimers with Fos and ATF proteins. Likewise, ATF proteins also form homodimers and heterodimers with Jun and ATF members (Karin et al., 1997). The Fos proteins do not form homodimers, but can couple with the Jun proteins (Halazonetis et al., 1988; Kouzarides and Ziff, 1988).

Jun/Jun or Jun/Fos dimers preferentially bind to the TPA-response element (TRE), a consensus sequence of TGACTCA, while Jun/ATF or ATF/ATF dimers, associate preferentially with the cAMP-response element (CRE), a consensus sequence of TGACCTCA (Karin et al., 1997; Shaulian and Karin, 2002).

Various stimuli can regulate AP-1 activity by affecting the phosphorylation, the expression, and/or the combination of AP-1 proteins (Karin, 1995; Karin et al., 1997; Shaulian and Karin, 2002). Among the Jun subfamily, c-Jun is the most potent activator of transcription (Ryseck and Bravo, 1991), and it has been suggested to play a central role in AP-1 signaling (Karin and Hunter, 1995; Karin, 1995). The regulation of c-Jun provides a good example of AP-1 regulation. c-Jun is expressed in many cell types at low levels, and its expression is increased in response to many stimuli (Karin and Hunter, 1995; Karin, 1995). Although c-Jun can form

Jun/Jun dimers, its association with c-Fos gives rise to a more stable dimeric complex, which has a higher affinity for DNA and elevated transcription activity (Halazonetis et al., 1988; Kouzarides and Ziff, 1988). In contrast to c-Fos, JunB antagonizes c-Jun function as their association attenuates AP-1 activity (Bakiri et al., 2000; Chiu et al., 1989; Passegue and Wagner, 2000; Szabowski et al., 2000). c-Jun can be phosphorylated by c-Jun amino-terminal kinase (JNK). Phosphorylated c-Jun is not only more stable, but also shows higher transcriptional activity than unmodified protein (Hibi et al., 1993; Smeal et al., 1994; Treier et al., 1994).

Studies have shown that Jun and Fos members are differentially expressed in the epidermis. During mouse development, c-Jun and JunB are first detected in the epidermis at E17.5. While the expression pattern of c-Jun is ill defined, JunB is restricted to the suprabasal cells (Wilkinson et al., 1989). Among the Fos subfamily, c-Fos and Fra-2 are expressed in a number of tissues including the epidermis, while Fra-1 is detected at low levels with no tissue specificity (Carrasco and Bravo, 1995; Dony and Gruss, 1987). Detailed expression pattern of Fra-2 in the epidermis has been studied at E16.5, and is found specifically in the basal and granular layers (Carrasco and Bravo, 1995).

In the epidermis of newborn mice, a distinct expression pattern of the Jun and Fos proteins has been described. The basal layer expresses almost all of these proteins, including c-Jun, JunB, JunD, c-Fos, Fra-1 and Fra-2. c-Jun, JunD, and Fra-1 are also expressed in the spinous layer, while JunB and

Fra-2 are also detected in the granular layer (Carrasco and Bravo, 1995; Dony and Gruss, 1987; Rutberg et al., 2000). It is also worth noting that expression of the Jun and Fos members in the neonatal human epidermis is different from that in the newborn mouse epidermis in many respects (Eckert and Welter, 1996; Welter and Eckert, 1995)

Ablation of c-Jun causes early embryonic death (Hilberg et al., 1993; Johnson et al., 1993), while conditional knockout in the epidermis results in impaired eyelid development due to reduced EGFR expression. Despite this, these conditional knockout mice do not display any obvious skin defects, although wound-healing defects have been noted (Li et al., 2003; Zenz et al., 2003). These studies suggest that c-Jun is not essential for epidermal development, consistent with the fact that c-Jun starts to be expressed in the epidermis during late embryonic development (Wilkinson et al., 1989).

JunB null embryos die early owing to defects in the placenta and cannot be supported past E12.5 by tetraploid aggregation (Schorpp-Kistner et al., 1999). Therefore, whether JunB affects epidermal development remains unknown.

JunD null mice are viable, and males exhibit impaired growth and age-dependent reproduction defects. No skin phenotype has been noted (Carrasco and Bravo, 1995; Thepot et al., 2000).

c-Fos null mice are viable and fertile, and exhibit multiple defects including osteopetrosis. There are no skin defects associated with c-Fos

deficiency, although disruption of c-Fos inhibits malignant conversion of skin tumors (Johnson et al., 1992; Wang et al., 1992; Saez et al., 1995).

Mice deficient in FosB develop normally, but adult females show nurturing problems probably due to the malfunction of hypothalamus. No skin defects are found in the mutant mice (Brown et al., 1996; Gruda et al., 1996).

Fra-1$^{-/-}$ embryos, which die early because of the defects in the placenta and the yolk sac, can be rescued up to birth by tetraploid aggregation methods, and the rescued Fra-1$^{-/-}$ pups display no overt skin defects (Schreiber et al., 2000).

Many genes critical for epidermal homeostasis, such as TGs and many keratins, including K1, K5, K6 and K14, are AP-1 targets, suggesting that AP-1 likely plays important roles in the epidermis (Angel et al., 2001). However, single knockout of the Jun and Fos members (with the exception of JunB and Fra-2; ablation of JunB causes early death during embryonic development, while Fra-2 knockout is still unavailable) does not cause any overt skin defects. One explanation for this is that functional redundancy between AP-1 members may exist (Angel et al., 2001; Jochum et al., 2001).

6. Cutaneous squamous cell carcinoma

In general, cancer cells exhibit modifications in genome composition, ranging from subtle point mutations to dramatic gain or loss of genetic material (aneuploidy) (Hanahan and Weinberg, 2000; Pihan and Doxsey, 2003). As a result of genetic and epigenetic alterations, cancer cells display

many features distinct from normal cells such as growth factor independence, insensitivity to growth inhibitory and apoptogenic signals, limitless replicative potential, stimulation of angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000).

6.1 Human squamous cell carcinoma

Nonmelanoma skin cancers are the most common cancers among the Caucasian populations in North America, with over 1 million cases diagnosed each year in the United States. Roughly 80% of nonmelanoma skin cancers are basal cell carcinomas (BCCs) and about 20% are squamous cell carcinomas (SCCs) (Alam and Ratner, 2001). Skin BCCs are rarely fatal, although they can be locally invasive and tissue destructive. By contrast, SCCs are invasive and more than 10% will metastasize. In fact, more than 1,500 people die from SCCs every year in the US (Stratton et al., 2000).

Exposure to solar UV radiation is the most common cause of skin SCCs. It is believed that UV radiation directly causes gene mutations, especially in the p53 tumor suppressor gene, which favors clonal expansion of mutated cells (Marks, 1995).

6.2 Experimental models for SCC

Cancer development is a multistage and multifactorial phenomenon (Hanahan and Weinberg, 2000; Kinzler and Vogelstein, 1996). Mouse skin carcinogenesis can be induced by sequential application of chemical

carcinogens (Figure 1-5). When normal keratinocytes are induced into SCCs through such protocols, the phenotypic alterations are associated with reproducible genetic and epigenetic changes. This experimental model is, therefore, a convenient and powerful tool to study at least some of the biological processes associated with cancer development (Yuspa, 1994).

In the two-step chemical carcinogenesis protocol, a subthreshold dose of DNA mutagen, such as 7, 12-dimethylbenz[a]anthracene (DMBA), is applied directly onto the mouse skin, causing irreversible genetic changes in keratinocytes corresponding to tumor initiation. Subsequently, tumor promoters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), are repeatedly administered onto the DMBA-treated skin, providing a stimulus conducive for the clonal expansion of initiated cells. The function of tumor promoters is epigenetic, and, therefore, is reversible (Yuspa, 1994).

Tumor initiation and promotion give rise to squamous papillomas. Only a few of the papillomas will progress to malignancy, while most will regress with time. Premalignant progression is the major time-consuming phase of tumor pathogenesis and is, in general, a spontaneous process that is not influenced by most tumor promoters (Yuspa, 1994). The major genetic abnormalities associated with tumor progression are those related to generalized genomic instability, namely chromosomal imbalance and aneuploidy (Aldaz et al., 1988; Aldaz et al., 1989). Trisomies of chromosome 6 and 7 and aneuploidy are developed as diploid squamous papillomas progress toward malignancy (Aldaz et al., 1989). Malignant conversion is

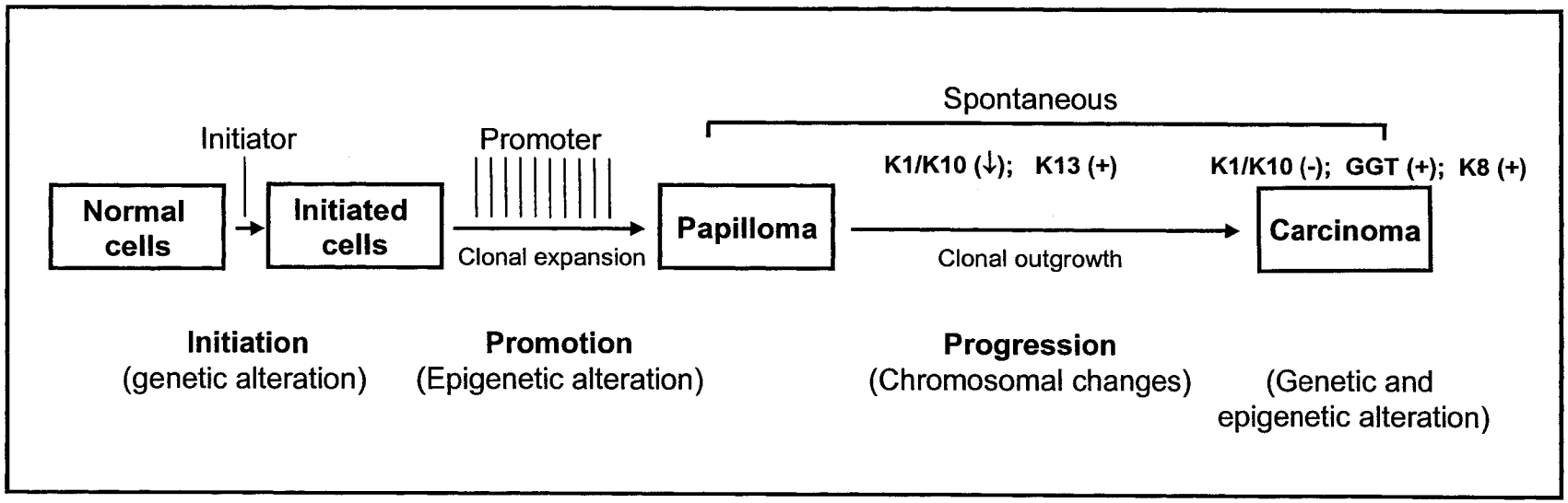


Figure 1-5. Diagrammatic representation of multistage mouse skin carcinogenesis (Modified from Yuspa, 1994).

accompanied by many further genetic and epigenetic alterations (Yuspa, 1994).

An oncogenic mutation in the Ha-ras gene is found in more than 90% of the epidermal tumors initiated by DMBA (Balmain et al., 1984). A causal role of oncogenic Ras for skin tumor initiation has been established by a number of studies (Yuspa, 1994; Slaga et al., 1996; Brown et al., 1990). Indeed, wild type keratinocytes, when infected with a retrovirus expressing v-Ha-Ras, form squamous papillomas after grafted onto nude mice that phenotypically resemble those induced using the DMBA/TPA protocol (Roop et al., 1986).

Additional evidence supporting a role of Ras activation in skin tumorigenesis comes from studies on transgenic mice expressing v-Ha-Ras. When the transgene is driven by the K10 promoter, mice appear with neonatal skin hyperplasia, spontaneously develop papillomas with time, and are very sensitive to tumor promotion (Greenhalgh et al., 1993). When the transgene is driven by a ζ-globin promoter, mice develop papillomas at areas of skin abrasion, and are also very sensitive to tumor promotion (Leder et al., 1990). Furthermore, transgenic mice expressing a dominant active form of Son of sevenless (SOS-F), a guanine nucleotide exchange factor that promotes Ras activation by facilitating GDP-GTP exchange, spontaneously develop squamous papillomas soon after birth (Sibilia et al., 2000).

In the chemical carcinogenesis model, the mutated Ha-ras gene is heterozygous in papillomas, but frequently homozygous in carcinomas

(Bianchi et al., 1990; Quintanilla et al., 1986). In fact, exogenous oncogenic Ha-ras can cause malignant conversion of papilloma cells with a heterozygous Ha-ras mutation, suggesting that Ha-ras gene dosage is important in determining the neoplastic phenotype (Greenhalgh et al., 1989; Harper et al., 1986).

Oncogenic Ras causes enhanced expression of EGFR ligands including TGFα (transforming growth factor α), amphiregulin, β-cellulin and HB-EGF (heparin-binding EGF-like growth factor) (Dlugosz et al., 1995). Overexpression of TGFα by transgenic targeting to mouse epidermis followed by topical promotion with TPA is also sufficient to induce skin papillomas (Dominey et al., 1993; Vassar et al., 1992).

Upregulation of EGFR is frequently detected in skin papillomas and SCCs (Yuspa, 1994). Consistently, when grafted onto nude mice, v-Ha-ras-infected $EGFR^{-/-}$ keratinocytes form squamous papillomas with significantly reduced size (Dlugosz et al., 1997). Ablation of EGFR also impairs skin tumorigenesis in SOS-F transgenic mice (Sibilia et al., 2000). These observations suggest an important role for this receptor in tumor growth.

Upregulation of phosphorylated c-Jun, Fra-1, Fra-2, and ATF-2 and downregulation of JunB correlates with epidermal malignancy, suggesting an important role for AP-1 in skin tumorigenesis (Joseloff and Bowden, 1997; Zoumpourlis et al., 2000). Among the AP-1 members, c-Jun is essential for tumor promotion and progression. Expression of a dominant negative c-Jun in a malignant keratinocyte cell line blocks tumor formation when the transfected

cells are grafted onto nude mice (Bowden et al., 1994). Moreover, targeted expression of a dominant negative c-Jun (TAM67) in the epidermis inhibits tumorigenesis after DMBA/TPA treatment (Young et al., 1999). Consistent with these findings, disruption of c-Jun in the epidermis attenuates the ability of SOS-F to induce benign epidermal lesions (Zenz et al., 2003). Interestingly, ablation of Jnk-2 (c-Jun N-terminal kinase-2) also blocks skin tumorigenesis. By contrast, $Jnk1^{-/-}$ offspring show enhanced sensitivity to DMBA/TPA tumor induction. These outcomes correlate with reduced activation of extracellular signal-regulated kinase (ERK) and AP-1 in $Jnk\text{-}2^{-/-}$ mice, and increased activity in $Jnk\text{-}1^{-/-}$ mice (She et al., 2002). Activation of ERKs is important for skin tumorigenesis, since expression of a dominant negative ERK2 inhibits AP-1 transactivation and neoplastic transformation (Watts et al., 1998).

Although disruption and interference with c-Jun function apparently inhibits skin tumorigenesis, deletion of c-fos is not sufficient. c-fos is, however, required for malignant conversion of squamous papillomas as ablation of c-fos blocks the formation of SCCs (Saez et al., 1995). In addition, malignant conversion of a benign skin tumor cell line can be achieved by overexpression of c-Fos (Greenhalgh and Yuspa, 1988).

About 5% of squamous papillomas will spontaneously convert to SCCs. The risk factor of malignant conversion among squamous papillomas is different. A subset of them appear early, grow large, do not regress when promotion is withdrawn, and are at high risk to evolve into SCCs (Yuspa, 1994; Yuspa, 1998). These high risk papillomas lose K1/K10 expression and

gain K13 expression (Warren et al., 1993). Loss of K1 and K10 expression is also associated with malignant SCCs, and has been considered a hallmark for malignant conversion (Yuspa, 1994; Yuspa, 1998).

Ectopic expression of K10 in the basal keratinocytes compromises cell proliferation and inhibits skin tumorigenesis in mice, an effect that may be mediated by inhibition of Akt activation by K10 (Santos et al., 2002). In agreement with this, elevated Akt activity has been found in chemically induced skin tumors at every stage, and overexpression of Akt accelerates tumor formation (Segrelles et al., 2002).

Other changes in gene expression are also linked to epidermal tumor promotion. For example, $\alpha 6\beta 4$ integrin expression is extended into suprabasal keratinocytes in both the high risk papillomas and skin SCCs, while expression is only seen at the basal surface of the basal keratinocytes in both normal skin and low risk papillomas (Tennenbaum et al., 1993).

Loss of TGFβ1 and TGFβ2 expression is another distinct feature associated with high risk papillomas and SCCs (Glick et al., 1993). TGFβ shows biphasic action during multistage skin carcinogenesis, acting early to inhibit tumor progression but later to promote malignancy (Wang, 2001).

Mutations in the p53 gene are rare in chemically induced papillomas. By contrast, p53 mutation and loss of heterozygosity (LOH) at the p53 locus are frequently detected in SCCs (Ruggeri et al., 1991). Consistent with this, p53 null mice develop SCCs very rapidly after DMBA/TPA treatment,

suggesting that loss of p53 promotes malignant progression (Kemp et al., 1993).

γ-glutamyl transpeptidase (GTT), an enzyme catalyzing the transfer of glutamyl groups between peptides, and K8, a simple epithelial keratin, are not detected in squamous papillomas, but are expressed in malignant SCCs. In particular, K8 expression is associated with advanced SCCs (Aldaz et al., 1988; Diaz-Guerra et al., 1992; Larcher et al., 1992).

As reviewed above, mouse skin carcinogenesis models not only establish a link between genetic pathways and stages of tumor evolution but also are tractable model systems to study the roles of genes in the tumorigenic process. Using these experimental models, many other genes have been found to be involved in skin tumorigenesis. For instance, both human SCC and experimental SCC express high level of cyclin D1 and have elevated CDK4 activity (Rodriguez-Puebla et al., 1999). Inactivation of cyclin D1 attenuates oncogenic Ras function, and compromises skin tumor formation (Robles et al., 1998). Furthermore, overexpression of cyclin D1 in the epidermis causes enhanced skin carcinogenesis in mice (Yamamoto et al., 2002). Consistent with these data, disruption of CDK4 inhibits chemically induced skin tumors, and human keratinocytes co-transfected with CDK4 and Ras develop SCCs very rapidly in nude mice (Lazarov et al., 2002; Rodriguez-Puebla et al., 2002).

CDK activity is also regulated by CDK inhibitors, such as $p21^{cip1}$ and $p27^{kip1}$. Inactivation of $p21^{cip1}$ does not affect either papilloma formation or

malignant conversion in the DMBA/TPA induced carcinogenesis model. Instead, $p21^{cip1}$ deficiency enhances the yields of undifferentiated SCC, an advanced form of epidermal neoplastic lesion, suggesting a role of $p21^{cip1}$ in tumor differentiation (Philipp et al., 1999; Topley et al., 1999). Ablation of $p27^{kip1}$ causes enhanced tumor growth and a slight increase in malignant conversion, indicating that $p27^{kip1}$ may be involved in regulating clonal expansion of the initiated cells during tumor promotion (Philipp et al., 1999).

Section 2. Retinoid signaling

The biological importance of vitamin A (retinol) has long been known, as vitamin A deprivation or excess vitamin A can cause severe developmental and post-partum defects (Chambon, 1996; Ross et al., 2000).

Vitamin A and its derivatives are collectively called retinoids. Retinoic acid (RA), a carboxylic acid derivative of vitamin A, is the major biologically active retinoid, and its functions are mediated by two groups of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (Chambon, 1996).

1. Absorption, metabolism and transportation of vitamin A

The body acquires vitamin A from the diet either as preformed vitamin A or as carotenoids (Figure 1-6a) (Gottesman et al., 2001; Marill et al., 2003). Preformed dietary vitamin A is usually from animal-derived food products, and consists primarily of retinyl esters that are hydrolyzed to retinol in the small intestine. β-carotene is the most common previtamin A, which is usually derived from leafy green vegetables. Dietary β-carotene is converted into retinol in the intestine via a two-step catalytic process, with oxidative cleavage of one molecule of β-carotene generating two molecules of retinaldehyde, which are subsequently reduced to retinol by aldehyde reductases. Retinol is then taken up by the intestinal mucosa and esterified by lecithin:retinol acyltransferase (LRAT) (Gottesman et al., 2001; Marill et al., 2003).

The newly formed retinyl esters are subsequently packaged into chylomicrons, which enter into and travel along the lymphatic system. About 75% of chylomicron retinyl ester are taken up by hepatocytes, and either stored in stellate cells (lipocytes) or hydrolyzed to retinol, which is then secreted into circulation bound to retinol-binding protein (RBP) (Gottesman et al., 2001; Marill et al., 2003).

Most tissues obtain vitamin A primarily from RBP-bound retinol, the predominant form of vitamin A in fasting blood (Gottesman et al., 2001; Marill et al., 2003). Although RBP null mice show reduced levels of plasma retinol and neonatal animals display impaired retinal function, the mutant mice are viable and fertile, suggesting that RBP is not vital for most vitamin A-dependent functions (Quadro et al., 1999).

Once inside the cells, retinol associates with the cellular retinol-binding proteins (CRBPI, -II, and III) (Gottesman et al., 2001; Napoli, 1999; Ong, 1994; Vogel et al., 2001; Conforti et al., 2000). In mouse, CRBPI is expressed in multiple tissues of embryos and adults, being particularly abundant in the liver (Ong and Chytil, 1978; Ong, 1984). By contrast, CRBPII is detected mainly in the intestine of adults, although it is also expressed transiently in several other tissues including the liver (Crow and Ong, 1985; E X et al., 2002; Hind et al., 2002; Levin et al., 1987). CRBPIII, a newly identified member of this family, is expressed in muscle and adipose tissues (Conforti et al., 2000; Vogel et al., 2001).

Mice deficient for CRBP I or CRBP II grow and reproduce normally, although the hepatic stores of retinyl esters are reduced, suggesting that they play roles in retinoid homeostasis, but are largely dispensable (Conforti et al., 2000; Ghyselinck et al., 1999; Conforti et al., 2000; E X et al., 2002).

RA binds to another class of retinoid binding proteins, the cellular retinoic acid binding proteins (CRABP I and –II) (Donovan et al., 1995; Ross et al., 2000). During embryogenesis, both proteins are widely expressed. In the adults, CRABP I is nearly ubiquitously detectable, while CRABP II is restricted to several tissues including the skin, uterus, ovary and brain (Dolle et al., 1990; Fiorella et al., 1993; Perez-Castro et al., 1989; Ruberte et al., 1991).

CRABPs are thought to enhance the solubility of RA in the aqueous cytosol and facilitate RA trafficking between different cellular compartments (Dolle et al., 1990; Fiorella et al., 1993; Perez-Castro et al., 1989; Ruberte et al., 1991). CRABP I may also promote RA degradation by presenting RA to microsomal enzymes such as Cyp26 (Fiorella and Napoli, 1991; Fujii et al., 1997; White et al., 1996). CRABP II interacts with nuclear retinoid receptors (RAR and RXR, see below) in a ligand-dependent manner, suggesting that this protein may act as a chaperone for RA by transporting it from the cytosol to the nucleus and presentation to RXR/RAR dimers (Delva et al., 1999).

CRABP I null mice are completely normal, while CRABP II null and CRABP I/CRABP II double null animals only display a mild limb phenotype,

suggesting that both proteins are not essential for either embryogenesis or adult life (Fawcett et al., 1995; Gorry et al., 1994; Lampron et al., 1995).

2. Biosynthesis and catabolism of RA

A two-step oxidation process is involved in the generation of RA from retinol (Figure 1-6a). The first step, oxidation from retinol to all-trans retinaldehyde is a reversible reaction and can be catalyzed by various alcohol dehydrogenases (ADHs) or short-chain dehydrogenases (SDRs). The next step, conversion from all-trans retinaldehyde to all-trans RA, is an irreversible reaction, mediated by retinaldehyde dehydrogenases (RALDHs). All-trans RA can give rise to 9-cis RA and 13-cis RA either by spontaneous isomerization or, potentially, by uncharacterized isomerases (Gottesman et al., 2001; Marill et al., 2003).

Embryonic development and adult health require a balance between synthesis and degradation of RA to maintain proper concentrations. RA catabolism is catalyzed by cytochrome P450 enzymes, Cyp26A1, -B1 and -C1. These enzymes can oxidize RA to more polar compounds, such as 4-hydroxy RA and 4-oxo RA, as well as other hydroxylated derivatives (Gottesman et al., 2001; Marill et al., 2003). Although a number of studies suggest that these molecules may have some biological effects, recent genetic studies on Cyp26A1 and RALDH2 compound knockout mice argue against any biological significance (Niederreither et al., 2002; Perlmann, 2002).

3. Retinoid receptors

The RA signal is mediated by the RARs and the RXRs, which are members of the nuclear receptor superfamily (Chambon, 1996; Mangelsdorf et al., 1995). RA binds to RARs, while 9-cis RA binds to both RARs and RXRs (Giguere et al., 1987; Levin et al., 1992; Petkovich et al., 1987; Mangelsdorf et al., 1992). Although RA has been revealed to be involved in regulating many biological processes, the biological significance of 9-cis RA is unknown, and it cannot be detected in all species (Altucci and Gronemeyer, 2001; Chambon, 1996; Mangelsdorf et al., 1995; Ross et al., 2000).

3.1 The nuclear receptor superfamily

Nuclear receptors are ligand-activated transcription factors, which can be divided into three groups based on their ligand-binding, DNA-binding and dimerization properties (McKenna et al., 1999). Group I receptors, the classic steroid hormone receptors, include receptors for glucocorticoids (GR), estrogens (ER), progestins (PR), androgens (AR), and mineralocorticoids (MR). These receptors bind to palindromic DNA sequences as homodimers in a ligand-dependent manner. Group II receptors consist of retinoid receptors (RARs and RXRs), vitamin D receptors (VDR), and thyroid hormone receptors (TR), among others. Receptors in this group form heterodimers with RXRs and bind to direct DNA repeats, repressing transcription in the absence of ligands and activating transcription upon ligand-binding. Group III

receptors, or "orphan receptors", are nuclear receptors, of which the ligands are still unknown.

Nuclear receptors consist of modular domains (Figure 1-6b) (Aranda and Pascual, 2001). The N-terminal A/B domain is the least conserved among receptors and contains an autonomous activation function termed activation function-1 (AF-1). The C domain, spanning roughly 60-70 amino acid residues, is the most highly conserved domain and confers DNA-binding. In this region, 8 conserved cysteines tetrahedrally coordinate two zinc ions, forming two zinc-finger motifs that bind to DNA. The P-box at the base of the first zinc finger is responsible for DNA sequence-specific binding, while the D-box at the base of the second zinc-finger is involved in receptor dimerization. The D domain, also called the hinge region, connects C and E regions and contains a nuclear localization signal. This region also participates in interaction with certain nuclear receptor co-regulators. The E region encompasses the ligand-binding domain (LBD), which is multifunctional and responsible for ligand-binding, receptor dimerization, and interaction with other proteins. This region also harbors a ligand-dependent transactivation function termed AF-2.

3.2 RARs and RXRs

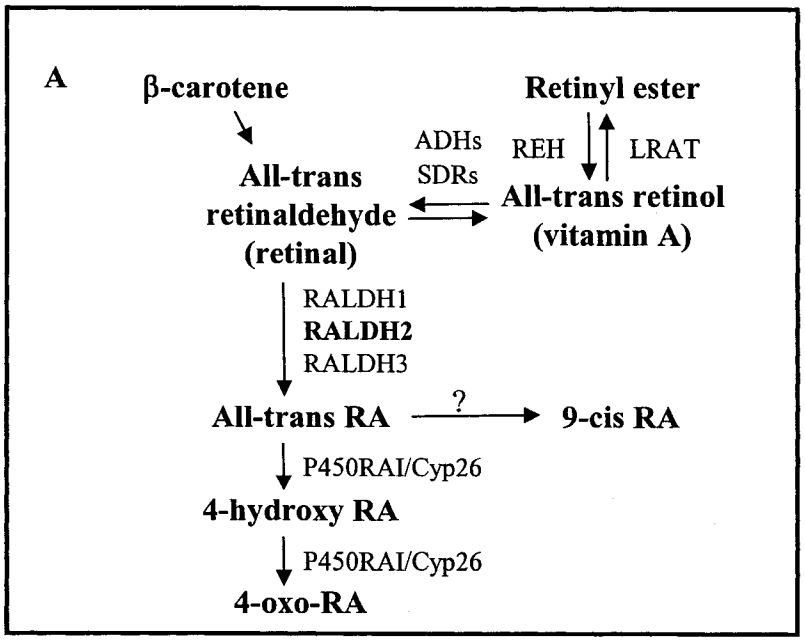
Both RARs and RXRs are expressed as α, β and γ subtypes that are encoded by different genes. In addition, each subtype has several isoforms as a result of differential promoter usage and alternative splicing. There are

two major isoforms for RARα (α1 and α2) and RARγ (γ1 and γ2), and four for RARβ2 (β1-β4). Several isoforms have also been identified for RXRα (α1 and α2), RXRβ (β1 and β2), and RXRγ (γ1 and γ2). At the protein level, all isoforms differ only with the respect to the N-terminal A domain (Mangelsdorf et al., 1995; Chambon, 1996).

RXR/RAR heterodimers can bind to specific DNA sequences referred to as RA response elements (RAREs), which are characterized by direct repeats of (A/G)GGTCA, in the promoter/enhancer region of the target genes (Chambon, 1996; Mangelsdorf et al., 1995). The spacing between the repeats of two half RARE consensus sequences is variable, but a separation by five (DR5) or two (DR2) nucleotides seems to be optimal for RXR/RAR binding (Mader et al., 1993). Non-consensus, complex RAREs, including palindromic and everted repeats, have also been described, and the sequences flanking the RAREs can also influence receptor binding (Chambon, 1996; Giguere, 1994; Ross et al., 2000).

When bound to DR2 or DR5 RAREs, RXR binds to the 5' half site while RAR occupies the 3' half site (Kurokawa et al., 1993; Perlmann et al., 1993; Zechel et al., 1994). As an exception, the heterodimers can also bind to atypical DR1-RAREs in the opposite orientation (Kurokawa et al., 1995; Mangelsdorf et al., 1991).

Figure 1-6. Mechanisms of RAR-mediated transcription. A) Schematic diagram of vitamin A metabolism (Modified from Perlmann, 2002). ADH, alcohol dehydrogenase; LRAT, lethicin:retinol acyltransferase; RALDH, retinaldehyde dehydrogenase; REH, retinyl easter hydrolase; SDR, short-chain dehydrogenase/reductase. B) Structural organization of the nuclear receptors. AF-1, activation function-1; AF-2, activation function-2; DBD, DNA-binding domain; LBD, ligand-binding domain. C) RAR-mediated transcriptional repression and activation (Adopted from Altucci and Gronemeyer, 2001). Apo-LBD, unliganded-LBD; CoR, transcription coactivator; DRIP, vitamin D receptor-interacting protein; HAT, histone acetyltransferase; HDAC, histone deacetylase; Holo-LBD, liganded-LBD; RARE, RA response element; SMCC, Srb and mediator protein-containing complex; TRAP, thyroid hormone receptor-associated protein.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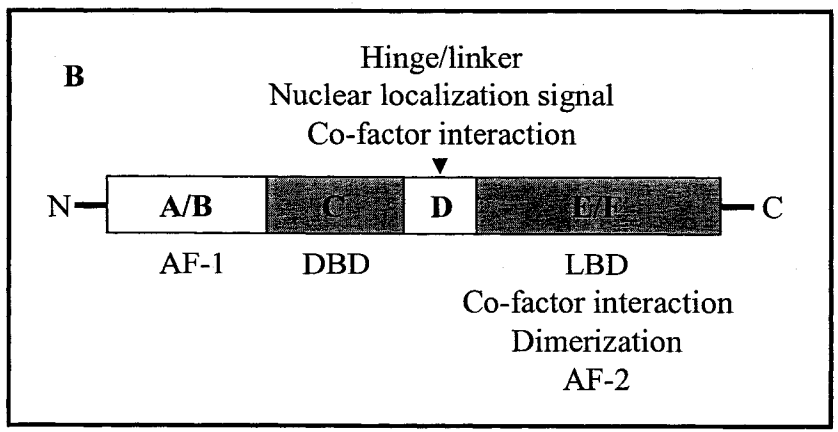
β-carotene
Retinyl ester
ADHs
SDRs
REH
LRAT
All-trans retinaldehyde (retinal)
All-trans retinol (vitamin A)
RALDH1
RALDH2
RALDH3
All-trans RA
?
9-cis RA
P450RAI/Cyp26
4-hydroxy RA
P450RAI/Cyp26
4-oxo-RA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Hinge/linker
Nuclear localization signal
Co-factor interaction
N
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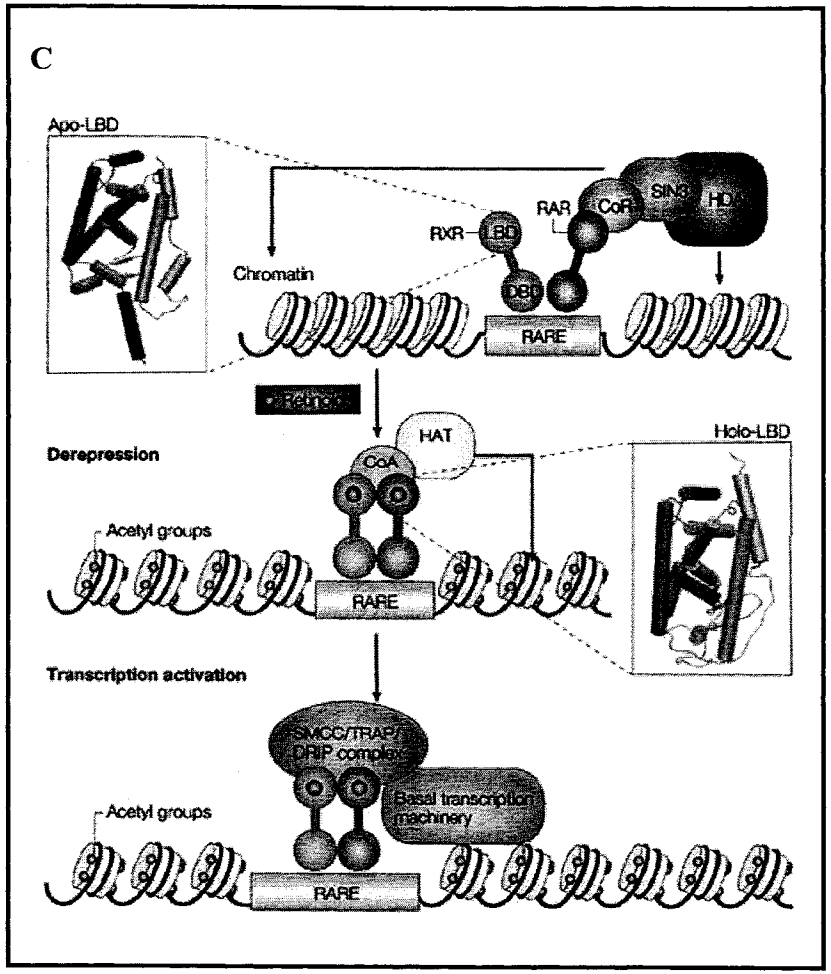
E/F
C
AF-1
DBD
LBD
Co-factor interaction
Dimerization
AF-2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Apo-LBD
Chromatin
RXR
LBD
DBD
RAR
CoR
SIN3
HD
RARE
Retinoic
HAT
Holo-LBD
Derepression
CoA
Acetyl groups
RARE
Transcription activation
SMCC/TRAP/
DRIP complex
Basal transcription machinery
Acetyl groups
RARE

3.3 Retinoid-mediated gene expression

RARs form heterodimers with RXRs as DNA-binding factors, acting to either repress or activate gene expression in a ligand-dependent manner (Figure 1-6c) (Altucci and Gronemeyer, 2001; Aranda and Pascual, 2001; Rosenfeld and Glass, 2001; Wei, 2003). In the absence of ligands, RXR/RAR heterodimers interact with transcription corepressors (CoRs), such as the nuclear receptor corepressor (NcoR) and the silencing mediator for retinoid and thyroid hormone receptors (SMRT) (Aranda and Pascual, 2001; Chen and Evans, 1995; Horlein et al., 1995).

NcoR and SMRT are related proteins, sharing similarities in terms of their primary sequence, domain structure and function. Both proteins contain multiple repression domains (RDs) and nuclear receptor-interacting domains (RIDs) (Burke and Baniahmad, 2000; Hu and Lazar, 2000; Ordentlich et al., 2001; Wei, 2003). RIDs are located at the C-terminus of both NcoR and SMRT. A conserved motif, known as the CoRNR box of consensus LXXXIXXXI/L (where L is leucine, I isoleucine, and X any amino acid), forms an extended α-helix and binds to a hydrophobic groove formed by helices 3, -4, -5, and -6 of the unliganded LBDs of nuclear receptors (Perissi et al., 1999; Zhu et al., 1999). Consistent with this, mutations in the LBDs which change the affinity between nuclear receptors and corepressors often impact on receptor-dependent transcription (Kao et al., 2003).

Through their amino terminal RD1, NcoR/SMRT directly interact with the corepressor Sin3, which in turn recruits histone deacetylases (HDACs),

resulting in the formation of a repressive complex (Alland et al., 1997; Heinzel et al., 1997; Nagy et al., 1997). Recently, NcoR and SMRT have been found to interact with class II HDACs directly, suggesting that a Sin3-independent repression mechanism may also exist in terms of NcoR/SMRT-HDAC association (Huang et al., 2000).

DNA is packaged into chromatin, of which the basic unit is the nucleosome. A nucleosome is composed of an octamer of four core histones (H), i.e. an H3/H4 tetramer and two H2A/H2B dimers, surrounded by 146 bp of DNA (Strahl and Allis, 2000). Chromatin architecture has been recognized as an important factor in regulating gene expression, and histone acetylation is an important post-transcriptional modification event which is known to influence chromatin structures (Narlikar et al., 2002; Strahl and Allis, 2000). Acetylation of lysine residues in the N-terminal histone tails is associated with all core histones *in vivo*. However, H3/H4 modification is much more extensively characterized than that of H2A/H2B. Steady-state levels of histone acetylation are dependent on the balance between the opposing activities of histone acetyltransferses (HATs) and HDACs. In general, histone hyperacetylation correlates with increased transcriptional activity, while hypoacetylation is associated with repression (Narlikar et al., 2002; Strahl and Allis, 2000; Wade, 2001).

Transcription repression by the corepressor complex is thought to be mediated by HDACs. HDACs remove acetyl groups from nucleosomal histones, causing local condensation of chromatin. The resultant compact

chromatin is relatively inaccessible to the basal transcription machinery, resulting in reduced transcription efficiency (Aranda and Pascual, 2001; Rosenfeld and Glass, 2001).

HDACs identified to date fall into three main classes based on their homology to yeast HDACs (de Ruijter et al., 2003; Khochbin et al., 2001; Narlikar et al., 2002). Class I HDACs (HDAC 1, -2, -3, -8) are closely related to the yeast RPD3 protein. Class II HDACs (HDAC 4, -5, -6, -7, -9, and -10) are homologues to yeast deacetylase HDA1. Class III HDACs (sirtuins, SIRT1-7) are analogous to another yeast protein, SIR2.

Ligand-binding to RARs induces a major shift of helix 12, which contains the AF-2 core, to cover the hydrophobic binding pocket for CoRs, which is formed by helices 3, -4, and –5, and mediates their release (Wurtz et al., 1996). The conformational change also exposes a charged interface on helix 12 running along the length of the hydrophobic groove, which interacts strongly with the RID motifs of transcription coactivators (CoAs) (Aranda and Pascual, 2001; Rosenfeld and Glass, 2001).

Several types of CoAs, such as CREB-binding protein (CBP)/p300, the p160 family of CoAs, and p300/CBP-associated factors (p/CAF), are able to interact with RXR/RAR heterodimers in such a ligand-dependent manner. A common feature shared by the CBP/p300 and p160 CoAs is the presence of a LXXLL motif, which is often repeated in the RID domains of these CoAs, and which interacts directly with helix 12 of nuclear receptors (Aranda and Pascual, 2001; Rosenfeld and Glass, 2001; Wei, 2003).

CBP and p300 are highly related proteins (Chan and La Thangue, 2001; Goodman and Smolik, 2000; Chakravarti et al., 1996; Kamei et al., 1996). The N-terminus of these factors host a nuclear receptor interacting domain (Chan and La Thangue, 2001; Goodman and Smolik, 2000). CBP/p300 specifically associates with the LBD of the RARs in a ligand-dependent manner, and such an interaction results in an elevated activation of RAREs in reporter assays (Chakravarti et al., 1996; Kamei et al., 1996). The role of CBP/p300 in RAR-mediated gene expression is further highlighted by the fact that exogenous CBP enhances transcription mediated by liganded-RARs, and in $p300^{-/-}$ embryonic fibroblasts such a function of RARs is compromised (Dilworth et al., 1999; Rosenfeld and Glass, 2001).

The central region of CBP and p300 possesses intrinsic HAT activity (Chan and La Thangue, 2001; Goodman and Smolik, 2000; Bannister and Kouzarides, 1996; Ogryzko et al., 1996). It has been proposed that CBP/p300 confers transcription activation by RARs *via* their HAT activities (Glass and Rosenfeld, 2000). In support of this hypothesis, exogenous CBP enhances liganded-RAR-mediated gene expression specifically from a chromatinized reporter construct (Dilworth et al., 1999). Furthermore, RA treatment causes a rapid but transient increase in histone acetylation, which is associated with the activation of RA-target genes (Chen et al., 1999b).

It is also worth noting that CBP and p300 acetylate substrates other than histones (Chan and La Thangue, 2001; Goodman and Smolik, 2000).

For instance, CBP/p300 acetylates p53, resulting in an increased affinity of p53 for DNA, potentiating its transcriptional activity (Gu et al., 1999).

In addition to the association with RARs, CBP/p300 also interact with a variety of other transcription factors and with components of the basal transcriptional machinery, including TATA-binding proteins (TBPs), TFIIB, TFIIE, and TFIIF. It has also been proposed that CBP/p300 may also function as a bridge between a transcriptional factor and the basal transcriptional machinery. Such an adaptor function may facilitate the assembly of the basal transcriptional machinery, thus promoting gene expression (Chan and La Thangue, 2001; Goodman and Smolik, 2000).

The p160 family of CoRs consists of three members including SRC-1/NcoA1 (steroid receptor coactivator-1/nuclear receptor coactivator 1), TIF2/GRIP1/NcoA2 (transcriptional intermediary factor 2/glucocorticoid receptor-interacting protein 1/nuclear receptor coactivator 2) and p/CIP/AIB1/ACTR/TRAM-1 (p300/CBP-cointegrator-associated protein/ amplified in breast cancer 1 /activator for thyroid hormone and retinoid receptors/receptor-associated coactivator/thyroid hormone receptor-activator molecule 1) (Aranda and Pascual, 2001; Rosenfeld and Glass, 2001).

Besides their RID domains, the p160 proteins also possess conserved motifs necessary for interaction with CBP/p300 (Koh et al., 2001; Chen et al., 1999a). It has been suggested that the p160 family acts as coactivators, at least in part, by serving as adapter molecules to recruit CBP/p300 complexes

to nuclear receptors in a ligand-dependent manner (Kurokawa et al., 1998; Torchia et al., 1997).

The carboxyl terminus of some p160 members, such as SRC-1 and ACTR, is also reported to have intrinsic HAT activity, which is, however, much weaker than the HAT activity of CBP/p300 or p/CAF (see below), and the carboxyl terminus of SRC-1 and ACTR does not appear to contain features corresponding to the acetyl CoA binding site of p/CAF, or yeast GCN5 HAT domains (Clements et al., 1999; Trievel et al., 1999; Chen et al., 1997; Spencer et al., 1997).

p300/CBP-associated factor (p/CAF), the mammalian homolog of yeast Gcn5, is another coactivator with HAT activity (Yang et al., 1996). Like CBP/p300 and the p160 proteins, p/CAF also interacts with the LBD of RARs in a ligand-dependent manner, and is also found in a complex consisting of multiple proteins including TBP-associated factors (TAFs) and some of the TFIID components (Blanco et al., 1998; Chen et al., 1997; Korzus et al., 1998).

Some other coactivators without HAT activity, such as the TRAP/DRIP (thyroid hormone receptor-associated protein/vitamin D receptor-interacting protein) complex, have also been identified (Aranda and Pascual, 2001; Rosenfeld and Glass, 2001; Wei, 2003). The TRAP/DRIP complex is composed of multiple subunits, and interacts with nuclear receptors primarily through a single component, TRAP220/DRIP205, in a ligand-dependent manner (Fondell et al., 1996a; Fondell et al., 1996b; Rachez et al., 1998;

Yuan et al., 1998). The TRAP220/DRIP205 component contains two alternatively utilized LXXLL nuclear receptor interaction domains (Lee et al., 1995; Zhu et al., 1997).

Although the TRAP/DRIP complex lacks intrinsic HAT activity, it potentiates transcription from chromatinized templates (Rachez et al., 1999). The molecular basis underlying this observation remains unknown. Since some components of this complex are also found in other activator complexes, such as the yeast Mediator complex (Gu et al., 1999), which interacts with RNA polymerase II, it is thought that the TRAP/DRIP complex may act to enhance RNA polymerase II recruitment to the nuclear receptor-occupied promoter (Glass and Rosenfeld, 2000; Rosenfeld and Glass, 2001; Rachez et al., 1999).

3.4 Interference with AP-1 activity by RARs

Transrepression of gene expression by RARs can also be achieved through interference with a number of signaling pathways, including AP-1 (Altucci and Gronemeyer, 2001; Chambon, 1996).

The precise mechanism of transrepression of AP-1 by RA remains poorly understood, although several models have been suggested. First, liganded-RARs have been reported to be able to directly associate with c-Jun and c-Fos and thereby titrate them from *cis*-regulatory DNA sequences (Pfahl, 1993). Second, liganded-RARs may compete with AP-1 for limiting coactivators, such as CBP/p300 (Kamei et al., 1996; Lee et al., 1998b). Third,

RA can inhibit JNK activity by enhancing the activity of MAPK phosphatase 1 (MKP-1) (Caelles et al., 1997; Lee et al., 1998a). Finally, it has also been reported that RA inhibits AP-1 through promoting the association of AP-1 with SMRT (Suzukawa and Colburn, 2002).

4. RA and epidermal development and homeostasis

A critical role for vitamin A in epithelial homeostasis has long been known. Vitamin A deficiency (VAD) causes squamous metaplasia of diverse mucosal epithelia in animals, and cutaneous hyperkeratosis in human (Frazier and Hu, 1931; Goodwin, 1934; Wolbach and Howe, 1925). Consistent with these findings, when cultured with vitamin A, the epidermis loses its keratinized phenotype and elicits characteristics typical of mucosal epithelia (Barnett and Szabo, 1973; Fell and Mellanby, 1953; Yuspa and Harris, 1974). In contrast to these observations, a hyperkeratotic skin phenotype that exhibits hyperproliferation of the basal keratinocytes and thickened suprabasal epidermal layers can be induced by topical application of vitamin A (retinol) or RA (Kang et al., 1995; Fisher and Voorhees, 1996). These seemingly contradictory outcomes may be caused by a difference in vitamin A/RA concentrations in the epidermis between these studies. This hypothesis is supported by an organ-culture experiment showing that optimum epidermal morphology is achieved only with nanomolar concentrations of extracellular RA. RA concentrations above or below this range causes abnormal keratinocyte differentiation (Asselineau et al., 1989).

As reviewed above, RA is the major biologically active form of vitamin A. RA concentration in the epidermis is in the nanomolar range, near that of plasma, which ranges between 4nM-14nM (De Leenheer et al., 1982; Eckhoff and Nau, 1990). About 70% of RA in the epidermis exists as 13-cis RA and about 30% as all-trans RA, while 9-cis RA is undetectable (Duell et al., 1996).

The epidermis expresses RARα, RARγ, RXRα and RXRβ, with RARγ and RXRα being the predominant receptor types (Darwiche et al., 1995; Fisher and Voorhees, 1996). Therefore, these receptors likely play an important role in regulating epidermal development and homeostasis. In agreement with such a notion, targeted expression of a dominant negative (dn) RARα (Saitou et al., 1994) in the epidermis using the K14 promoter results in a severe skin phenotype. Transgenic newborn skin appears thin, shiny, and without wrinkles. Histologically, the suprabasal layers of the epidermis are significantly reduced, suggesting a blockage of differentiation (Saitou et al., 1995). *In vitro*, interference of RAR function by dnRARs also inhibits keratinocyte differentiation. In particular, in organotypic cultures, keratinocytes transfected with a dnRARγ fail to form suprabasal layers (Aneskievich and Fuchs, 1995), consistent with the transgenic data (Saitou et al., 1995).

In contrast to the findings using dnRARs, ablation of both RARα and RARγ, the total RAR subtypes expressed in the epidermis, does not profoundly affect skin development. Although some minor histological alterations in the granular epidermis have been observed, growth and

differentiation appears essentially unchanged. This suggests that RARs may not play an essential role in epidermal development (Chapellier et al., 2002; Ghyselinck et al., 2002; Lohnes et al., 1994).

Although several mechanisms have been proposed to explain the discrepancy between the outcome of ablation of RARαγ and the effects of dnRARα on skin development (Andersen and Rosenfeld, 1995), the basis underlying these differences still remains unsolved. RXRs act as heterodimeric partners for other nuclear receptors expressed in the epidermis such as VDR, TR and peroxisome proliferator activated receptors (PPAR), and dnRAR has been suggested to affect signaling mediated by some of these nuclear receptors through sequestering RXRs. For instance, expression of a dnRAR in the suprabasal epidermis using the K10 promoter abolishes the lipid multilamellar structures of the stratum corneum, thereby compromising the barrier functions of the skin. This effect is thought to be caused by abnormal PPAR signaling as a result of overexpression of this dnRARα (Attar et al., 1997; Imakado et al., 1995). In support of this, an organ-culture model shows that activation of PPARs is able to accelerate the development of the fetal epidermal permeability barrier in rats (Hanley et al., 1997). Compelling evidence also suggests that PPAR agonists can promote keratinocyte differentiation *in vitro* (Kuenzli and Saurat, 2003).

Targeted disruption of RXRα, the prevalent RXR subtype expressed in the epidermis, causes early embryonic death (Kastner et al., 1994; Sucov et al., 1994). Conditional deletion of RXRα in the epidermis does not appear to

affect epidermal development, although mutants transiently exhibit a shiny skin at birth. However, with time the epidermis appears scaly and exhibits hyperproliferation and abnormal differentiation possibly due to an inflammatory response. The most significant effect of RXRα loss, however, is on hair follicles. There is a delay in hair formation in newborns, and with time these mice eventually develop alopecia due to hair follicle degeneration and cysts of hair follicle origin (Li et al., 2000; Li et al., 2001b). Since similar hair follicle abnormalities are also seen in VDR null mice, RXRα may affect hair follicles through RXR/VDR signaling (Li et al., 2000; Li et al., 2001b; Li et al., 1997; Li et al., 1998b; Yoshizawa et al., 1997).

Ablation of RXRα and RXRβ, the total RXRs expressed in the epidermis, results in a skin phenotype similar to the RXRα conditional null mice, although the phenotype is slightly more severe (Li et al., 2000).

5. RA, RARs and epidermal tumorigenesis

Many experimental, epidemiological and clinical observations suggest that retinoids are promising agents for cancer prevention or treatment (Altucci and Gronemeyer, 2001). The first evidence suggesting that vitamin A may have a tumor suppressive effect comes from the observations made by Wolbach and Howe (1925) who found that vitamin A deficient rats develop squamous metaplasia of various mucosal epithelial tissues reminiscent of some precancerous lesions in human. Subsequently, similar observations have been made in other animal models (Moon et al., 1994).

In vitro, retinoids can inhibit cell growth and/or induce apoptosis of many human cancer cell types, suggesting a broad preventive or therapeutic promise for retinoids against human malignancies (Altucci and Gronemeyer, 2001). Epidemiological studies also support an inverse relationship between the incidence of numerous cancers and serum retinoid levels (Hong and Itri, 1994). Clinically, RA and its analogs have been successfully used to treat some precancerous lesions including leukoplakia, cervical dysplasia, actinic keratoses (AK) and xeroderma pigmentosum (XP) (Hong and Itri, 1994; Niles, 2002), and certain malignancies, such as acute promyelocytic leukemia (APL) (Altucci and Gronemeyer, 2001).

The role of retinoids in epidermal tumorigenesis has been subject to extensive studies. Retinoids are effective in preventing carcinoma development from chemically induced murine papillomas, suggesting that retinoids inhibit carcinogenesis at promotion and/or progression stages (Chen et al., 1994; Chen et al., 1995; De Luca et al., 1993; De Luca et al., 1996). As reviewed in Section I of this Chapter, AP-1 is involved in skin carcinogenesis, and trans-repression of AP-1 has been suggested to underlie the tumor suppressive effect of RA in the chemical skin carcinogenesis model (Huang et al., 1997).

In support of the notion that retinoids act to inhibit tumor promotion/progression during carcinogenesis, RA and its analogs have been found to be effective in treatment of several precancerous skin lesions and for prevention of some secondary tumors. For instance, 13-*cis* RA is widely used

to treat certain skin disease such as cystic acne, and can significantly reduce the malignant conversion rate of XP, an inherited skin disease at a high risk for skin cancer (Niles, 2002). AKs are precursors of human SCCs, and treatment with all-*trans* RA can decrease the number and size of Aks (Misiewicz et al., 1991; Moriarty et al., 1982).

Despite some encouraging results from clinical trails, the promise of retinoids in treating human cancers, notably solid tumors, has not yet been realized (Altucci and Gronemeyer, 2001). The major problems in current RA-based treatments include toxicity and resistance. Many patients receiving systemic RA treatment suffers from side effects, the so-called RA syndrome (Altucci and Gronemeyer, 2001; Fontana and Rishi, 2002). The typical clinical signs of RA syndrome include fever, respiratory distress, weight gain, oedema of the lower extremities, pleural or pericardial effusions, hypotension and, occasionally, renal failure.

To overcome the side effects of RA, many synthetic retinoid molecules have been developed; N-(4-hydroxyphenyl) retinamide (4-HPR) is one of the most promising such retinoids. Many lines of evidence suggest that it has increased effectiveness as an anti-tumor agent and reduced toxicity compared to RA (Altucci and Gronemeyer, 2001; Fontana and Rishi, 2002; Wu et al., 2001). Studies using animal models have demonstrated that 4-HPR treatment can prevent chemically induced malignancies of the breast, prostate, bladder and skin (Ohshima et al., 1985; Pollard et al., 1991; Slawin et al., 1993; Ulukaya and Wood, 1999), while clinical trials have shown that 4-

HPR has efficacy against head and neck SCCs, ovarian adenocarcinomas and breast adenocarcinomas (Fontana and Rishi, 2002; Wu et al., 2001). Interestingly, 4-HPR can suppress the development of ovarian carcinomas and prevent secondary breast malignancies in pre-menopausal women, but not in post-menopausal women (Camerini et al., 2001).

4-HPR can induce apoptosis of many cancer cell types, and its potent apoptotic ability is thought to underlie its activity against cancers (Fontana and Rishi, 2002; Wu et al., 2001). 4-HPR can bind and activate RARγ, as well as RARβ albeit to a lesser extent (Fanjul et al., 1996). However, both RAR-dependent and independent mechanisms have been suggested to mediate 4-HPR-induced apoptosis, and cell type-specific functions of 4-HPR have also been revealed (Fontana and Rishi, 2002; Wu et al., 2001).

RA resistance is another limiting factor for the clinical use of RA. The etiology for RA resistance appears to be multifactorial, and may be partially caused by enhanced activity of P450 enzymes and lipoxygenase induced by RA (Muindi and Young, 1993; Muindi et al., 1992; Zhou et al., 1998). RA also can induce expression of CRABPII, which may sequester RA from its nuclear receptors (Cornic et al., 1992; Degos et al., 1995; Delva et al., 1993; Muindi et al., 1992).

As mentioned in Section I of this Chapter, cancers are associated with various genetic and epigenetic alterations. In this regard, aberrant expression of RARs has been found in many experimental and clinical malignancies (Sun and Lotan, 2002). One of the most notable examples is APL.

APL is a distinct type of acute myeloid leukemia characterized by a blast cell morphology (Piazza et al., 2001). Expression of the chimeric PML (promeolocytic leukemia gene)-RARα protein caused by a t(15;17) translocation plays an etiological role in leukemogenesis in most APL cases. Fusion of RARα gene to the promeolocytic leukemia zinc finger (PLZF) gene, the nucleophosmin (NPM) gene or the signal transducer and activator of transcription 5b (STAT5b) have also been detected in some APL cases (Piazza et al., 2001).

The chimeric PML-RARα protein functions in a dominant negative manner to inhibit wild type RAR and binds to corepressor-HDAC complexes with higher affinity than the wild type receptor. Physiological concentrations of RA are unable to dissociate the corepressor complex, thereby leading to constitutive repression of some target genes essential for granulocytic maturation. By contrast, exposure to pharmacological doses of RA are able to effect cofactor exchange, restoring the expression of those target genes and thereby causing the remission of APL (Grignani et al., 1998; He et al., 1998; Lin et al., 1998). Interestingly, targeted expression of PML-RARα in the bone marrow through the MRP8 promoter not only recapitulates the APL phenotype, but also causes skin defects affecting both hair follicles and IFE. Moreover, some severely affected mice develop squamous papillomas (Brown et al., 1997; Hansen et al., 2003).

Other functions of PML-RARα have also been suggested, such as inhibition of wild type PML function (Piazza et al., 2001).

Loss of RARβ expression is also associated with a diverse range of solid tumors, such as head and neck SCC (HNSCC), non-small cell lung cancers (NSCLCs), breast, prostatic and cervical cancers (Sun and Lotan, 2002). Moreover, induced expression of RARβ correlates with the growth-inhibitory effects of RA on these solid tumors, and in many cell lines derived from them, suggesting that RARβ may act as a tumor suppressor (Sun and Lotan, 2002). Consistent with this, transfection of RARβ decreases the tumorigenicity of a human lung cancer cell line, while transgenic mice expressing anti-sense RARβ2 develop spontaneous lung cancers (Berard et al., 1996; Houle et al., 1993). Methylation-mediated gene silencing is a frequent mechanism of RARβ repression, and tumor-specific hypermethylation of the RARβ2 promoter has been found in diverse epithelial malignancies (Hayashi et al., 2001; Ivanova et al., 2002; Kwong et al., 2002; Nakayama et al., 2001).

Although it may act as a tumor suppressor in a broad range of epithelial tissues (Sun and Lotan, 2002), such a function of RARβ can not impact on the epidermis, since it lacks RARβ expression (Fisher and Voorhees, 1996). As reviewed above, the epidermis expresses both RARα and RARγ, with RARγ the prevalent receptor type (Darwiche et al., 1995; Fisher and Voorhees, 1996). Progressive loss of RAR expression is associated with mouse chemical skin carcinogenesis. Although both RARα and RARγ are detectable in papillomas and differentiated SCC, their

expression is lost in more advanced malignancies such as undifferentiated SCC and spindle cell carcinomas (Darwiche et al., 1995).

Topical application of TPA to the skin causes a downregulation of both RAR types, indicating that a PKC-dependent mechanism may be involved in RAR silencing (Kumar et al., 1994). As a support, inhibition of PKC activity can restore RAR expression in keratinocyte cultures harboring oncogenic Ras (Darwiche et al., 1996).

Progressive loss of RAR expression is also associated with human epidermal carcinogenesis (Xu et al., 2001). Consistent with this, solar UV radiation, the major etiological factor for human skin cancers, decreases RAR expression in the epidermis (Wang et al., 1999).

In contrast to RARs, RXR expression is not affected during mouse chemical carcinogenesis, although conditional ablation of RXRα, the predominant RXR expressed in the epidermis, sensitizes the epidermis to DMBA/TPA induced tumor formation (Altucci and Gronemeyer, 2001; Darwiche et al., 1995).

Systematic studies on the role of vitamin A in embryonic development and adult health began in early 1900s (Frazier and Hu, 1931; Goodwin, 1934; Wolbach and Howe, 1925). Since then, vitamin A has been found to play an important role in many biological processes, and RA is recognized as its major biologically active derivative (Chambon, 1996; Ross et al., 2000). In recent decades, our understanding of vitamin A/RA function has been deepened since the discovery of retinoid receptors (RARs and RXRs). As the

mediators of RA signal, retinoid receptors influence biological events through modulating gene expression either as DNA-binding transcription factors or by interfering with pathways mediated by other transcription factors (Chambon, 1996; Ross et al., 2000; Wei, 2003).

Retinoids have been used to treat some human disorders, and are promising anti-tumor agents (Altucci and Gronemeyer, 2001; Chambon, 1996; Ross et al., 2000). To design a better treatment regime and to realize their preventive and therapeutic potential, a better understanding of their function is needed. To this end, my Ph.D. research project focused on studying the roles of RARs in epidermal tumorigenesis and development.

Section III. Experimental Rational

As reviewed in the previous sections, retinoids elicit tumor suppressive effects in many settings (Altucci and Gronemeyer, 2001), and progressive loss of RARs is associated with epidermal carcinogenesis (Darwiche et al., 1995; Xu et al., 2001). These observations indicate a link between RAR repression and skin tumorigenesis. However, the biological significance of loss of RAR expression in skin tumorigenesis has not been explicitly assessed. To this end, I investigated whether ablation of RARs predisposes the epidermis to v-Ha-Ras-induced tumorigenesis.

Ablation of RARα, RARγ, or both, affect mouse viability (Lohnes et al., 1993; Lufkin et al., 1993; Wendling et al., 2001), and thus the classical chemical carcinogenesis model is not applicable for our purpose. To this end, keratinocyte cell lines deficient in various RARs were derived from corresponding RAR null fetuses, and a keratinocyte-based tumorigenesis protocol was introduced to address the role of RARs in epidermal tumorigenesis. In Chapter II, *in vitro* characterization of the keratinocytes with various RAR deficiencies is described, and in Chapter III, the effect of loss of these RARs on skin tumorigenesis is presented.

Ablation of RARs only causes a minor epidermal abnormality (Chapellier et al., 2002; Ghyselinck et al., 2002; Lohnes et al., 1994), while targeted expression of a dnRARα in basal keratinocytes results in profound epidermal defects (Saitou et al., 1995). Although several mechanisms underlying the discrepancy between these two models have been proposed,

no direct *in vivo* evidence has been presented to support these hypotheses. To this end, transgenic mice expressing a dnRARα, or its DBD mutant, were generated. Phenotypic studies on these animals have suggested a molecular basis explaining the different outcomes resulting from RARαγ deficiency or expression of dnRAR, as described in Chapter IV.

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Chapter II

Characterization of retinoic acid receptor-deficient keratinocytes

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Preface

Among the RARs (RARα, -β, and –γ), the epidermis expresses RARα and RARγ. Loss of these RARs is associated with skin carcinogenesis both in human and animals. This study is aimed at investigating the biological significance of loss of these RARs in skin tumorigenesis. Because targeted deletion of RARα and/or RARγ causes embryonic or perinatal death, we were unable to employ the classical chemical carcinogenesis protocol to address our questions. To this end, we established keratinocytes and used an alternative model to study the role of loss of RARs in skin tumorigenesis. This Chapter presents the experimental results showing the effects of RAR disruption on a number of RA-regulated cellular events in keratinocyte culture.

Dr. Philippe Goyette is the first author of this work. Under the supervision of Dr. David Lohnes, the candidate was heavily involved in the establishment of keratinocyte cell lines and in the studies on the roles of RAR subtypes in RA-induced inhibition of cell growth and AP-1 activity. Wei Wang provided assistance in cell culture, while Francois Seguin did some preliminary work for this study. This paper was drafted by Dr. Philippe Goyette, and revised by Dr. David Lohnes.

Summary

Retinoids are essential for normal epidermal growth and differentiation and show potential for the prevention or treatment of various epithelial neoplasms. The retinoic acid receptors (RARα, -β and -γ) are transducers of the retinoid signal. The epidermis expresses RARγ and RARα, both of which are potential mediators of the effects of retinoids in the epidermis. To further investigate the role(s) of these receptors, we derived transformed keratinocyte lines from wild-type, RARα, RARγ and RARαγ null mice and investigated their response to retinoids, including growth inhibition, markers of growth and differentiation, and AP-1 activity. Our results indicate that RARγ is the principle receptor contributing to all-*trans*-retinoic acid (RA)-mediated growth arrest in this system. This effect partially correlated with inhibition of AP-1 activity. In the absence of RARs, the synthetic retinoid *N*-(4-hydroxyphenyl)-retinamide inhibited growth; this was not observed with RA, 9-*cis* RA or the synthetic retinoid (E)-4-[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl] benzoic acid. Finally, both RARα and RARγ differently affected the expression of some genes, suggesting both specific and overlapping roles for the RARs in keratinocytes.

Introduction

Vitamin A derivatives (retinoids) play central roles in embryonic development and maintenance of various tissues in the adult (1-3). Retinoids also exhibit potent antitumorigenic properties in diverse model systems, and show potential for the treatment of a number of human malignancies, including diverse epithelial cancers or pre-cancerous lesions (4-9).

The retinoid signal is transduced by two families of nuclear receptors, the retinoic acid (RA) receptors (RARα, -β and -γ and their isoforms) and the retinoid X receptors (RXRα, -β and -γ) (10-13). RARs function as ligand-inducible transcription regulators by binding, together with an RXR partner, to specific *cis*-acting response elements (RAREs). RARs can be activated by both RA and its stereoisomer, 9-*cis* RA, whereas RXRs are activated only by 9-*cis* RA (14). RXRs are also essential heterodimeric partners for a number of other nuclear receptor signalling pathways, including thyroid hormone, vitamin D, and certain orphan receptors (15;16). Although 9-*cis* RA is not obligatory for transcriptional regulation via these pathways, some results suggest that RXR-specific ligands can elicit transcriptional activation in certain settings (17-20).

RARs, like several other nuclear receptors, can function in a ligand-dependent manner to inhibit AP-1 activity, and it has been suggested that the effect of retinoids on the growth of transformed cells may occur through this *trans*-repression mechanism (21-23). This inhibition is believed to be due, at least in part, to competition for limiting amounts of transcriptional co-factors,

such as CBP and/or its homologue p300, common to both pathways (24;25). Other mechanisms, such as inhibition of the expression of AP-1 family members or c-Jun N-terminal kinase (JNK), may also contribute to this cross-talk (26-29).

Gene targeting of the various RARs has revealed essential and diverse roles for these receptors (1;30;31). However, due to perinatal or embryonic lethality inherent to many of these RAR null backgrounds, there is a void in our knowledge of RAR function in a number of contexts, such as tumorigenesis.

Exogenous retinoids can attenuate the effects of tumor promoters in the two-stage skin carcinogenesis protocol (8;32). Among the retinoid receptors, normal epidermis expresses RARγ and RARα as well as RXRα and RXRβ, with RARγ and RXRα as the predominant heterodimer (33-35). This pattern of expression prompted us to investigate the roles of RARα and RARγ in mediating the antitumorigenic effects of retinoids in epithelial keratinocytes. To this end, we established RARα, RARγ and RARαγ null keratinocyte lines by transformation with a dominant-negative p53 expression vector and compared the properties of these various lines. Our results demonstrate that RARα and RARγ affect different aspects of retinoid response in these transformed cells, with RARγ being the primary mediator of RA-induced growth inhibition. However, other synthetic ligands affected proliferation independent of the RARs. RAR-dependent, but not -independent, growth inhibitory effects generally correlated with attenuation of AP-1

transcriptional activity. Finally, the effects of RARα and RARγ on expression of certain keratinocyte markers suggests that each RAR may perform a subset of specific functions, which cannot be entirely fulfilled by other RARs in this cell type.

Experimental procedures

Primary keratinocyte culture and immortalization

The RAR null mice used in these studies have been described previously (36;37). RARα, RARγ and RARαγ mutants were generated from the appropriate mating, whereas wild-type offspring were obtained from $RAR\gamma^{+/-}$ intercrosses. Fetuses were procured by Caesarean section at 18.5 days post coitus, and genotype was determined by polymerase chain reaction as described (38). Primary keratinocyte cultures were established from the epidermis by standard means (39), and cultured in S-minimal essential medium with 10% chelex-treated fetal calf serum (calcium concentration of 0.5mM), insulin (5μg/ml), hydrocortisone (0.5μM), $MgCl_2$ (1.5mM), cholera toxin ($1.2x10^{-11}$M), adenine (24μg/ml), and gentimycin (10μg/ml). The next day, the cells were fed with media further supplemented with epidermal growth factor (10ng/ml) and expanded for several days. Cultures were treated at 3-5 days post-plating with versene (0.5mM EDTA in phosphate-buffered saline) to remove contaminating fibroblasts. The cells were subcultured at a 1:3 ratio at most 2 times prior to transformation.

A single 10-cm plate of cells (~2 X 10^6) of each genotype was harvested, and cells were resuspended in 800μl of medium. The cells were then electroporated (250 mV, 960 μF in a 0.4-cm gap cuvette) with 25μg of a linearized expression vector harboring a mutated p53 from the Friend erythroleukemia cell line CB7 (40). Cells were plated and routinely

subcultured until past crisis. All experiments were performed using cultures between passage 16 and 26.

Growth assays

Transformed keratinocytes were seeded into 96-well plates at a cell density of 500 cells/well and were treated the following day with vehicle (Me_2SO) or the appropriate retinoid (RA, 9-*cis* RA, 4-HPR or TTNPB). Medium was replenished every second day. Growth was assessed either in response to varying concentrations of retinoid at eight days post-plating, or over time in response to 10^{-6}M ligand. DNA content was assessed as a measure of cell growth using crystal violet staining as previously described (41). Relative dye binding was assessed by O.D. at 590nm using a microplate reader. Results were expressed either as A_{590} values or as growth relative to untreated controls and were derived from the mean (+/- S.D.) of four replicate wells.

Transient transfection and AP-1 activity assay

Transfections were performed using Lipofect ACE reagent (Life Technologies, Inc.). Briefly, cells were plated in 6-well cluster plates at $4x10^4$ cells/well. Transfections consisted of 0.5μg of AP-1 reporter or appropriate control (42), either alone or with expression vectors encoding c-Fos, c-Jun, CBP, p300 or RARs. Total DNA (5μg; normalized with KS+) was mixed with 10μl lipid and added to 100μl of serum-free S-minimal essential medium. The

lipid/DNA mixture was then added to the cells in 1ml complete medium and incubated at 37°C overnight. Medium was changed daily, and luciferase activity assessed 48 hours post-transfection. Results were corrected for protein concentration and are expressed as the mean (+/- S.D.) from three independent transfections. All experiments were repeated at least three times with comparable results.

Electrophoretic mobility shift assays (EMSA) and Western blot analysis

Cells were cultured in 10-cm plates in the presence of RA (10^{-6}M) or vehicle for 48 hours prior to harvest. Nuclear proteins were isolated from each cell line and protein concentration was determined using the DC protein assay kit (BioRad). Electrophoretic mobility shift assays were performed essentially as before (43). Briefly, binding reactions containing ~2ng of probe (50,000cpm) and 5μg of nuclear protein were separated by electrophoresis through a 6% polyacrylamide gel containing 0.25X Tris borate and EDTA. Specificity of binding was assessed by competition with a 10-fold excess of unlabelled RARE (5' GGGTAGGGTTCACCGAAAGTTCACTCGCA) or AP-1 (5' GATCCGATGAGTCAGCCA) double-stranded oligonucleotides.

For Western blot analysis, 40μg of nuclear protein from the various cell lines were size fractionated on a 10% SDS-polyacrylamide gel electrophoresis and electroblotted to Immobilon-P polyvinylidene difluoride membrane as recommended by the supplier (Millipore). Proteins of interest were detected by incubation with the desired antibodies and detection with an

ECL kit (Amersham Pharmacia Biotech) as per the manufacturer's instructions. Antibodies were purchased from Santa Cruz Biotechnology.

Northern blot analysis

Fifteen microgram of total RNA, isolated by Trizol reagent (Life Technologies, Inc.), were size fractionated on a 1% agarose-formaldehyde gel in MOPS buffer and transferred to a MAGNA nylon membrane (MSI). Fragments were isolated by restriction digestion of cDNAs followed by purification by Geneclean, and used to generate probes by labeling with ^{32}P-α-ATP by random priming with an oligo labeling kit (Amersham Pharmacia Biotech). Membranes were hybridized according to the manufacturers directions.

Results

Generation of RAR null cell lines

Primary cultures of wild-type and RAR null keratinocytes showed no major differences in morphology, growth or immortalization with dominant-negative p53. All lines grew well for at least 40 passages, suggesting that they were immortalized. None of the lines formed colonies in soft agar or were tumorigenic in nude mice (unpublished observations).

Electrophoretic mobility shift assay revealed that, relative to wild-type extracts, disruption of RARα, and to a greater extent RARγ, decreased specific binding to an RARE, and association was completely abolished in extracts from RARαγ double null cultures (Fig. 2-1). Northern blot analysis confirmed the disruption of RARα and/or RARγ message in the appropriate cell line (data not shown). RARβ transcripts were undetectable in all lines by Northern blot or polymerase chain reaction approaches, consistent with previous studies indicating that this receptor type is not expressed in epidermal keratinocytes (33;44). These data suggest that there is no compensatory upregulation of the remaining receptors in response to disruption of a given RAR.

Contribution of specific RARs to retinoid-mediated growth inhibition

All transformed cell lines exhibited similar morphology and growth characteristics in the absence of retinoid treatment (Fig. 2-2). However, wild-type and RARα$^{-/-}$ cultures were growth inhibited by 10^{-6}M RA (Fig. 2-2). In

Figure 2-1. Electrophoretic mobility shift assay of an RARE sequence by nuclear extracts from transformed keratinocyte lines. Nuclear extracts (5μg) were incubated with a labeled double-stranded RARE oligonucleotide probe (50,000cpm) and bound and free probe separated by polyacrylamide gel electrophoreses. Binding was competed with 10-fold excess RARE probe (*C*) but not by a non-specific probe comprised of an AP-1 recognition sequence (*NSC*). Note the presence of a non-specific complex (*N/S*) migrating slightly faster than the RAR-containing complex (*RAR*). *WT*, wild-type.

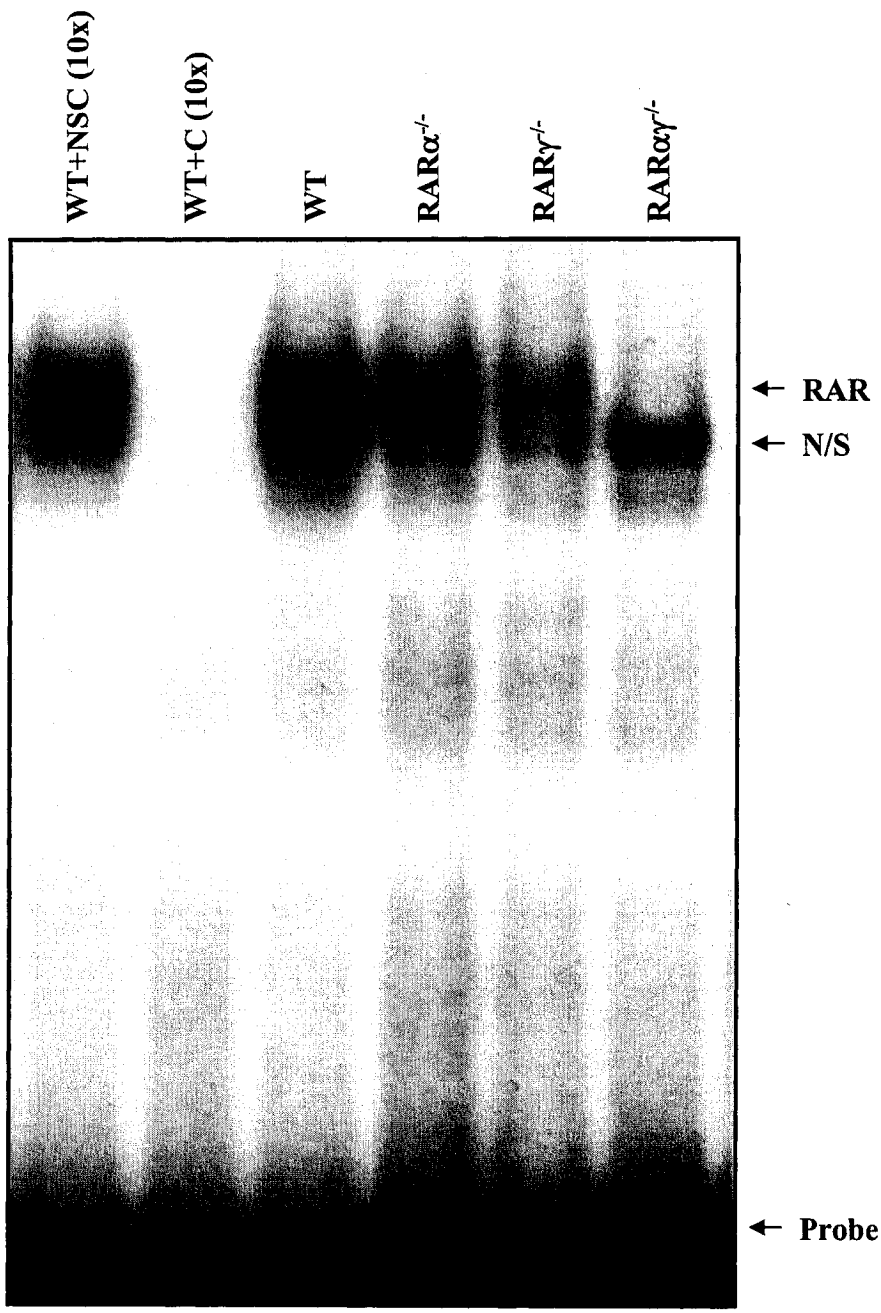
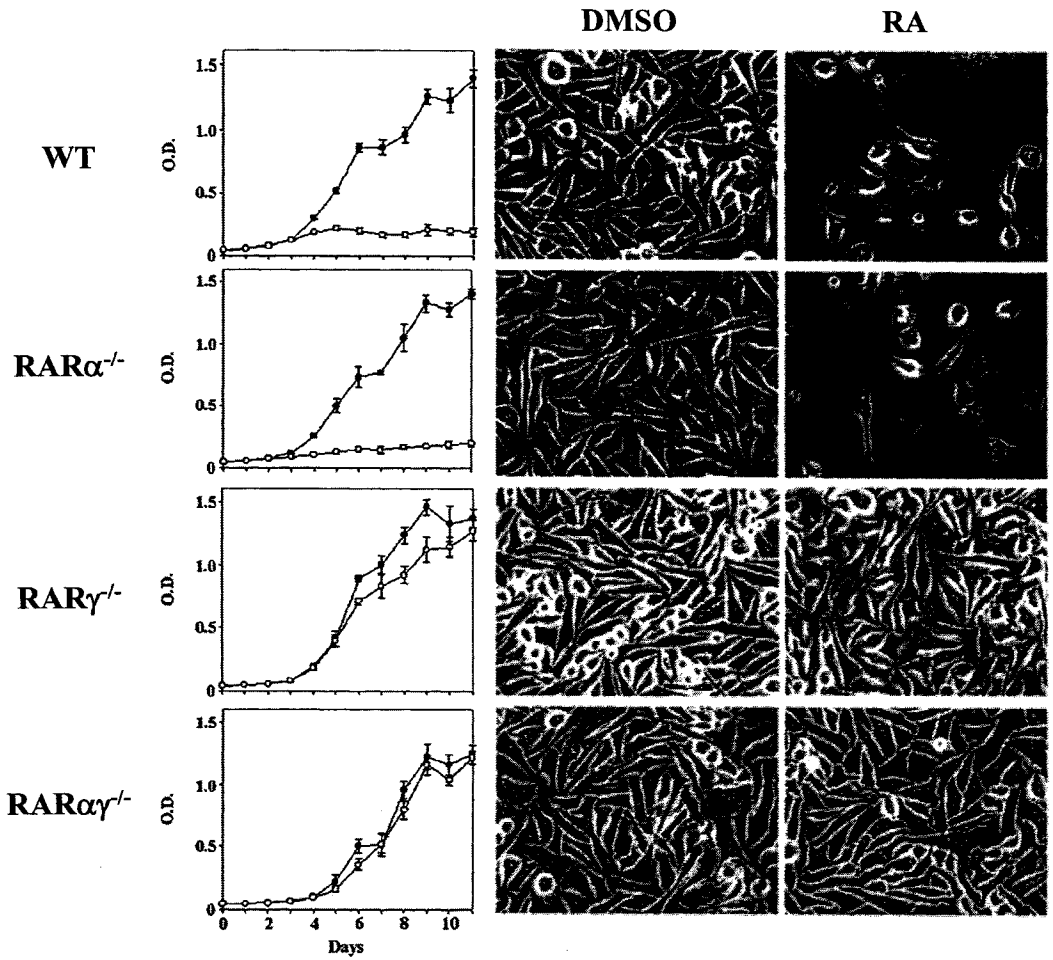

WT+NSC (10x)
WT+C (10x)
WT
RARα-/-
RARγ-/-
RARαγ-/-
RAR
N/S
Probe

Figure 2-2. Growth of transformed keratinocyte lines. The plots indicate growth of transformed keratinocyte lines in the absence (*closed circles*) or presence of 10^{-6}M RA (*open circles*) over 11 days. Microphotographs show representative cultures after six days of growth in the presence of vehicle (DMSO, Me_2SO) or 10^{-6}M RA as denoted on the top of the columns. Genotypes of the cultures are indicated to the left. *WT*, wild-type.

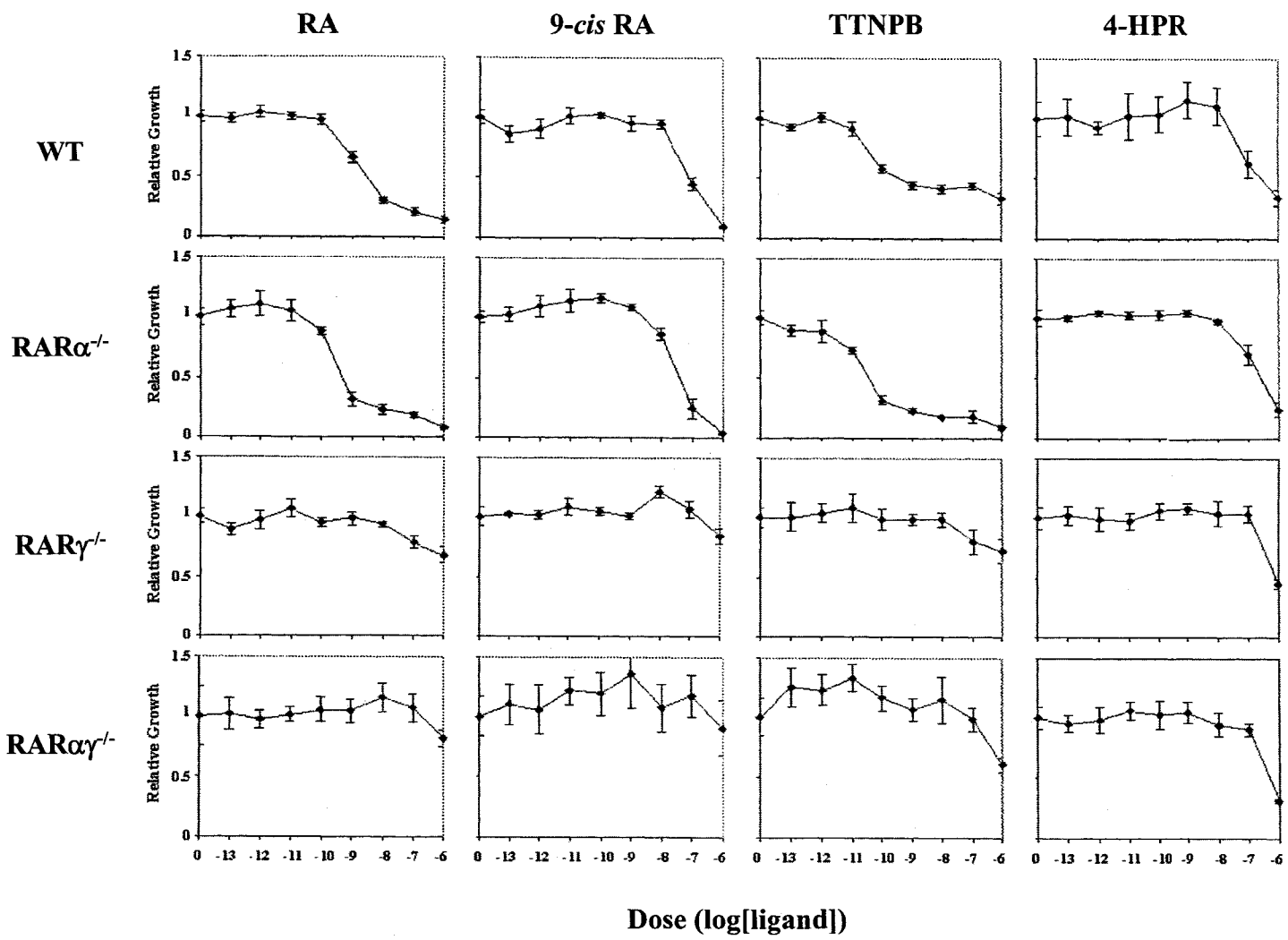

DMSO
RA
WT
RARα-/-
RARγ-/-
RARαγ-/-
O.D.
1.5
1.0
0.5
0
0
2
4
6
8
10
Days

marked contrast, RAR$\gamma^{-/-}$ cells were highly resistant and RAR$\alpha\gamma^{-/-}$ cultures were completely resistant to these effects. The growth arrest observed in wild-type and RARα null cultures was likely because of the inhibition of proliferation as opposed to apoptosis, as judged by thymidine incorporation and programmed cell death assays (data not shown).

Dose-response experiments were performed to determine the relative sensitivity of the various cell lines to growth arrest by RA, 9-*cis* RA, or the synthetic retinoids TTNPB or 4-HPR. As shown in Fig. 2-3, wild-type keratinocytes exhibited a significant reduction in proliferation at 10^{-9} M RA, with the maximal effect at 10^{-7} – 10^{-6}M RA. RAR$\alpha^{-/-}$ keratinocytes exhibited a similar profile, although their response to RA was slightly more pronounced than wild-type cultures. Consistent with time-course analysis, RAR$\gamma^{-/-}$ keratinocytes were only marginally inhibited by the highest dose of RA examined (10^{-6}M), and RAR$\alpha\gamma^{-/-}$ keratinocytes were not significantly affected by RA at any dose tested.

9-*cis* RA is a ligand for both RARs and RXRs, and RXR-agonists have been shown to induce effects on growth or differentiation in several model systems. Proliferation of both wild-type and RAR$\alpha^{-/-}$ cultures was inhibited by 9-*cis* RA, although higher concentrations were required compared with RA (Fig. 2-3). Interestingly, 9-*cis* RA had no significant outcome on the growth of either RAR$\gamma^{-/-}$ or RAR$\alpha\gamma^{-/-}$ cultures. This finding suggests that RXR activation does not lead to growth arrest in this model system, at least in the absence of

Figure 2-3. Analysis of keratinocyte proliferation in response to RA, 9-*cis* RA, TTNPB and 4-HPR. Cells were grown in the presence of vehicle or retinoids (from 10^{-13}M to 10^{-6}M) for 8 days. Growth was assayed by DNA content as described under "Experimental Procedures". Results are the mean +/- S.D. of quadruplicate samples, and are expressed relative to untreated cultures for each line. *WT*, wild-type.


RA
9-cis RA
TTNPB
4-HPR
WT
RARα-/-
RARγ-/-
RARαγ-/-
Relative Growth
1.5
1
0.5
0
0 -13 -12 -11 -10 -9 -8 -7 -6
Dose (log[ligand])

RARs. Whether RXR-specific signaling has other biological consequences remains to be investigated.

The RAR agonist TTNPB was a very potent inhibitor of growth in wild-type or RARα$^{-/-}$ cultures with an effect evident at 10^{-11}-10^{-10}M (Fig. 2-3). However, TTNPB affected RARγ$^{-/-}$ and RARαγ$^{-/-}$ cultures only at the highest dose tested (10^{-6}M). Whether this is indicative of effects on other pathways or is due to non-specific cytotoxicity is unknown.

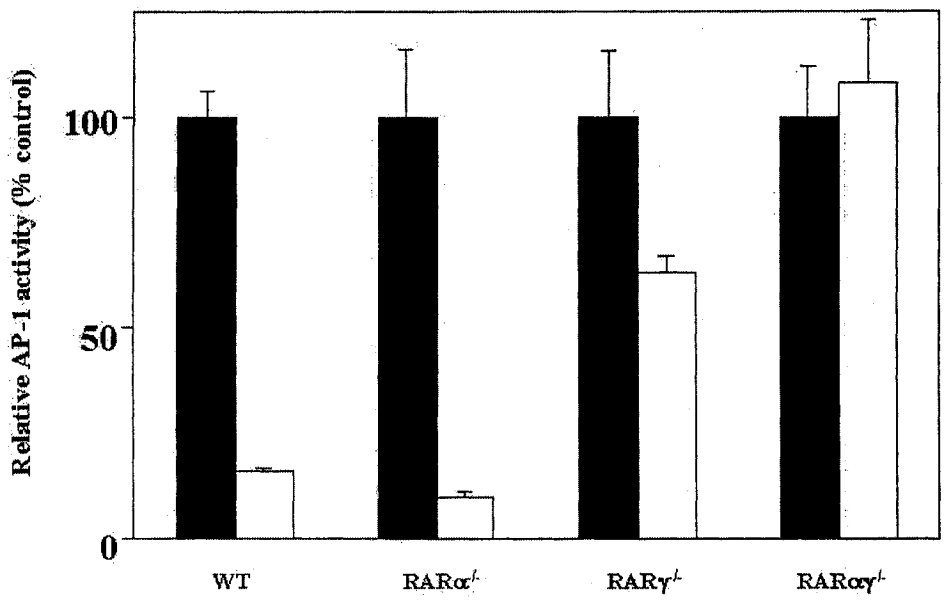
The synthetic retinoid 4-HPR has been shown to be a potent inducer of growth arrest and/or apoptosis in several model systems (45-47). This compound was the least efficient of all those tested in inhibiting proliferation of wild-type and RARα$^{-/-}$ cultures (Fig. 2-3). However, in marked contrast to the other retinoids, 4-HPR affected the growth of RARγ and RARαγ cultures at high doses, consistent with receptor-dependent and -independent mechanisms of action for this compound (48-53).

RAR regulation of AP-1 transcriptional activity

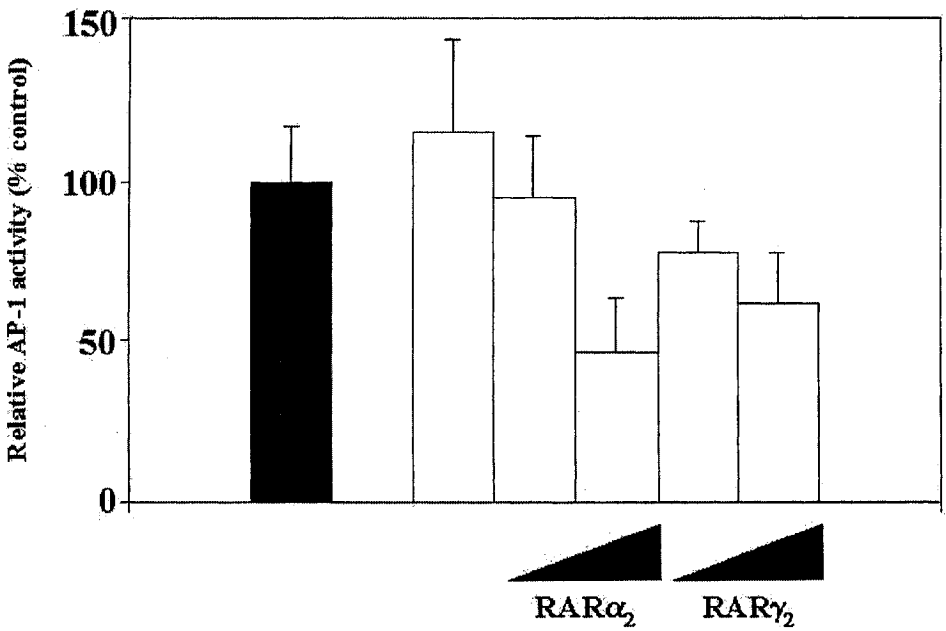
RARs can repress AP-1 transcriptional activity, and this mechanism of action has been proposed to underlie at least some of the antitumorigenic effects of retinoids (8;44;46;54). In transient transfection assays, we found that RA (10^{-6}M) inhibited AP-1 activity 8-10 fold in wild-type and RARα$^{-/-}$ cultures (Fig. 2-4A). AP-1 activity in RARγ$^{-/-}$ cultures was more modestly

Figure 2-4. Effects of RA on AP-1 activity. A) AP-1 reporter vector (0.5μg/well) was transfected into transformed keratinocyte lines and luciferase activity assessed following treatment with vehicle (*closed bars*) or RA (10^{-6}M; *open bars*) as described under "Experimental Procedures". B) Empty vector or RARα2 or RARγ2 expression vectors were transfected into RARαγ null keratinocytes, and cells treated as above. The *closed bars* represent expression in untreated RARαγ null cultures, and *open bars* are values from cells treated with RA. Samples transfected with RAR expression vectors (0.1 or 0.5μg/well) are denoted by the *triangles* and RAR type at the bottom of the figure. For both A and B, results are the mean +/- S.D. of three independent transfections and are expressed as percentage activity relative to untreated controls. *WT*, wild-type.

A


Relative AP-1 activity (% control)
100
50
0
WT
RARα-/-
RARγ-/-
RARαγ-/-

B

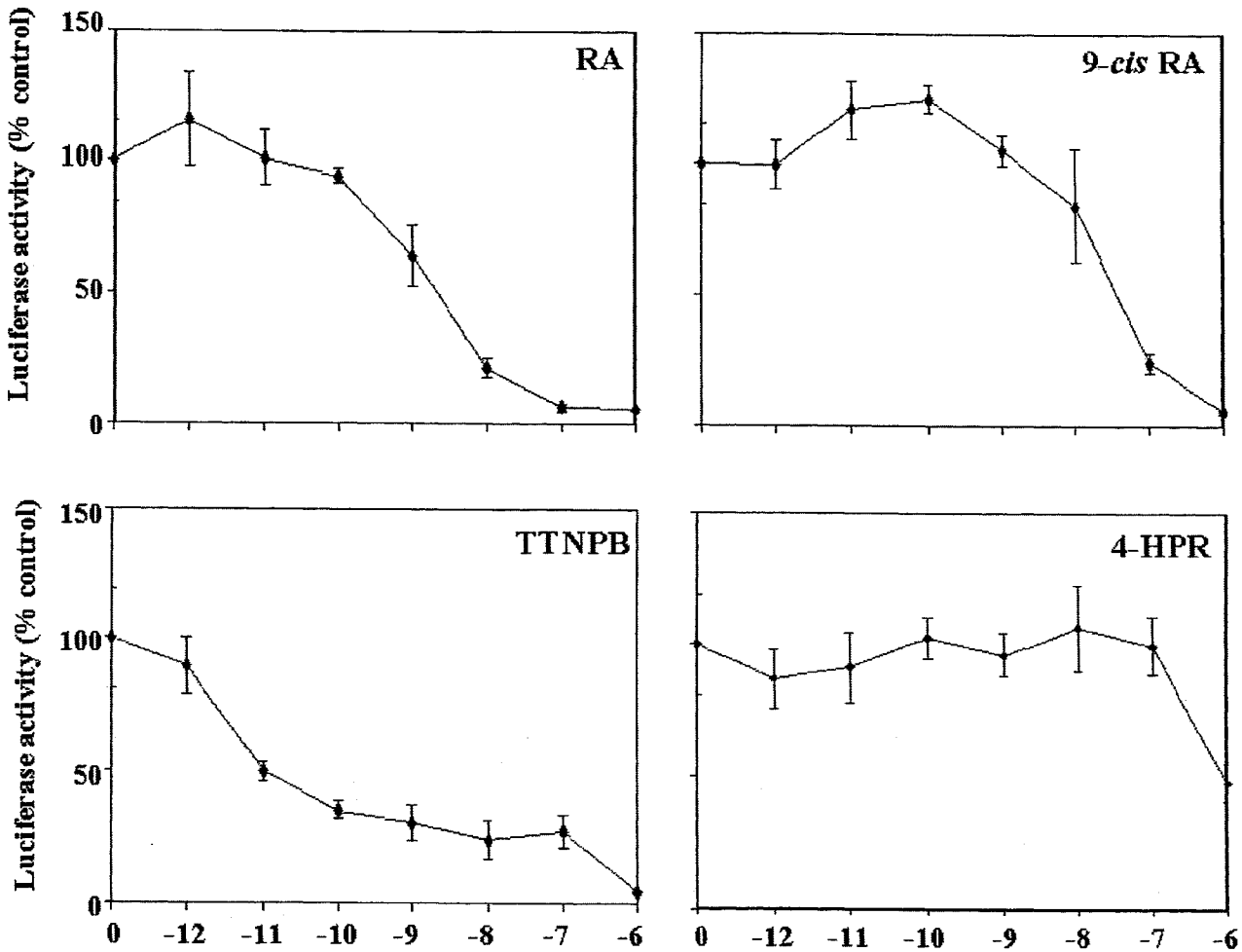

Relative AP-1 activity (% control)
150
100
50
0
RARα2
RARγ2

affected, typically exhibiting 10-30% reduction, whereas activity in RARαγ$^{-/-}$ cultures was not affected. The latter line was capable of response following re-introduction of either RARα or RARγ by transient transfection (Fig. 2-4B). Thus, attenuation of AP-1 activity requires the presence of at least one functional RAR, although there does not appear to be discrimination between receptor types for this outcome.

Dose-response studies revealed a close parallel between AP-1 activity and growth inhibition mediated by all four compounds in wild-type cultures (Fig. 2-5). However, growth arrest induced by 4-HPR in RARγ and RARαγ mutant lines never correlated with a reduction of AP-1 activity (data not shown). This finding underscores a unique and unknown mechanism of action for this retinoid in affecting proliferation.

We next determined the effect of RA on the expression of AP-1 members in wild-type and RAR null lines. Both the basal mRNA levels and RA-response of several of the AP-1 members varied across the different RAR null lines. In untreated cells, *c-fos* expression was comparable across all four lines, although it was slightly reduced in RARαγ null cells (Fig. 2-6). RA strongly inhibited *c-fos* in both wild-type and RARα$^{-/-}$ lines but had no effect in RARγ or RARαγ null cultures. This pattern was also observed at the protein level (Fig. 2-7). In contrast, treatment affected *c-jun* expression only in RARα null cultures, although basal mRNA levels varied across the lines. However, c-Jun protein did not reflect its cognate mRNA levels, and was reduced by RA treatment in wild-type, RARα$^{-/-}$ and RARγ$^{-/-}$ cultures. Phosphorylated c-Jun (P-

Figure 2-5. Effects of RA, 9-*cis* RA, TTNPB and 4-HPR on AP-1 reporter activity in wild-type cultures. Transfection was performed as under "Experimental Procedures". Cells were treated with carrier or with the indicated concentrations of the retinoids, and luciferase activity was assessed 48 hours post-transfection. Results are the mean value of triplicate samples +/- S.D. and are expressed as percentage values relative to untreated control.


RA
9-cis RA
TTNPB
4-HPR
Luciferase activity (% control)
Luciferase activity (% control)
150
100
50
0
150
100
50
0
0
-12
-11
-10
-9
-8
-7
-6
0
-12
-11
-10
-9
-8
-7
-6

Jun) levels paralleled those of c-Jun, suggesting that variations in phosphorylation were because of alterations in total c-Jun levels, rather than effects on JNK activity.

Fra-1 expression was barely detectable in wild-type, RARα$^{-/-}$ and RARαγ$^{-/-}$ lines but was elevated in RARγ$^{-/-}$ cultures; Western blot analysis was inconclusive, as the signal was too weak to be distinguished (data not shown). *junB* and *junD* expression did not vary significantly with the exception that *JunB* levels were slightly reduced in the RARαγ$^{-/-}$ line (Fig. 2-6 and data not shown).

A number of mechanisms have been suggested to underlie retinoid-repression of AP-1 activity. These include inhibition of JNK activity, which is unlikely given the observation that P-Jun levels appear to change as a function of c-Jun levels. Alternatively, the observed down regulation of c-Fos and/or c-Jun proteins might play a role, especially if either of them are limiting. A third mechanism, involves competition for limiting ancillary factors common to both RAR and AP-1 transcriptional complexes, such as p300/CBP (25). We addressed the latter two possibilities by assessing the ability of exogenous CBP, p300, c-Fos or c-Jun to negate the effects of RA treatment on AP-1 activity.

CBP or p300 transfection in wild-type cells resulted in a dose-dependent increase in AP-1 activity in the absence of RA (Fig. 2-8A). Interestingly, p300 appeared to be more potent in affecting AP-1 activity, suggesting that it may be preferred over CBP in this context. Despite this

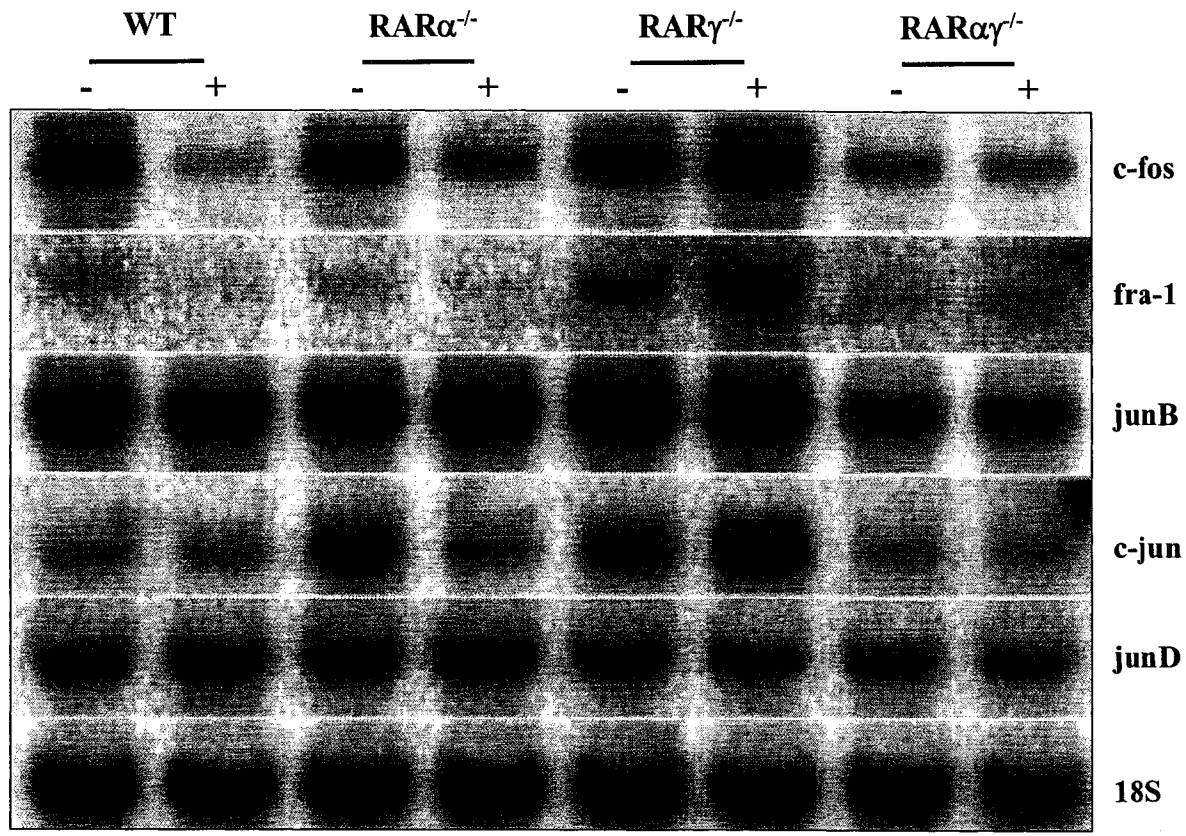
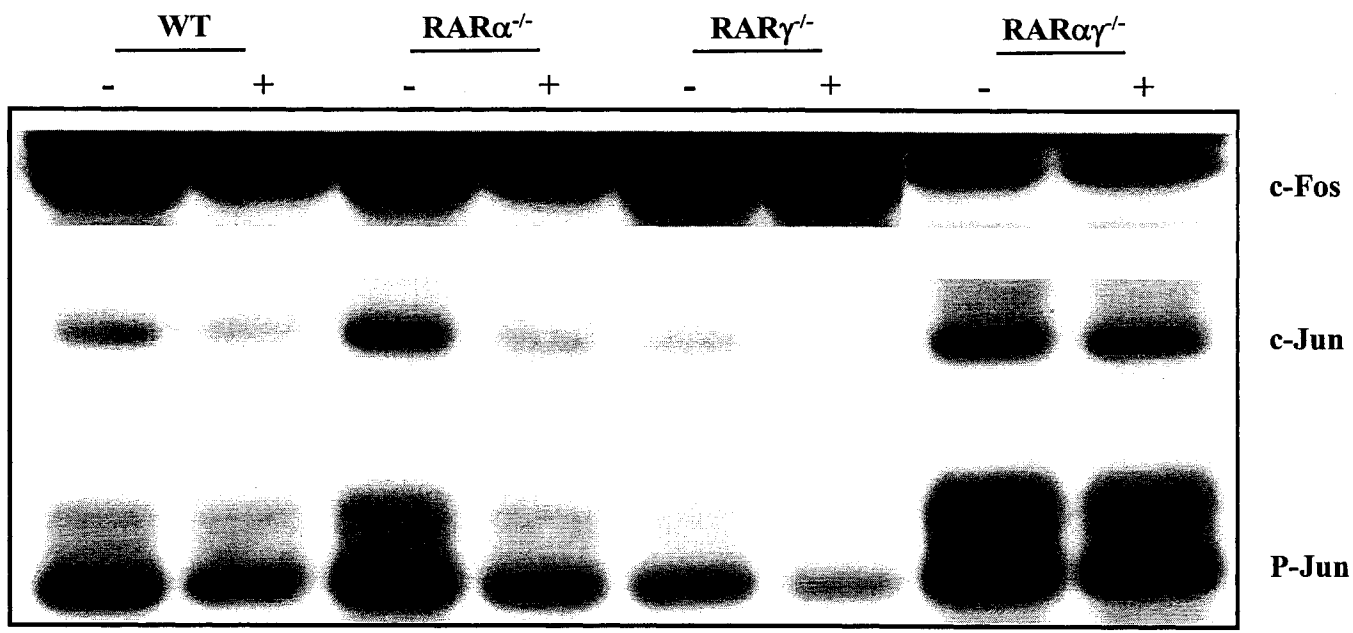

WT
RARα-/-
RARγ-/-
RARαγ-/-
- + - + - + - +
c-fos
fra-1
junB
c-jun
junD
18S

Figure 2-7. Effects of RAR ablation and RA treatment on c-Fos, c-Jun and P-Jun protein levels. Nuclear protein extracts (40μg) from cells treated for 48 hrs with carrier (-) or 10^{-6}M RA (+) were used to prepare a Western blot. Specific antibodies used to probe the blots are denoted to the *right*, and the various cell lines are noted at the *top*. *WT*, wild-type.


WT
RARα-/-
RARγ-/-
RARαγ-/-
- + - + - + - +
c-Fos
c-Jun
P-Jun

increase in activity, expressing the data as fold inhibition indicated that both factors resulted in only a modest reversal of inhibition (Fig. 2-8B).

Overexpression of either c-Fos or c-Jun also resulted in an increase in basal AP-1 activity, again with only a marginal reduction in fold-repression mediated by RA (Fig. 2-8). Although this rescue effect was more pronounced when both c-Jun and c-Fos were co-transfected, repression was not completely abolished (Fig. 2-8B). These observations suggest that several mechanisms, including titration of limiting co-factors and inhibition of expression of AP-1 family members, act in concert in a RAR-dependent manner to attenuate AP-1 activity in these transformants.

Effect of RAR ablation on gene expression

Northern blot analysis was performed to study the effect of receptor disruption on the expression levels of several genes implicated in keratinocyte growth and differentiation. The major integrin isoforms found in epidermis are integrin α_2, α_3, α_6, β_1 and β_4. These are expressed in basal keratinocytes, and a decrease in integrin expression is generally correlated with differentiation and loss of proliferative potential (55;56). Northern blot analysis revealed that, with the exception of the α_3 isoform, all integrins were down-regulated by RA treatment in wild-type, RAR$\alpha^{-/-}$ and RAR$\gamma^{-/-}$ cultures in a manner that correlated with the effects of treatment on proliferation (Fig. 2-9). Moreover, although integrin expression was not affected by RA treatment in RAR$\alpha\gamma$ null keratinocyte cultures, basal expression of integrin α_2, α_3 and β_4 were

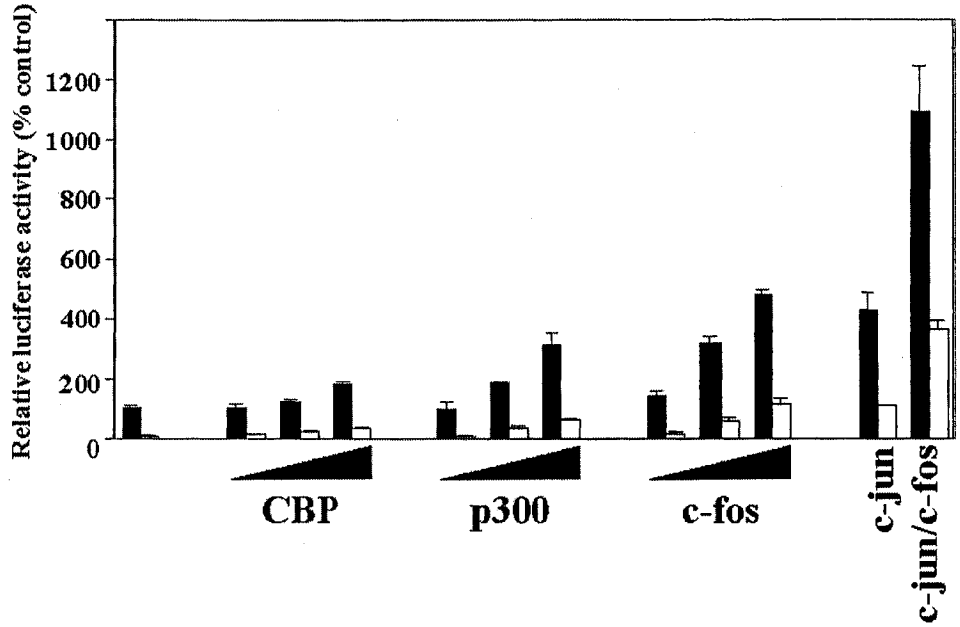
substantially reduced. The seemingly contradictory observation that both RA excess and RAR loss can reduce expression of several integrins is perhaps indicative of an altered differentiation state in the double null mutant line. Interestingly, integrin β_1 expression decreased in untreated RAR$\alpha^{-/-}$ cells and was upregulated in untreated RARγ mutant cultures.

Keratin expression patterns reflect the differentiation states of various epithelial strata (55;56). K5/K14 are expressed in basal epidermal cells, whereas K1/K10 are associated with early differentiation steps and predominate in suprabasal cells. K6 and K19 are not expressed in normal epidermal keratinocytes but are often observed in situations of aberrant proliferation, such as psoriasis, wound healing, and propagation in tissue culture. With the exception of RAR$\alpha\gamma$ null cultures, RA treatment repressed expression of K10 (Fig. 2-9). This observation may be related to the fact that RA excess can inhibit keratinocyte differentiation (57;58). However, RAR disruption did not result in upregulation of this differentiation marker, suggesting that K10 is not normally regulated by the RARs, but responds to pharmacological levels of RA.

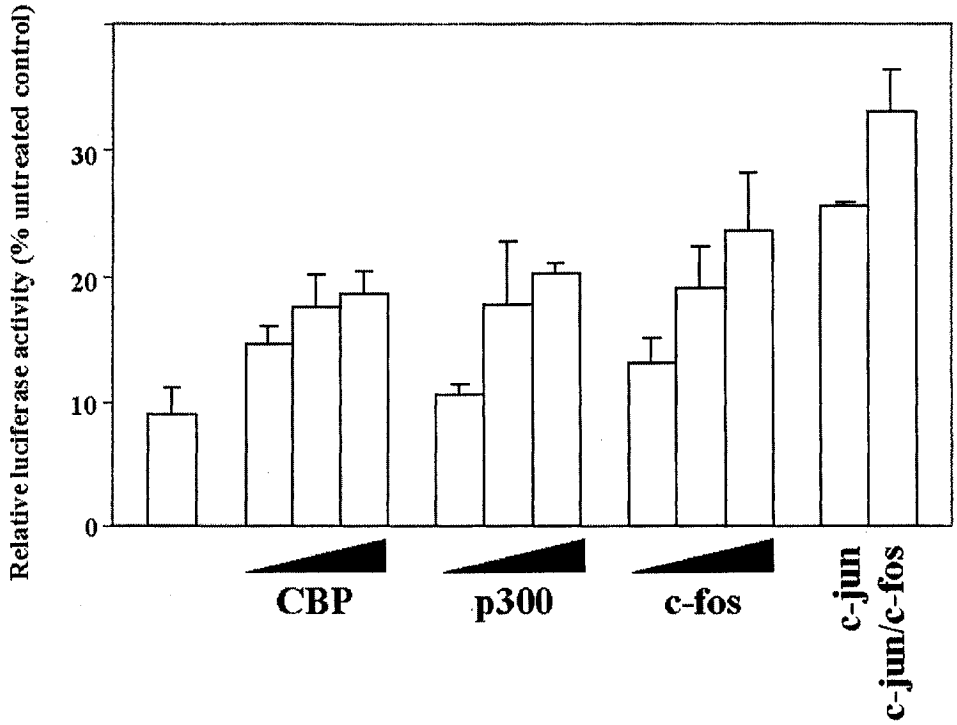
RA suppressed K6 expression in wild-type, RAR$\alpha^{-/-}$, and (to a lesser extent) RAR$\gamma^{-/-}$ cultures, consistent with the effect of treatment on both AP-1 activity and proliferation. K19 expression was induced in wild-type and RAR$\alpha^{-/-}$ cultures. Interestingly, this gene was also upregulated in RAR$\gamma^{-/-}$ cells in the absence of treatment. These data indicate that, as for the integrins, the roles of the various RARs on keratin expression vary depending on both the

Figure 2-8. Rescue of AP-1 repression. Wild-type keratinocytes were transfected with an AP-1 reporter in the absence or presence of various amounts of expression vectors encoding CBP, p300, c-Fos (0.1, 0.5 or 1.0μg) or c-Jun (0.5μg), or c-Fos plus c-Jun (0.5μg each). A) Cells were treated with vehicle (*closed bars*) or 10^{-6}M RA (*open bars*) and luciferase activity assessed 48 hours post-transfection. Results are expressed as percentage activity relative to untreated wild-type control. B) The results from A were expressed as fold AP-1 activity relative to untreated transfected cultures. Results are the mean +/- S.D. of three independent transfections for both A and B.

A

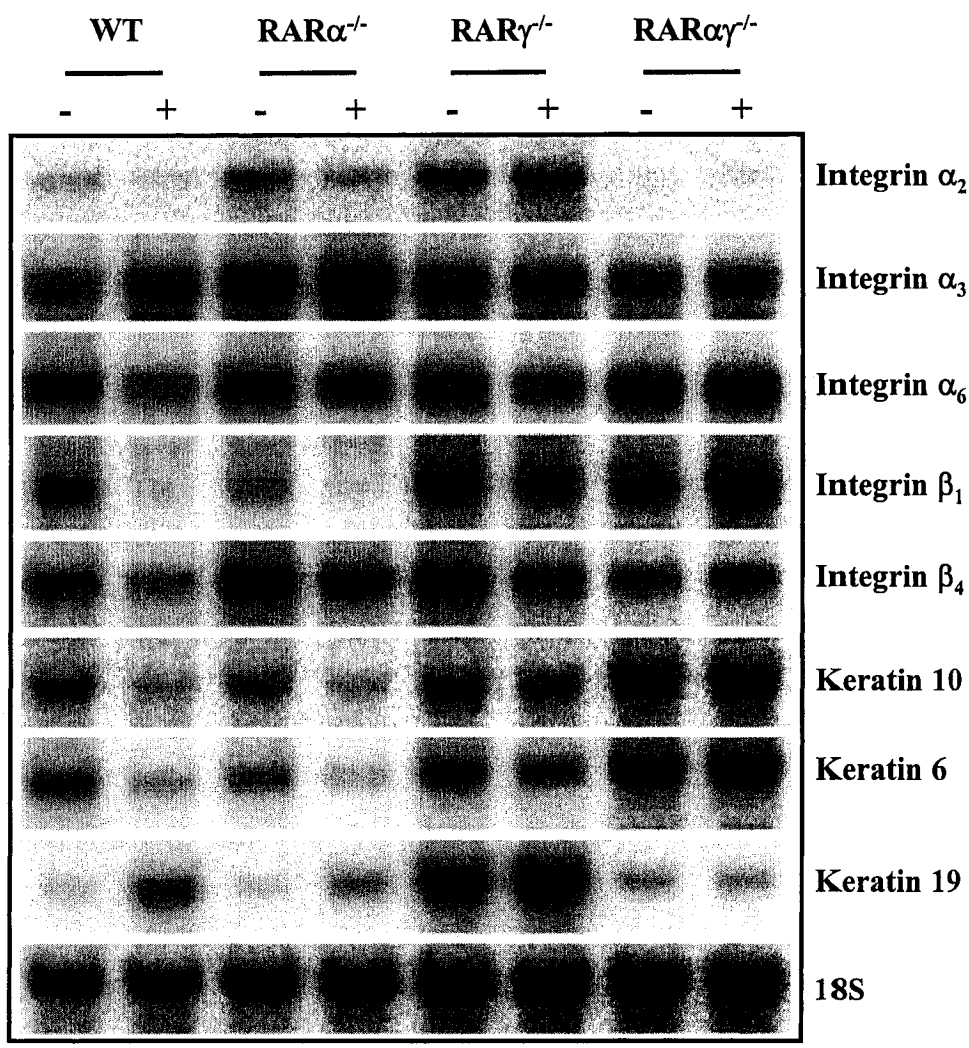

Relative luciferase activity (% control)
1200
1000
800
600
400
200
0
CBP
p300
c-fos
c-jun
c-jun/c-fos

B


Relative luciferase activity (% untreated control)
30
20
10
0
CBP
p300
c-fos
c-jun
c-jun/c-fos

receptor and the gene of interest. Moreover, most of the integrin and keratin markers were affected in both RARα and RARγ null cultures. This demonstrates that both receptor types transduce effects on expression of many responsive genes.

Figure 2-9. Northern blot analysis of keratinocyte gene expression. Wild-type and RAR null keratinocytes were treated with carrier (-) or 10^{-6}M RA (+) for 48 hours. 15μg of total RNA from each cell line was used to prepare Northern blots, which were probed with cDNAs encoding various keratinocyte markers, as denoted to the *right* of the figure. Ribosomal RNA (*18S*) was used as loading control. The results are typical of at least two experiments.


WT
RARα-/-
RARγ-/-
RARαγ-/-
- + - + - + - +
Integrin α2
Integrin α3
Integrin α6
Integrin β1
Integrin β4
Keratin 10
Keratin 6
Keratin 19
18S

Discussion

We present, for the first time, the effects of RAR disruption on the characteristics and RA response of transformed epidermal keratinocytes. These data indicate that each RAR type plays both specific as well as overlapping roles in events related to keratinocyte growth and gene expression.

RARs are not necessary for survival and growth of transformed keratinocytes in culture

Previous work using a dominant-negative RARα under the control of a basal-keratinocyte specific promoter suggested that RA signaling is essential for normal keratinocyte differentiation (59). We found that wild-type and RARαγ null keratinocytes are comparable in regards to growth and morphology, although expression of some markers, such as integrin α_2, did differ. It is unlikely that RARβ plays any compensatory role in these cells, as we have never observed expression of this receptor in these cultures irrespective of RAR status. Moreover, the RARαγ null line was completely resistant to excess RA with respect to all outcomes examined. Thus, these cells are likely completely devoid of functional RARs. The difference between the relatively mild effects observed in the present study, compared with a dominant-negative RAR (59), suggests that transgene expression affects other pathways, perhaps by sequestration of RXRs. Alternatively, we cannot

exclude an unrecognized compensation mechanism in the RAR null animals and derivative cells that may mask certain roles for these receptors in skin.

RARγ is the principle mediator of growth arrest in transformed keratinocytes

Analysis of the effects of the various RARs on growth inhibition suggests that the major player in transducing this effect is RARγ with only a negligible contribution by RARα. This may simply be due to the prevalence of the former receptor type in keratinocytes (4) rather than indicative of receptor-specific function. Nevertheless, irrespective of the basis for this finding, these data suggest that targeting RARγ is a logical strategy to affect disorders of keratinocyte proliferation.

In the absence of the RARs, the RXR ligand 9-*cis* RA had no effect on proliferation, suggesting that liganded RXRs do not impact on keratinocyte growth, at least in the absence of RARs. This is in contrast to previous reports, which suggests growth inhibitory effects mediated by RXR-selective agonists in several other transformed cell types (17;19;60-64). This suggests either that RXR ligands inhibit growth in a cell type-specific manner or that these synthetic agonists have effects that cannot be mimicked by 9-*cis* RA. It will be of interest to determine if such ligands exert an effect in the RARαγ null line.

Although 9-*cis* RA inhibited the growth of wild-type and RARα null lines, it was consistently less potent than RA or TTNPB. Because both RA

and 9-*cis* RA have comparable affinities for the RARs (53), this observation suggests either that 9-*cis* is more labile than RA or is titered away from RAR signaling. In contrast to 9-*cis* RA, the synthetic RAR-agonist TTNPB was a strong inhibitor of growth. As displacement assays suggest that TTNPB binds to the RARs with a lower affinity than RA, the greater relative potency of this analog likely lies in its enhanced stability and/or weaker association with CRABPs relative to RA (65). Moreover, although full manifestation of growth inhibition by TTNPB required the RARs, a slight inhibition at the highest dose used was seen in RARγ and RAR$\alpha\gamma$ null cultures, suggesting either nonspecific cytotoxicity or effects via nonreceptor-mediated pathways.

Evidence for RAR-independent mechanisms of growth inhibition by 4-HPR

4-HPR can inhibit growth and induce apoptosis in a number of model systems (35;45;66;67). Although this compound can act directly via the RARs, some of its effects may be mediated by receptor-independent mechanisms (48;64;66). Indeed, we found that 4-HPR inhibited proliferation in all lines assessed but that this occurred at lower concentrations in RAR-positive cultures. This is consistent with 4-HPR acting through both RAR-dependent and -independent mechanisms. As 4-HPR is well tolerated at high doses (66), it should mediate effects even in those epithelial tumors that lack retinoid responsiveness to "pure" RAR agonists.

RARs and inhibition of AP-1 activity

RA is a potent inhibitor of AP-1, and this mechanism of action has been proposed to underlie at least some of the anti-tumorigenic effects of retinoids (1;52;67-71). We found that both RARα and RARγ can repress AP-1 activity in p53-transformed keratinocytes in culture. However, attenuation of AP-1 reporter activity in RARγ null cultures (~30%) did not agree well with growth inhibition in this cell line, which was minimal. A lack of correlation was especially notable in the case of 4-HPR, which never repressed AP-1 reporter activity in RARγ or RARαγ null cultures at concentrations that inhibited their growth. This observation supports the existence of retinoid-mediated mechanisms of growth inhibition unrelated to AP-1 activity, at least in this model.

Multiple pathways for RA in inhibition of AP-1 activity

Several mechanisms have been proposed to underlie the crosstalk between AP-1 and RA signaling pathways. These include titration of common transcriptional co-regulators, such as CBP/p300 (25), effects on expression of AP-1 family members (27;72;73) or inhibition of JNK activity (26). We found evidence that supports the two former possibilities in our model.

RA inhibited c-Fos and c-Jun (P-Jun) protein levels in a manner that partially correlated with the observed attenuation of AP-1 activity. Although c-Jun levels paralleled the effect of treatment on AP-1 across the various cell lines, c-Fos was not affected by RA in RARγ or RARαγ null cultures. These

data underscore a specific role for RARγ in inhibiting c-Fos expression, whereas either RARα or RARγ affected c-Jun. In this regard, it is interesting to note that c-Jun protein was also elevated in untreated RARα and RARαγ null lines, indicating that RARα, presumably in its unliganded state, represses c-Jun expression. Consistent with this possibility, prior studies also suggests that certain RARs may be antagonistic to one another in F9 cells (74;75).

Transfection of either CBP or p300 induced basal AP-1 expression in wild-type keratinocytes and modestly attenuated the effects of RA on AP-1 activity. The persistent inhibition of AP-1 by RA, even following transfection of higher levels of CBP/p300 expression vector, suggests that the RARs remain in excess. Alternatively, as c-Fos and/or c-Jun also appear to contribute to this relationship, the combination of several distinct events underlies retinoid-antagonism of AP-1.

RARα and RARγ play both overlapping and specific roles in keratinocytes

The phenotype of RAR mutant mice clearly indicates that these receptors are highly but not completely redundant. This is supported by work using RAR-null F9 embryocarcinoma cells (*e.g.* Refs. 71 and 76). Our present findings suggest that specificity also exists regarding RAR function in keratinocytes. Although certain aspects of this selectivity may be explained by the relative levels of expression, with RARγ being the predominant receptor

type, this is not always the case. For example, repression of integrin α_6 and integrin β_4 was more affected by the loss of RARα than the loss of RARγ.

In addition to specific functions, it is also interesting to note that RARα may actually attenuate the effects of RARγ in certain instances. For example, growth inhibition mediated by RA, 9-*cis* RA or TTNPB was greater in RARα null cells relative to wild-type cultures, suggesting that RARγ is a more efficient inducer of growth arrest in the absence of RARα. Other complex interactions were also observed. RA induced Keratin 19 expression in wild-type cells, and this induction was greatly reduced in RARα null lines, indicating that RARα positively regulates this gene. However, in RARγ null cultures, K19 basal expression was induced, and RA induction was lost. One mechanistic explanation for this is that RARγ normally mediates repression of this gene in the absence of RA, and that RARα induces its expression in the presence of ligand. Consistent with this, K19 expression in RAR$\alpha\gamma^{-/-}$ cells returns to basal values, and regulation is lost, supporting a model for opposing function between RARα and RARγ in regulating this gene; such antagonism has been suggested previously (75). Although analysis of the regulatory sequences governing this response is necessary to understand the nature of these observations, these examples offer strong evidence that RARα and RARγ are not completely functionally equivalent in this model system.

Abbreviations

RA, all-trans retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; TTNPB, (E)-4-[2-(5,5,8,8 tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl] benzoic acid; 4-HPR, N-(4-hydroxyphenyl)-retinamide; JNK, c-Jun N-terminal kinase; EMSA, electrophoretic mobility shift assay; DMSO, dimethyl sulfoxide; CBP, CREB binding protein

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Chapter III

RARγ acts as a tumor suppressor in mouse keratinocytes

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Oncogene, in press

Preface

As described in Chapter II, keratinocytes deficient in RARα, RARγ or both were established. Using these RAR-deficient keratinocytes, RARγ was demonstrated as the major RAR subtype mediating RA-induced growth inhibition and AP-1 repression in vitro. In this Chapter, evidence is provided to define the roles of RARα and RARγ in skin tumorigenesis.

The candidate under the supervision of Dr. David Lohnes carried out most of the work in this study. Dr. Philippe Goyette was involved in studying the effects of RAR disruption on Ras downstream targets. This manuscript was drafted by the candidate, and revised by Dr. David Lohnes.

Abstract

Retinoic acid (RA), the principle biologically active form of vitamin A, is essential for many developmental processes as well as homeostasis in the adult. Many lines of evidence also suggest that RA, acting through the RA receptors (RARs), can also suppress growth of tumors of diverse origin. To directly assess the role of retinoid receptors in a model of epidermal tumorigenesis, we investigated the incidence of tumor formation using keratinocytes lacking specific RAR types. Our data suggest that loss of RARγ, but not RARα, predisposed keratinocytes to *v*-Ha-Ras-induced squamous cell carcinoma. We also found that ablation of RARγ, but not RARα, abolished RA-induced cell cycle arrest and apoptosis in these keratinocytes. Reconstitution of receptor expression into RAR null cells restored sensitivity to RA, and reversed the tumorigenic potential of receptor-deficient keratinocytes. These data strongly support tumor suppressor effects for RARs, in particular endogenous RARγ, in murine keratinocytes.

Introduction

Retinoic acid (RA), the major biologically active form of vitamin A, is an important regulator of vertebrate development, and is essential for maintenance of many tissues, including the skin (8,21,67). The biological functions of RA are mediated by two members of the nuclear receptor family, the retinoic acid receptors (RARα, RARβ and RARγ and their isoforms) and the retinoid X receptors (RXRα, RXRβ and RXRγ and their isoforms) (2,8,77).

RARs modulate gene expression by binding to the *cis*-acting response elements (RAREs) in the promoter/enhancer region of target genes as heterodimers with RXRs (8,37,77). In the absence of ligand, these heterodimers associate with transcriptional co-repressor complexes, including histone deactyltransferases, resulting in chromatin condensation and suppression of gene transcription. Ligand-binding mediates conformational changes in the receptor leading to disassociation of co-repressors and recruitment of co-activators, which either have intrinsic histone acetyltransferase (HAT) activity or associate with HATs. HAT function leads to de-condensation of chromatin, facilitating gene transcription (77,85).

In addition to direct regulation of target genes, RARs, like several other nuclear receptors, can impact on gene expression by trans-repression of other transcription factors, such as AP-1 (2,51). AP-1 activity has been closely linked to epidermal tumorigenesis in vivo and cellular transformation in vitro (35). While the basis for this effect is not fully understood, *trans*-

repression of AP-1 has been suggested to be responsible for the growth inhibitory effects of RA in many cell types, including keratinocytes (26,36).

RA has long been known to influence epidermal development and differentiation under pharmacological or pathological conditions (21). Among the retinoid receptors, normal cutaneous epidermis and cultured keratinocytes express RARα and RARγ, as well as RXRα and RXRβ, with RARγ and RXRα being the predominant RAR and RXR types (14,21). Pharmacological concentrations of RA can inhibit the growth of transformed keratinocytes in culture, and this effect is mediated largely through RARγ (26), suggesting a key role for this receptor in keratinocyte growth and differentiation. However, in contrast to observations using exogenous retinoid administration, murine knockout models suggest that the RARs have only a minor effect on development and homeostasis of the skin under normal conditions (9,48). These findings, however, do not preclude a role for retinoid signaling in pharmacological settings or in pathological conditions.

A role for retinoid signaling in epithelial tumorigenesis has been suggested by numerous observations. Treatment with certain tumor promoters, such as TPA or merazein, or exposure to ultraviolet radiation, the major causative factor of non-melanoma skin cancers (50), leads to a decrease in expression of both RARα and RARγ in the epidermis (14,42,75). Expression of these receptors is also gradually lost with progression of skin tumors from a benign lesion to malignant squamous cell carcinoma (14,87). Similar relationships between extinction of RAR expression and

tumorigenesis have been observed for a number of other carcinomas, including lung, breast and head and neck cancers (2,17,71,84,86).

We previously found that RA inhibits the growth of transformed keratinocytes in culture primarily through RARγ (26). In this study, we assessed the consequence of RAR ablation on *v*-Ha-Ras-induced keratinocyte tumorigenesis. We found that cells lacking RARγ, but not RARα, predisposed keratinocytes to Ras-induced tumorigenesis. This effect correlated with a loss of RA-induced cell cycle arrest and apoptosis as well as inhibition of AP-1 activity. Taken together, our findings are consistent with a role for the RARs, and in particular RARγ, as tumor suppressors in murine keratinocytes.

Materials and methods

Cell culture

Establishment of the wild type, RARα$^{-/-}$, RARγ$^{-/-}$, and RARαγ$^{-/-}$ keratinocyte lines has been described previously (26). Unless otherwise noted, cells were maintained in complete media comprised of S-minimal essential medium (S-MEM, Invitrogen), supplemented with L-glutamine (2mM), sodium pyruvate (1mM, Invitrogen), 10% chelex (Bio-Rad)-treated fetal calf serum (Invitrogen), epidermal growth factor (Sigma), insulin (5μg/ml, Sigma), hydrocortisone (0.5μM, Sigma), $MgCl_2$ (1.5mM), cholera toxin ($1.2x10^{-11}$M, Sigma), adenine sulfate (24μg/ml), and gentamicin (10μg/ml), at a final calcium concentration of 0.05mM as previously described.

Growth assay

Cells were seeded on 96-well tissue culture plates (Nunc) at a density of 800 cells/well. Twenty four hours post-plating, media was changed to include either vehicle (DMSO; 0.1%, v/v) or RA (1μM) with daily replenishment for 10-14 days. DNA content was assessed using crystal violet staining (41), with dye binding measured using a microplate reader (Molecular Devices) at 595nm. Growth was expressed as the mean absorbance value at 595nm (A_{595}) of four independent determinations.

Cell cycle analysis

Cells were seeded on 100mm tissue culture dishes (Nunc). After 24 hours in complete media, cells were synchronized by culture in media 0.1% in serum for 30 hours, and subsequently treated with RA (5μM) or vehicle in media 10% in serum for 48 hours. Cells were then harvested, fixed in 75% ethanol at -20°C overnight and stained with propidium iodide (PI, Sigma) in the presence of RNase A. Cell cycle populations were assessed by FACScan analysis (Becton Dickinson).

Apoptosis analysis

Cells were seeded on 100mm tissue culture dishes. After 24 hours of culture in complete media, cells were washed twice with PBS and treated with RA (5μM, Sigma) or vehicle in S-MEM containing 10% serum. Cells were harvested 48 hours post-treatment and apoptosis assessed by Hoechst 33258 (Sigma) staining and annexin V (BD Biosciences) binding.

RARE and AP-1 reporter assays

Cells were seeded on 6-well tissue culture dishes (Becton Dickinson). After 24 hours of culture in complete media, cells were transiently transfected using Lipofect ACE reagents (Invitrogen) with luciferase reporter plasmids (1μg/well) harboring either a 3xβRARE or a 3xTRE as described previously (3,26). A LacZ expression vector was included in all samples to control for transfection efficiency. Cells were subsequently treated with RA (1μM) or vehicle and luciferase activity assessed 24-48 hours post-treatment as

described previously (26). Reporter activity was expressed as the mean +/- the standard deviation of independent triplicate transfections.

Northern blot analysis

Total RNA was extracted from cells or tumors using Trizol reagent (Invitrogen) according to the manufacturer's directions. 10-15μg of RNA was fractionated on a 1% agarose-formaldehyde gel, transferred to Hybond-N^+ membrane (Amersham), and hybridized with ^{32}P-labelled cDNAs specific for keratin (K) 8, K10, K13 or β-actin according to the manufacture's instructions.

Western blot analysis

Cells were cultured on 100mm plates in the presence of RA (10^{-6}M) or vehicle. Proteins were isolated from each cell line and protein concentration determined using the DC protein assay kit (BioRad). Forty micrograms of protein were solubilized in loading buffer, fractionated on a SDS-PAGE gel and electroblotted to an immobilon-P PVDF membrane as recommended by the supplier (Millipore). Proteins of interest were detected using the appropriate antibodies and detection with ECL (Amersham).

To assess *v*-Ha-Ras expression, proteins were extracted from cultures 2 days post-infection. Primary antibody against Ha-Ras was purchased from Santa Cruz. For kinase activation assays, cells were starved for 30 hours in S-MEM (Invitrogen) lacking serum, EGF, and insulin, and subsequently stimulated with serum (10%), EGF (10ng/ml), or insulin (5μg/ml) for 5-20 min

prior to lyses. Monoclonal antibodies against phospho-ERK1/2 (New England), phospho-JNK (Santa Cruz), or phospho-p38 MAP kinase (CalBiochem), or a polyclonal antibody against phospho-Akt (Cell signaling) were used. For cell cycle analysis, primary antibodies against $p21^{cip1}$, $p27^{kip1}$, cyclinD1, pRb (from Santa Cruz), and anti-phosphorylated Rb (Pharmingen) were employed. Caspase-3 cleavage was detected using an antibody against the active form of the enzyme (Cell Signaling).

Reconstitution of RAR expression

RAR$\alpha\gamma$ double-null keratinocytes were electroporated with linearized plasmids encoding RARα1 or RARγ1 in pIRESpuro (Clontech), and puromicin resistant clones isolated. RAR function and expression was assessed by RARE reporter assay and by EMSA.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously (26,32). Briefly, nuclear protein extracts (3μg) from cultures were incubated in 13.4mM Hepes (pH7.5), 30mM KCl, 1mM dithiothreitiol, 3% glycerol, 0.1% NP-40, 0.25μg/μl BSA, and 60ng/μl poly(dI-dC) for 30 min on ice, following which a ^{32}P-labled oligonucleotide probe encoding an RARE was added and incubation continued at room temperature for an additional 30 min. Protein-DNA complexes were resolved by polyacrylamide gel electrophoresis and products revealed by autoradiography.

Southern blot analysis

Genomic DNA (10-15μg) was restricted with appropriate enzymes, fractionated on a 1% agarose gel, and transferred to a Hybond-N^+ membrane (Amersham). A ^{32}P-labelled HindIII-PstI fragment from HB11 (ATCC(18)) was used as a probe to detect *v*-Ha-*ras* integrants. Hybridization was performed according to the manufacturer's instructions.

Retroviral infection and tumorigenicity assay

Two days after plating, keratinocytes were infected with a *v*-Ha-*ras* retrovirus at a MOI of 1-2 as described previously (66). Cultures were harvested 4 days after infection and re-suspended in PBS at a density of 10^7 cells/ml. $1x10^6$ cells were injected subcutaneously (*s.c.*) into athymic nude mice and tumor incidence and size noted 6-8 weeks post-injection. Tumors were subsequently processed for Northern or Southern blot analysis or were fixed in Bouin's solution (Sigma) and embedded in paraffin. For histopathological evaluation, tumors were sectioned and stained with hematoxylin and eosin.

Results

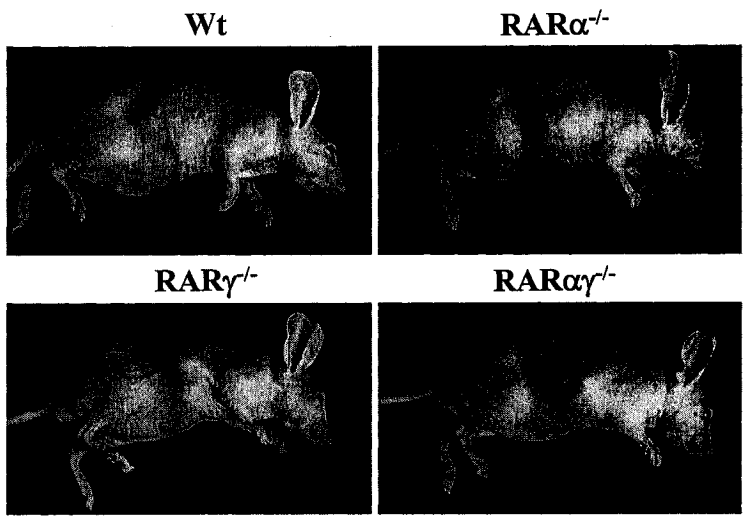
RARγ suppresses Ras-induced tumorigenesis

Previous studies revealed a correlation between loss of RARα and/or RARγ and development of papillomas and squamous cell carcinoma in both mouse and man (14,87). To further investigate this relationship, we assessed the effects of loss of these receptors on tumorigenesis. Because of the perinatal lethality caused by disruption of RARα and/or RARγ (47-49), we used immortalized keratinocyte cell lines lacking one or both of these receptor types as a model system (26).

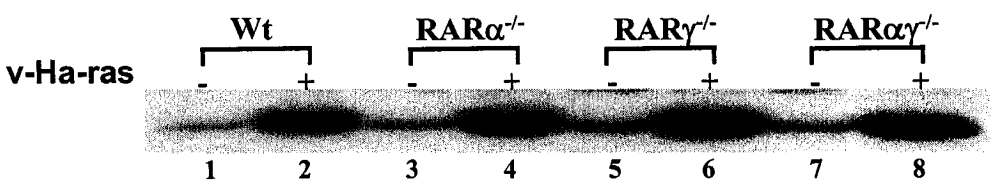
Neither the wild type nor any of the RAR null cells formed tumors following *s.c.* injection (data not shown). However, following transduction with a *v*-Ha-*ras* retrovirus (66), RARγ$^{-/-}$ and RARαγ$^{-/-}$ cultures, but not wild type or RARα null cells, gave rise to tumors at a high incidence (Figure 3-1A, Table 1). Western blot analysis indicated that the predisposition of RARγ$^{-/-}$ or RARαγ$^{-/-}$ cells to develop tumors was not due to variation in oncogene expression, as all lines exhibited similar levels of Ha-Ras following retroviral transduction irrespective of genotype (Figure 3-1B; compare lanes 2, 4, 6, and 8). Moreover, all tumors exhibited similar levels of *v*-Ha-*ras* integrants, and were of polyclonal origin as assessed by Southern blot (Figure 3-1C and data not shown).

Figure 3-1. Analysis of keratinocyte tumorigenicity. A) Tumorigenicity of RAR null keratinocytes. Cultures with genotypes noted were transduced with a *v*-Ha-*ras* retrovirus and injected subcutaneously into nude mice. Representative tumors 8 weeks post-injection are shown. B) Western blot analysis of Ras expression from RAR$\alpha^{-/-}$, RAR$\gamma^{-/-}$ and RAR$\alpha\gamma^{-/-}$ cell lines 48 hours post-transduction. C) Southern blot analysis of *v*-Ha-*ras* integrants from RAR$\gamma^{-/-}$ and RAR$\alpha\gamma^{-/-}$ tumors. Genomic DNAs from mock and *v*-Ha-*ras*-transduced RAR$\alpha\gamma^{-/-}$ cells (denoted v-ras) were used as controls.

A


Wt
RARα-/-
RARγ-/-
RARαγ-/-

B


v-Ha-ras
Wt
RARα-/-
RARγ-/-
RARαγ-/-
- + - + - + - +
1 2 3 4 5 6 7 8

C

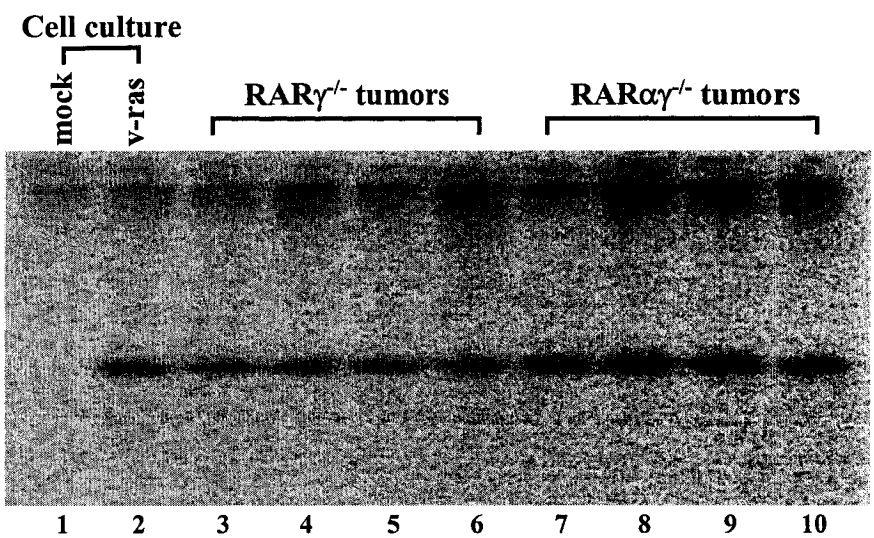

Cell culture
mock
v-ras
RARγ-/- tumors
RARαγ-/- tumors
1 2 3 4 5 6 7 8 9 10

Table 3-1. Tumor incidence

Genotype	Wt		RAR $\alpha^{-/-}$			RAR$\gamma^{-/-}$		RAR $\alpha\gamma^{-/-}$	
Independent Cell lines	1	2	1	2	3	1	2	1	2
#Tumors/ Injection	0/12	0/4	0/10	0/8	0/4	5/10	3/4	9/16	7/8
Total incidence	0/16		0/22			8/14		16/24	
Tumor volume (cm^3)	-		-			1.2		2.2	

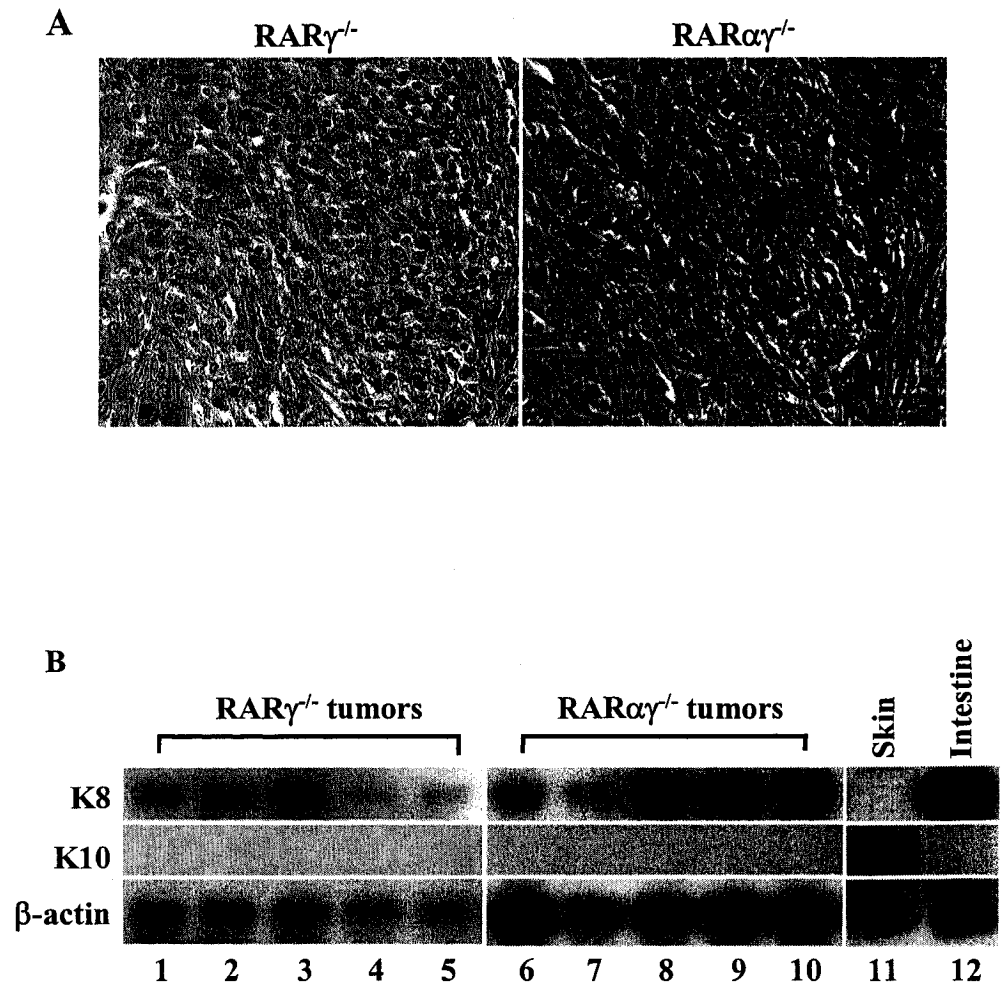
Histologically, tumors derived from both RARγ$^{-/-}$ and RARαγ$^{-/-}$ cultures exhibited characteristics consistent with squamous cell carcinoma (Figure 3-2A). In agreement with this, Northern blot analysis (Figure 3-2B) showed a lack of expression of K10, indicative of malignant conversion (58), and gain of expression of K8, a marker associated with the malignant epithelial phenotype (44). K13, a premalignant marker of skin tumor progression (25), was not detected in these samples (data not shown).

These studies suggest that loss of RARγ predisposed keratinocytes to Ras-induced tumorigenesis, and that RARγ may function to suppress both tumor formation and the malignant conversion of the epidermal tumors. Interestingly, although RARα$^{-/-}$ cells were not predisposed to tumorigenesis in this model system, tumors derived from RARαγ$^{-/-}$ cultures were significantly larger than those from RARγ$^{-/-}$ cells (Figure 3-1A and Table 3-1), suggesting that RARα can impact on tumor growth (see also below).

RAR disruption does not affect Ras-induced targets

Oncogenic ras, together with loss of RARγ, was necessary for in vivo tumor formation in this model. Activated Ras acts through multiple downstream kinases, such as ERK, JNK, p38 kinase, and Akt, contribute to cellular transformation (39,76,82). Certain of these targets have also been reported to be affected by RA in some cell types (5,24,45,88). It was therefore conceivable that loss of RARγ might affect tumor formation through altered expression or activation of these kinases. To investigate such a possibility, we

Figure 3-2. Characterization of tumors. A) Hematoxylin and eosin stained sections from representative tumors derived from the noted cell lines. B) Northern blot analysis from independent tumor samples. Note the loss of K10 and gain of K8 expression in all tumors analyzed. RNA from skin and intestine were used as positive controls for K10 and K8 expression, respectively.


A
RARγ-/-
RARαγ-/-
B
RARγ-/- tumors
RARαγ-/- tumors
Skin
Intestine
K8
K10
β-actin
1 2 3 4 5 6 7 8 9 10 11 12

investigated the effect of RAR ablation on activation of these intermediaries by various stimuli.

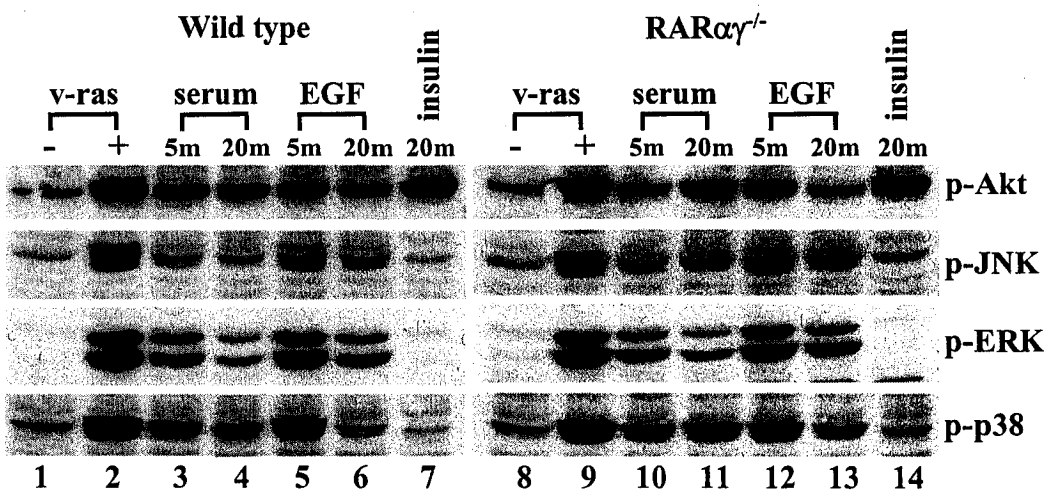
v-Ha-*ras* infection, serum or EGF stimulation enhanced phosphorylation of ERK, JNK, p38 and Akt comparably in both wild type and RAR$\alpha\gamma^{-/-}$ cells (Figure 3-3; compare lanes 2-6 with lane 1 and lanes 9-13 with lane 8). Insulin treatment likewise induced activation of Akt in a genotype-independent manner (Figure 3-3; compare lane 7 with lane 14). As these kinases represent the major mediators of Ras-induced tumorigenesis (39,68,76,82), it would appear unlikely that RAR loss impacted on tumor formation by altered activity of these intermediaries.

Ablation of RARγ attenuates RA-induced cell cycle arrest and apoptosis

Cellular transformation typically affects multiple cellular characteristics, including resistance to growth inhibitory and apoptogenic stimuli (27). RA can cause cell cycle arrest and apoptosis of many transformed cell types, and loss of RARs may affect these outcomes. We therefore tested whether RAR ablation compromised the ability of RA to induce cell cycle arrest and/or apoptosis in the transformed keratinocyte lines.

When cultured in complete media RA treatment does not cause significant cell death, although RARγ-dependent growth inhibition is observed (26). However, omission of EGF, insulin, hydrocortisone and cholera toxin from the complete media resulted in potent RA-induced cell-cycle arrest and apoptosis. Synchronized wild type and RARα null cultures treated with RA for

Figure 3-3. Western blot analysis of the effect of RAR disruption on Ras targets. Wild-type or RAR$\alpha\gamma^{-/-}$ cultures were either transduced with *v*-Ha-*ras* at an MOI of 1 or treated with noted stimuli and whole cell extracts subjected to Western blot analysis as described in Materials and Methods.


Wild type
RARαγ-/-
v-ras
serum
EGF
insulin
-
+
5m
20m
5m
20m
20m
v-ras
serum
EGF
insulin
-
+
5m
20m
5m
20m
20m
p-Akt
p-JNK
p-ERK
p-p38
1
2
3
4
5
6
7
8
9
10
11
12
13
14

48 hours exhibited an accumulation of cells in G0/G1 and a decrease in S+G2/M populations (Figure 3-4A). In marked contrast, G1 arrest was not seen in RAR$\gamma^{-/-}$ or RAR$\alpha\gamma^{-/-}$ cultures, which exhibited cell cycle profiles similar to vehicle-treated control cultures (Figure 3-4A). These observations suggest that loss of RARγ, but not RARα, abolished the inhibitory effect of RA on cell cycle progression.

We assessed expression of several cell cycle regulators involved in regulating the G1 to S phase transition, including $p21^{cip1}$, $p27^{kip1}$, cyclin D1, and pRb, each of which has been shown to be affected by exogenous RA in diverse cell types (4,7,57). Since no significant differences was seen between the wild type and RAR$\alpha^{-/-}$ keratinocytes or between the RAR$\gamma^{-/-}$ and RAR$\alpha\gamma^{-/-}$ keratinocytes as regards cell-cycle arrest, we compared the effect of RA on expression of these proteins in wild type and RAR$\alpha\gamma^{-/-}$ keratinocytes.

Phosphorylation of Rb by cyclin-dependent kinases (CDKs) in late G1 provides a checkpoint control for G1 to S phase transition *via* release of E2F proteins (13,70). In both wild type and RAR$\alpha\gamma$ null cultures, only the hyperphosphorylated form of Rb (ppRb) is seen 48 hours after addition of serum to starved cells (Figure 3-4B; lanes 1 and 3). RA treatment inhibited Rb hyperphosphorylation in the wild type cultures, but did not affect Rb expression, irrespective of receptor status (Figure 3-4B; lanes 2 and 4). In wild-type cells, RA treatment also caused an RAR-dependent increase in expression of both $p21^{cip1}$ and $p27^{kip1}$, both of which are negative regulators of G1 CDKs, and a decrease in the level of cyclin D1, a positive regulator of

G1 CDKs (54,78). In contrast, neither of these changes took place in RA-treated RARαγ$^{-/-}$ cells (Figure 3-4B; compared lane 2 with lane 1, and lane 4 with lane 3).

In addition to cell cycle arrest, RA can also induce apoptosis in many cell types. Consistent with this, RA induced morphological features of apoptosis in both wild type and RARα$^{-/-}$ cultures, such as membrane blebbing. Such effects were, however, not observed in the RA-treated RARγ$^{-/-}$ or RARαγ$^{-/-}$ cultures (data not shown). These observations were consistent with annexin V binding (Figure 3-5A) and Hoechst staining assays (Figure 3-5B), both of which suggest that RA induces significant apoptosis in wild type and RARα. In marked contrast, RA did not induce such changes in either RARγ$^{-/-}$ or RARαγ$^{-/-}$ cultures (Figure 3-5A and -5B).

Consistent with the results from apoptosis analysis, RA treatment of both wild type and RARα$^{-/-}$ cultures, but not RARγ$^{-/-}$ or RARαγ$^{-/-}$ line, was associated with processing of procaspase-3 to its active form (Figure 3-5C). To ensure that this block was not an inherent feature of these RAR null lines, we tested caspase-3 following treatment with 4-HPR (4-hydroxyphenretinamide), a synthetic retinoid which is able to induce cell growth arrest and apoptosis in an RAR-independent manner (26,83). In contrast to RA, treatment with 4-HPR resulted in apoptosis and activation of caspase-3 in both wild type and RARαγ$^{-/-}$ cultures (Figure 3-5C). This observation suggests that the lack of induction of cell cycle arrest and

Figure 3-4. RARγ mediates cell-cycle arrest. A) Effect of RA on cell-cycle progression. Synchronized cultures of wild-type (Wt) and RARαγ$^{-/-}$ cells were assessed for cell-cycle progression 48 hours after treatment as described in Materials and methods. B) Effect of RA on cell cycle regulators. Wild-type and RARαγ$^{-/-}$ cultures were treated with RA (5μM) or vehicle and whole-cell lysates assessed for the expression of hyperphosphorylated Rb, total Rb, cyclin D1, p27^{kip1} or p21^{cip1} by Western blot as described in Materials and methods. *hypophosphorylated pRb; **hyperphosphorylated pRb.

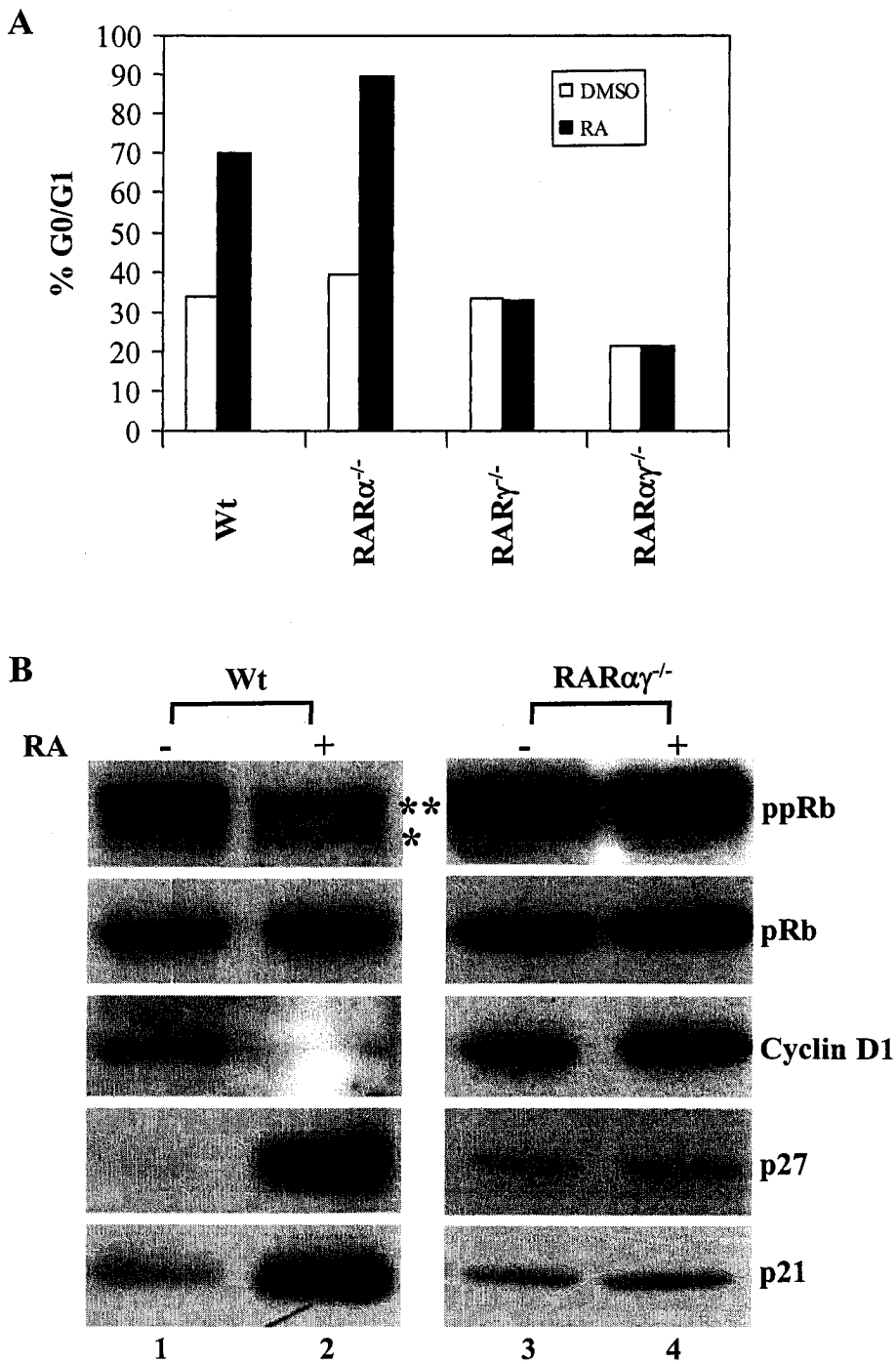
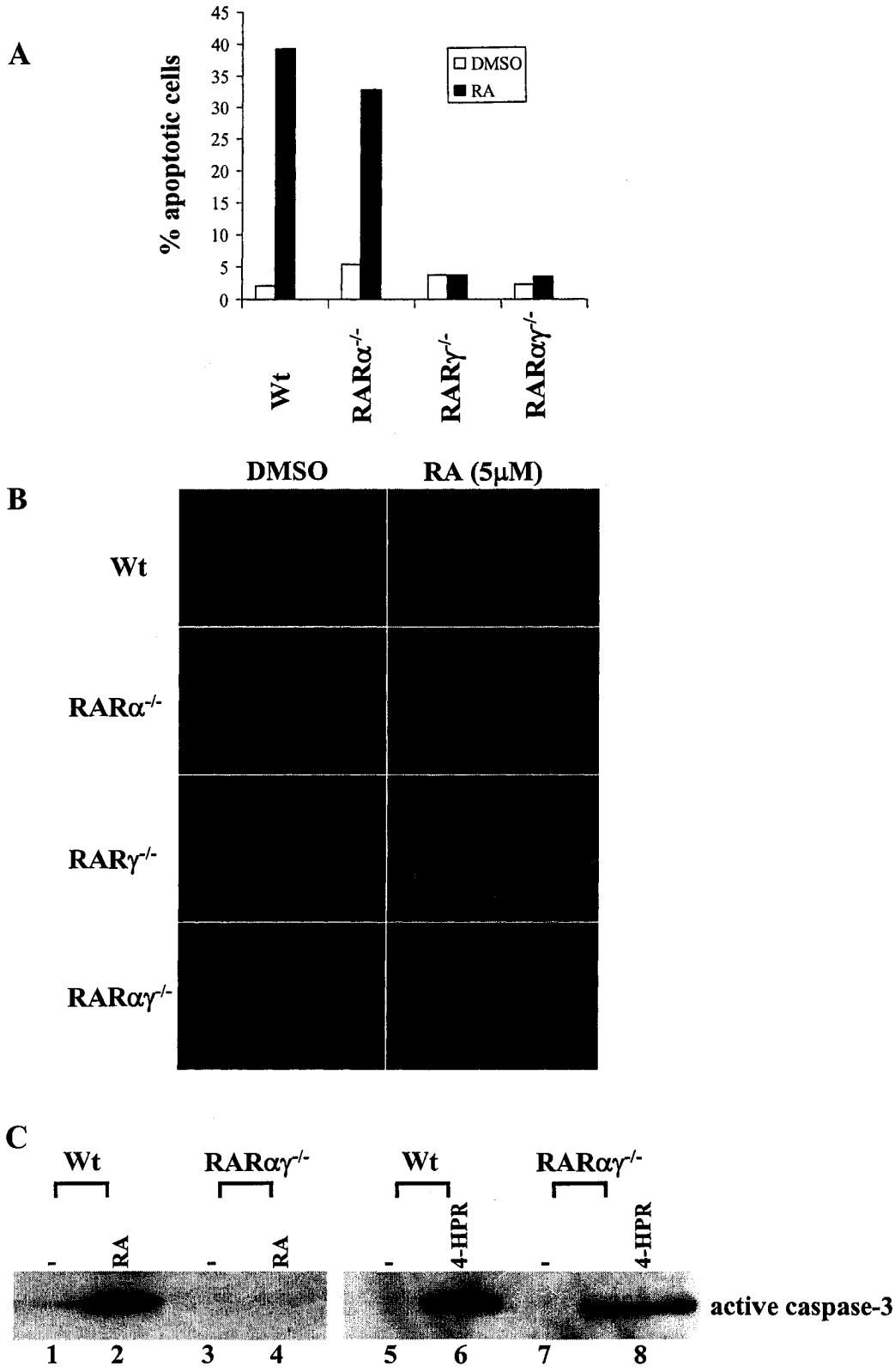

A
100
90
80
70
60
50
40
30
20
10
0
% G0/G1
DMSO
RA
Wt
RARα-/-
RARγ-/-
RARαγ-/-
B
Wt
RARαγ-/-
RA
-
+
-
+
**
*
ppRb
pRb
Cyclin D1
p27
p21
1
2
3
4

Figure 3-5. RARγ conveys a pro-apoptotic retinoid signal. A) Cells from wild-type (Wt) or RARαγ$^{-/-}$ cultures treated with RA (5μM) or vehicle were collected and processed for annexin V-binding as described in Materials and method. B) Hoechst staining of cultures treated in an identical manner to A. C) Western blot analysis of the active form of caspase-3 was performed on whole cell extracts from wild-type or RARαγ$^{-/-}$ cultures treated with RA (5μM), 4-HPR (5μM) or vehicle.


A
% apoptotic cells
45
40
35
30
25
20
15
10
5
0
DMSO
RA
Wt
RARα-/-
RARγ-/-
RARαγ-/-
B
DMSO
RA (5μM)
Wt
RARα-/-
RARγ-/-
RARαγ-/-
C
Wt
RARαγ-/-
Wt
RARαγ-/-
-
RA
-
RA
-
4-HPR
-
4-HPR
active caspase-3
1
2
3
4
5
6
7
8

apoptosis in RARαγ null cells is directly attributable to receptor loss, and is not an inherent defect of this particular line.

RAR reconstitution restores RA-responsiveness and reverses the tumorigenic potential of Ras-transduced RARαγ$^{-/-}$ keratinocytes

Decreased expression of RAR is seen in many cancer cell lines (84), and restoration of RAR expression can suppress tumor formation of some such lines *in vivo* (31). Moreover, although multiple, independent, RARγ and RARαγ null lines were all tumorigenic in our model, it is conceivable that RAR loss was not the primary basis underlying tumor formation. In order to further investigate this, we derived RARαγ$^{-/-}$ lines re-expressing RARα or RARγ and assessed their predisposition to *in vivo* tumor formation.

While a number of clones expressing various levels of RARα were readily derived, we were unable to recover clones expressing more than trace amounts of RARγ. It is notable that a similar phenomenon has been seen in some cancer cell lines which are incapable of re-expression of certain tumor suppressors (80).

Two clones re-expressing RARα, designated as IRES-RARα$^{\#2}$ and IRES-RARα$^{\#5}$, exhibited expression levels slightly greater than total RAR seen in wild type cells, as judged by EMSA (Figure 3-6A; lanes 9 and 10). Each of these reconstituted cell lines also mediated significant induction of an RARE reporter that was greater than that seen in wild type keratinocytes (Figure 3-6B). Two typical lines reconstituted with RARγ displayed much

lower levels of expression by EMSA (Figure 3-6A; lanes 11 and 12), and exhibited an induction of the RARE reporter greater than RAR null cells, but less than that seen in wild type cultures (Figure 3-6B).

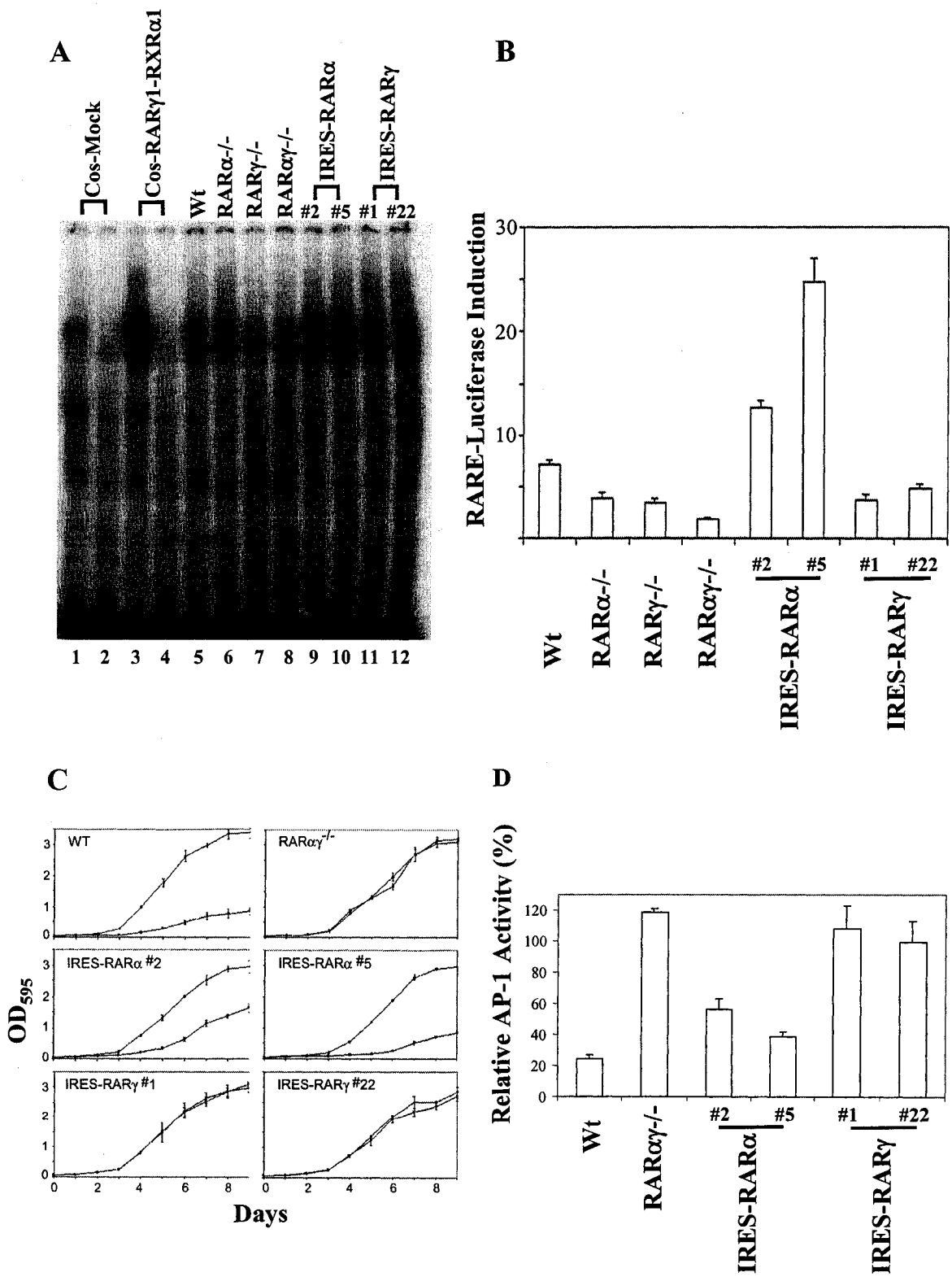
Exogenous RA mediates significant growth inhibition of wild type cells, and RAR$\alpha\gamma^{-/-}$ cells are completely resistant to this effect (Figure 3-6C) (26). Growth inhibition was partially (IRES-RAR$\alpha^{\#2}$) or completely (IRES-RAR$\alpha^{\#5}$) restored, relative to wild type cultures, in clones re-expressing RARα (Figure 3-6C). By contrast, lines expressing low levels of RARγ (Figure 3-6C) or RARα (data not shown) were resistant to growth inhibition, despite an intermediate level of induction of the RARE reporter.

Trans-repression of AP-1 activity by RA has been proposed to underlie the growth inhibitory effects of retinoids (2,22). Consistent with this, RA repressed the activity of an AP-1 reporter vector in wild type cells, but not in RAR null keratinocytes (Figure 3-6D). *Trans*-repression in RAR$\alpha\gamma^{-/-}$ cells was recovered in lines re-expressing high levels of RARα (Figure 3-6D), in good agreement with the growth inhibitory effects of RA (Figure 3-6C). Notably, however, *trans*-repression did not strictly correlate with either relative protein levels (Figure 3-6A) or activation of an RARE (Figure 3-6B). RA failed to repress AP-1 activity in all lines expressing low levels of RARα or RARγ (Figure 3-6D and data not shown).

To determine whether re-expression of RARs could repress the tumorigenic potential of RAR null keratinocytes, we compared tumor

Figure 3-6. RAR reconstitution in RARαγ$^{-/-}$ keratinocytes. A) EMSA analysis of RAR expression. Nuclear proteins (5μg) were extracted from the noted cell lines, incubated with a labeled human RARβ2 RARE oligonucleotide as described in Materials and methods and products resolved by nondenaturing PAGE. Nuclear proteins from mock and RARγ1/RXRα1 transfected COS-7 cells were used as negative and positive controls, respectively. Lanes 2 and 4 are binding reactions carried out in the presence of a 50-fold excess of unlabled RARE. IRES-RARα and IRES-RARγ represent RARαγ$^{-/-}$ clones re-expressing RARα or RARγ, respectlively. S; specific binding. NS; non-specific binding. B) Transcriptional regulation in parental and RAR-reconstituted cell lines. Wild type (Wt), RARα$^{-/-}$, RARγ$^{-/-}$, RARαγ$^{-/-}$, and RARαγ$^{-/-}$ cells re-expressing RARα (IRES-RARα #2 or #5) or RARγ (IRES-RARγ #1 or #22) were assessed for their ability to induce the activity of an RARE reporter plasmid as described in Materials and methods. Values are the means of independent triplicate determination, and expressed as fold induction by RA (1μm) relative to vehicle. C) Effects of RA on keratinocyte growth. Wild type (Wt), RARαγ$^{-/-}$, and RARαγ$^{-/-}$ cells re-expressing RARα or RARγ (as in panels a and b) were treated with either vehicle or RA (1μM) and cell growth assessed as described in Materials and methods. Results are expressed and the means +/- the standard deviations from four independent determinations. D) Effects of RA on AP-1 activity. Wild type (Wt), RARαγ$^{-/-}$, or RAR re-expressing lines were transfected with a 3xTRE reporter plasmid. Luciferase activity was assessed 48 h after

treatment with either vehicle or RA (1μM). Values are the mean +/- standard deviation of three determinations, and are expresses as percent activity of vehicle treated cultures.


A
Cos-Mock
Cos-RARγ1-RXRα1
Wt
RARα-/-
RARγ-/-
RARαγ-/-
IRES-RARα
IRES-RARγ
#2
#5
#1
#22
1 2 3 4 5 6 7 8 9 10 11 12
B
RARE-Luciferase Induction
30
20
10
Wt
RARα-/-
RARγ-/-
RARαγ-/-
#2
#5
IRES-RARα
#1
#22
IRES-RARγ
C
WT
RARαγ-/-
IRES-RARα #2
IRES-RARα #5
IRES-RARγ #1
IRES-RARγ #22
OD595
Days
D
Relative AP-1 Activity (%)
120
100
80
60
40
20
0
Wt
RARαγ-/-
#2
#5
IRES-RARα
#1
#22
IRES-RARγ

formation between parental RAR$\alpha\gamma^{-/-}$ cells and receptor-reconstituted clones. In marked contrast to the RAR$\alpha\gamma^{-/-}$ cells, which formed solid tumors at a high incidence, neither of the clones over-expressing RARα yielded tumors (Table 3-2). However, tumors were derived from clones expressing either RARα or RARγ at low levels at an incidence comparable to the parental control (Table 3-2 and data not shown).

Table 3-2. Tumor incidence of RAR-reconstituted keratinocytes

RAR Transgene	Clone (No.)	Solid Tumors/ Injections
IRES-RARα	**#2**	**0/10**
	#5	**0/10**
IRES-RARγ	**#1**	**6/10**
	#22	**7/10**
Control	**Wt**	**0/4**
	RARαγ$^{-/-}$	**5/6**

Discussion

Skin carcinogenesis is a multistage process, which includes tumor initiation, promotion, and progression (40,90). Dietary retinoid intake has been associated with a reduction in the incidence of a number of cancers, although recent clinical trials have raised some questions as to this validity of this relationship in at least some cases (30,34,53,56,60,61). In animal models, RA can inhibit papilloma formation and malignant conversion in chemical carcinogenesis protocols (10,16). In both man and mouse, RARα and RARγ are expressed in the epidermis, with RARγ the predominant receptor type (14,21). Expression of these RARs decreases progressively during skin carcinogenesis, suggesting that receptor loss may be an important causal step in some epidermal cancers (14,21). Similar observations have been made as regards extinction of RARβ expression in diverse neoplasms including lung and head and neck cancers (84).

RARγ functions as a tumor suppressor

To further investigate the relationship between RAR expression and epidermal carcinogenesis, we assessed the tumorigenic potential of a comprehensive panel of RAR-deficient keratinocyte lines (26). Loss of RARs alone did not predispose to a malignant phenotype. However, transduction with a v-Ha-ras retrovirus (66) revealed that loss of RARγ, but not RARα, predisposed keratinocytes to form tumors *in vivo,* suggesting that endogenous RARγ functions as a tumor suppressor in this cell type.

Tumors derived from both RARγ$^{-/-}$ and RARαγ$^{-/-}$ cultures exhibited histological properties consistent with squamous cell carcinoma. Loss of expression of K10, a hallmark of malignant conversion (58), and gain of K8, a marker associated with late stage malignancy (44) are consistent with this conclusion. The molecular phenotype of these tumors also implies that RARγ might affect both tumor progression and malignant conversion.

Loss of RARα alone did not affect the incidence of tumor formation. However, RARαγ$^{-/-}$ tumors were significantly larger than those derived from RARγ$^{-/-}$ cultures, suggesting that endogenous RARα might function to affect tumor growth. Moreover, re-introduction of RARα in RAR-null keratinocytes reverted the tumorigenic phenotype. As RARγ is the most prevalent RAR type expressed in keratinocytes (14,21,26), the simplest interpretation of these observations are that both receptor types functionally overlap, and that ablation of endogenous RARγ, but not RARα, results in loss of a critical threshold of receptor activity and subsequent sensitivity to Ras-induced tumor formation (see also below).

Loss of RARs affects retinoid-induced cell cycle arrest, apoptosis and AP-1 activity

Ras signals through multiple downstream targets (38,65), many of which are involved in Ras-induced oncogenesis (39,76,82). RA has been shown to affect the expression or activation of certain of these intermediaries (5,24,45,88), raising the possibility that loss of RARs might impact on

tumorigenesis through alterations in expression or function of these Ras targets. However, Western blot analysis revealed no appreciable differences between the wild type and RAR$\alpha\gamma^{-/-}$ cells in terms of expression or activation of any of these kinases.

Cancer cells acquire properties, including the ability to resist growth inhibitory and apoptogenic stimuli, which confer a selective growth advantage (27). We found that RA induced cell cycle arrest at G1 and apoptosis, and that these effects were mediated primarily through RARγ. At the molecular level, cell cycle arrest correlated with an upregulation of both $p21^{cip1}$ and $p27^{kip1}$, and loss of expression of cyclin D1; hypophosphorylation of pRb and subsequent arrest at G1 was likely secondary to these events. Apoptosis correlated with activation of caspase-3, a classic death effector (29). These outcomes were not due to an inherent resistance in RAR null keratinocytes, as both cell cycle arrest and apoptosis could be conferred either by reintroduction of RARα or by 4-HPR, a retinoid which can elicit receptor-independent effects (26,83).

While the principle retinoid target(s) mediating growth arrest is presently unknown, expression of $p21^{cip1}$, $p27^{kip1}$ and cyclin D1 is affected in a similar manner by RA in a number of systems. For example, $p21^{cip1}$ has been shown to be directly regulated by RARs, and this induction correlates with growth arrest (46). Retinoids have been shown to impact on expression of $p27^{kip1}$ and cyclin D1 in a post-translational manner, again correlating with growth inhibition (19,43). In a number of cases, retinoid-induced cell cycle

arrest has also been associated with reduced Rb hyperphosphorylation, and it has been proposed that this outcome is secondary to induction of cyclin-dependent kinase inhibitors, including $p21^{cip1}$ and/or $p27^{kip1}$ (57,91). Finally, retinoids have been shown to initiate apoptotic programs, including activation of caspase-3, in a number of cell types (62).

In the present study, $p21^{cip1}$, $p27^{kip1}$ and cyclin D1 were all affected by RA in a manner which correlated closely with growth inhibition, apoptosis and inversely with the tumorigenic potential of wild type and RAR null lines. While the identity of the primary target(s) involved in these events is unknown, it is conceivable that mis-regulation of one or more of these targets underlies the predisposition of RAR-null keratinocytes to ras-induced tumorigenesis. For example, cyclin D1 has been shown to play a key role in oncogenic ras-induced epidermal tumors (64), while Rb is frequently mutated in a number of epithelial tumors (13,59). Attenuation of $p21^{cip1}$ to Ras signaling has been associated with Ras-induced hyperplasia (1,63,74), while $p27^{kip}$ expression has been positively correlated with prognosis of a number of cancers (55).

Although retinoids have been shown to impact on the expression of a number of cyclins and cyclin dependent kinase inhibitors in diverse tissues and cell lines, the role of the various RARs on growth inhibition and expression of these cell cycle effectors appears to differ depending on the cell type examined. In F9 embryocarcinoma cells, which express all three RAR types following RA treatment, loss of RARβ, but not RARα or RARγ, leads to resistance to retinoid-mediated growth inhibition (20). Extinction of RARβ has

also been suggested to play a key role in head and neck, breast, ovarian and lung cancers (84). However, RARβ is not expressed in epidermal keratinocytes, and our data suggest that RARγ plays a pivotal role in retinoid-induced growth arrest (26) and tumorigenesis. By contrast, others have proposed that loss of RARα plays a critical role in epidermal tumorigenesis (15,28). In this regard, although we have found that loss of endogenous RARγ appears to be closely related to Ras-induced tumor formation, re-expression of exogenous RARα can suppress tumor formation when expressed at sufficient levels. It is therefore conceivable that both receptor types are functionally equivalent, at least as regards this particular outcome. Similar functional overlap has also been described for RAR-null mice (23,48,52) although such apparent redundancy may be an artifactual manifestation of gene disruption (72,73).

Retinoids can trans-regulate a number of signaling pathways, such as AP-1 (Fos-Jun) in diverse cell types (2,51). In this regard, it is interesting to note that AP-1 signalling is inhibited by exogenous RA in wild type, but not RAR-null keratinocytes (this study and (26)). This trans-repression effect is recovered in RAR-null keratinocytes after reintroduction of exogenous RARα, and correlates with resistance to Ras-induced tumorigenesis. A number of studies have demonstrated a key role for AP-1 signaling in epidermal tumorigenesis (33,35,89). AP-1 signaling has also been related to both cell cycle and apoptosis (69), and it is therefore tempting to speculate that RA-repression of this pathway may play a pivotal role in tumor suppression.

Receptor specificity or redundancy?

In an attempt to determine whether reconstitution of RAR expression in RAR-null keratinocytes could suppress Ras-induced tumorigenesis, we derived a number of cell lines re-expressing RARα or RARγ. It is notable that, although some lines expressing high levels of RARα were obtained, we were unable to recover clones expressing significant levels of RARγ. Similar observations have been made as regards stable expression of RARβ in certain cell types (W.E.C. Bradley, personal communication). This phenomenon resembles the effect of tumor suppressor gene hypersensitivity described for some cancer cell lines, whereby re-introduction of a tumor suppressor gene, which has been silenced or functionally mutated in the host line, is difficult to effect due to cell death or growth inhibition mediated by introduction of the exogenous gene (79-81). The fact that RARα clones expressing at appreciable levels of functional receptor were recovered may be indicative of some events related to growth inhibition or apoptosis, which are mediated preferentially by high levels of RARγ. Such functions, however, would not appear to be essential for RAR-dependent tumor suppression *in vivo*.

Although RARα, when expressed at sufficient levels, can suppress Ras-induced tumor formation, several observations suggest that it functionally differs from RARγ. For example, both RARα and RARγ null cell lines exhibit essentially equivalent potency in inducing an RARE reporter, although the

latter represents approximately 90% of all RAR protein in this cell type (Figure 6A and (26)). Conversely, endogenous RARγ is the major mediator of retinoid-induced growth arrest, tumor suppression and *trans*-repression of AP-1 activity (26). These observations are consistent with prior work suggesting that, while the RARs exhibit functional overlap, they may differ in their relative efficacy as regards certain parameters (6,11,12,72). Thus, while exogenous RARα can compensate for loss of RARγ, it also suggests that RARE induction is an irrelevant predictor of tumor suppression, while *trans*-suppression of AP-1 and growth inhibition appear to be more relevant parameters.

In conclusion, our data demonstrate that endogenous RARγ functions in a manner consistent with it being a tumor suppressor in the context of this model system. Whether RARγ functions in a similar manner in other protocols is presently under investigation. Moreover, while repression of AP-1 activity correlates well with RAR-mediated inhibition of tumorigenesis, the target genes mediating this effect are presently undefined.

Abbreviations

RA, all-*trans* retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; TPA, 12-*O*-tetradecanolphorbol-13-acetate; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; JNK, c-Jun amino-terminal kinase; RARE, retinoic acid responsive element; TRE, TPA responsive element; AP-1, activating protein 1; *s.c* injection, subcutaneous injection.

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Chapter IV

Dominant negative RARs elicit epidermal defects through a non-canonical pathway

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Preface

Studies on the roles of RARs in epidermal development have led to controversial conclusions. Targeted disruption of both RARα and RARγ, the total RARs expressed in the epidermis, does not result in any significant epidermal defects, suggesting that RARs are not essential for epidermal development. By contrast, targeted expression of dominant negative (dn) RARs in the basal epidermis causes developmental arrest of the epidermis, indicating that RARs are required for normal epidermal organogenesis. To explore the basis underling this discrepancy, a direct phenotypic comparison of these two models was made. This Chapter presents the experimental results showing that dnRARα downregulates a non-canonical RAR target, which may be responsible for the severe epidermal phenotypes of the dnRARα transgenic mice.

The candidate carried out all the experiments under the supervision of Dr. David Lohnes. This manuscript was drafted by the candidate, and revised by Dr. David Lohnes.

Abstract

Retinoic acid (RA) excess or deprivation affects many epithelial tissues including the skin. Consistent with this, transgenic mice expressing a dominant negative RARα in basal keratinocytes exhibit severe epidermal defects. By contrast, ablation of the entire complement of RA receptors (RARs) expressed in the epidermis (RARα and RARγ) results in only a mild skin phenotype. To begin to understand the basis for these differences, we compared the epidermal phenotypes of transgenic mice expressing various types of dominant negative RARs with the RARαγ null offspring. Consistent with previous results, transgenic offspring expressing a dominant negative RARα under the control of the K14 promoter exhibited severe epidermal defects. Further characterization revealed a close phenocopy to the defects characteristic of p63 null mice. Consistent with this, p63 expression was greatly reduced in the transgenic epidermis. RARαγ$^{-/-}$ epidermis, by contrast, exhibited normal expression of p63 and markers associated with normal skin skin phenotype. Since RARα and RARγ represent all RARs expressed in the epidermis, the effects elicited by the dominant negative RAR could conceivably be related to a non-canonical event. To assess this, we examined the effects of a comparable dominant-negative RAR that was incapable of DNA binding. We found that this receptor recapitulated the epidermal phenotype of the dominant-negative RAR transgene. These data are consistent with a model whereby dominant negative RARs interfere with

programs, such as p63 expression, *via* a crosstalk mechanism, which is independent of direct regulation of retinoid target genes.

Introduction

Retinoic acid (RA) has long been known to modulate cell growth and differentiation in many epithelial tissues, including the epidermis (1-8). Topical administration of pharmacological amounts of RA can cause epidermal hyperplasia (5;9;10), and impaired epidermal barrier function owing to reduced cohesiveness of the stratum corneum (11). Conversely, deprivation of dietary vitamin A, the precursor of RA, generally leads to hyperkeratosis of the skin (12;13).

The biological functions of RA are mediated *via* a family of ligand-inducible nuclear receptors, the RA receptors (RARα, β and γ and their isoforms). RA target genes are regulated by heterodimers between RARs and members of a second nuclear receptor family member, the RXRs (RXRα, β and γ). RXR-RAR heterodimers modulate gene expression by binding to cis-acting regulatory sequences (RAREs), usually consisting of direct repeats of the consensus PuG(G/T)TCA with 5 nucleotides intervening the repeats (DR5), although a number of variant motifs have been described (2;8;14;15). Unliganded RXR/RAR heterodimers associate with transcriptional co-repressor (CoR) complexes that are associated with histone deacetylase (HDAC) activity, resulting in chromatin condensation and gene silencing. Ligand-binding to the RAR moiety results in conformational changes in the ligand-binding domain of the receptor resulting in co-repressor release and recruitment of co-activators (CoA). Histone acetyltransferase (HAT) mediated

by such co-activator complexes results in chromatin decondensation and facilitates gene transcription (2;15-17).

The epidermis expresses RARα, RARγ, RXRα, and RXRβ, with RARγ and RXRα being the predominant receptor types (5;18;19). Although RA has strong effects on epidermal homeostasis under pharmacological conditions (5;9-13), the role of RARs in epidermal development and homeostasis under physiological conditions is less clear. Ablation of both RARα and RARγ in the epidermis gives rise to mice with only minor abnormalities affecting the granular layer, and major defects affecting keratinocyte differentiation are not apparent (9;20). In marked contrast, transgenic mice targeted expressing a dominant-negative (dn) RARα in basal keratinocytes exhibit severe epidermal abnormalities suggestive of an early block in differentiation (21). These disparate outcomes are not due to functional rescue by RARβ in the null mutants, which remains undetectable in RARαγ$^{-/-}$ epidermis (9), rather it has been suggested that the dnRAR may impact on epidermal development *via* a non-canonical pathway (22).

To further investigate the different effects of epidermal RAR ablation versus dnRAR, we compared the epidermal phenotypes of RARαγ double null mutants with transgenic offspring expressing either a dnRARα or an identical dominant-negative receptor incapable of DNA binding under the control of the K14 promoter. Consistent with prior work (21), dnRARα transgenic mice exhibited severe abnormalities of the epidermis, including a reduced suprabasal layer and abnormal expression of several differentiation markers.

Remarkably, these histological and molecular defects were recapitulated in transgenic offspring expressing the dnRARα mutated for DNA binding (denoted dnRARα^{DBD}). These data are consistent with an effect of these dominant-negative receptors on a pathway that is not related to direct retinoid target gene regulation.

The molecular phenotypes of the dnRAR transgenics bore similarity to the defects exhibited by p63 null mutants. p63 is a homolog of p53, and is essential for development and maintenance of a number of tissues, in particular, stratified epithelia including the epidermis (23-26). Consistent with this relationship, immunohistochemistry revealed that both (dn)RARα and dnRARα^{DBD} offspring had severely compromised p63 expression, suggesting that loss of p63 may be, at least in part, accountable for the observed epidermal abnormalities. By contrast, RARαγ$^{-/-}$ epidermis, which can be considered as a pan-RAR null material (9), was histologically indistinguishable from wild type littermates and exhibited normal expression of p63. These data suggest that, although p63 is not a RAR-target, interference with its expression may underlie the epidermal defects elicited by dnRARs.

Materials and methods

Generation and characterization of mutant RARs

A cysteine to alanine mutation in codon 88 (C88A) and a glycine to glutamic acid mutation in codon 303 (G303E) were introduced into the murine RARα1 cDNA using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene), and confirmed by sequencing. These mutations were predicted to disrupt DNA binding and to generate a dominant-negative RAR, respectively. A PstI-SmaI fragment from RARα1 (G303E; referred to hereafter as dnRARα) was used to replace the same region from RARα1 (C88A), generating the DNA-binding deficient, dominant-negative RARα referred to hereafter as $dnRAR^{DBD}$.

DNA binding of the RAR mutants was assessed by electrophoresis mobility shift assay (EMSA) as previously described (19). RA-induced transactivation potential of the mutagenized receptors was assessed by transient transfection assays in embryocarcinoma cells using a 3xβRARE reporter vector also as previously described (19).

Generation of mice

The RARα mutant cDNAs were excised from the parental vector by digestion with EcoRI, blunted by treatment with Klenow and inserted into pGEM3Z-K14 (a gift from E. Fuchs) linearized with BamHI and blunted with Klenow fragment. Transgenes were excised by digestion with EcoRI and HindIII and isolated by preparative gel electrophoresis. Purified DNA was

used to generate transgenic offspring by standard procedures. Offspring were recovered at embryonic day (E)18.5 and transgenics identified by Southern blot analysis using DNA prepared from tail biopsies. Dorsal skin from transgenic and control littermates was recovered and either fixed with Bouin's solution for histological immunohistochemical analysis, or frozen at –80°C.

Transgene expression was assessed by RT-PCR. Briefly, first strand cDNA was synthesized from 5μg of total RNA isolated from epidermal samples using TRIzol (Invitrogen) by reverse transcription with M-MLV reverse transcriptase (Invitrogen). Primers specific for dnRARα (CGCATCTACAAGCCTTG and CAAGTCGGTGAGGGGCT) or for dnRARα^{DBD} (CGCATCTACAAGCCTGC and CAAGTCGGTGAGGGGCT) were used to amplify the cDNA by PCR. As a control, expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also monitored by RT-PCR using the primers TCGGTGTGAACGGATTTG and ATTCTCGGCCTTGACTGT.

RARαγ double null mutants were generated by RARαγ$^{+/-}$ intercrosses and recovered by Cesarean section at E18.5. Offspring were genotyped by PCR as described (27;28).

Histology and immunohistochemistry

Dorsal skin samples from E18.5 offspring were fixed in Bouin's solution, embedded in paraffin and sectioned at 7μm. Immunohistochemistry was performed as described previously (29;30). Briefly, after deparaffination

and rehydration, sections were blocked with 10% serum (Sigma) in PBS/0.2% Tween-20 at room temperature for 1 hour and subsequently incubated with primary antibodies (K5 (Covance), 1:500 dilution; K6 (Covance), 1:50 dilution; K10 (Abcam), 1:25 dilution; or p63 (Santa Cruz), 1:25 dilution) at 4°C overnight. Slides were then washed three times with PBS/0.2% Tween-20 re-blocked with 10% serum and incubated with biotinylated secondary antibodies (1:150 dilution, Vector Laboratories) for one hour. Samples were then washed and incubated with horse radish peroxidase-coupled streptavidin (1:1000, NEN Life Sciences) and reactivity revealed by incubation with a diamino benzidine (DAB, Sigma) solution in TBS (DAB, 0.05%; imidazole, 0.01M; $NiCl_2$, 0.064%; H_2O_2, 0.009%) for 3-10 minutes. Specimens were counterstained with methyl blue (Sigma) before mounting.

Results

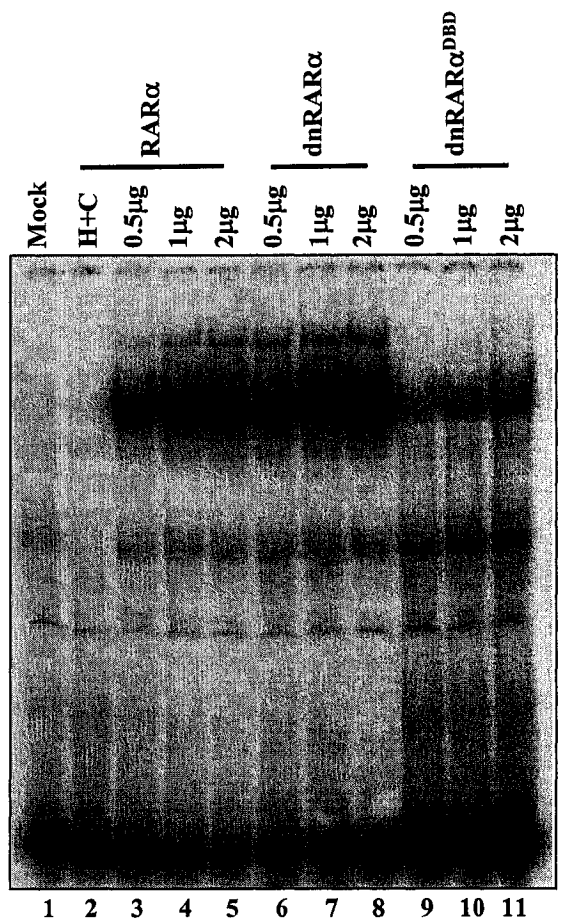
Characterization of dnRARα and dnRARα^{DBD}

The dominant negative RARα used in these studies was generated by a strategy identical to that used previously for the human receptor (31). As expected, binding of this dnRARα to a consensus DR-5 RARE did not differ from that of the wild type receptor as assessed by EMSA (Fig. 4-1A). Moreover, the dnRARα mutant was a potent inhibitor of the transactivation potential of RARα in transfection assays, attenuating greater than 90% of the activity of the wild type receptor at the lowest level tested (Fig 4-1B). A similar effect was also observed in wild type keratinoctyes, and this dominant negative was effective at attenuation transactivation mediated by either RARα or RARγ (data not shown).

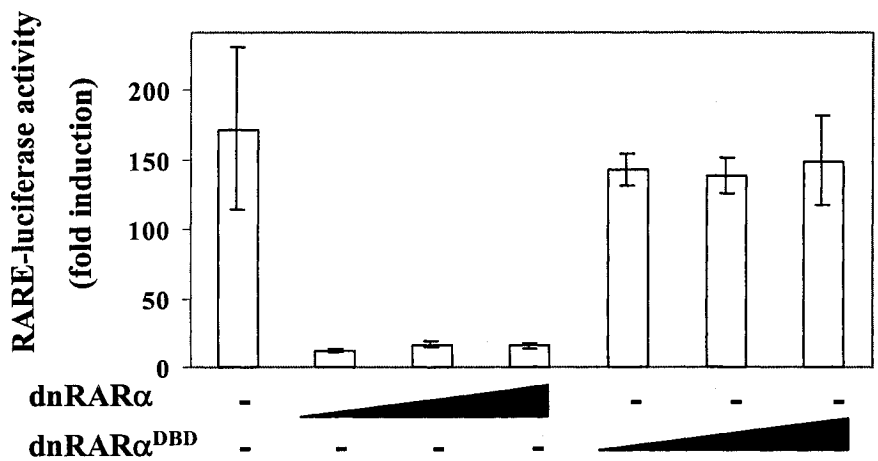
In order to create a DNA-binding-deficient RARα, we mutated codon 88 (in the first zinc finger) from cysteine to alanine. This mutation was then introduced into the dnRARα to generate dnRARα^{DBD}. This mutant did not exhibit detectable DNA-binding to the canonical RARE as assessed by EMSA (Figure 4-1A; compare lanes 9-11 with lanes 3-8). In transfection assays, dnRARα^{DBD} did not inhibit target gene transcription by wild type RARs in either P19 cells or keratinocytes (Figure 4-1B and data not shown). This mutant therefore does not appear to inhibit DNA-dependent transactivation functions.

Figure 4-1. Characterization of dominant-negative RARs. A) Electrophoresis mobility shift assay using nuclear proteins from Cos-7 cells transfected with either empty vector (lane 1), pSG5mRARα1 (lanes 3-5), pSG5dnRARα (lanes 6-8) or pSG5dnRARα^{DBD} (lanes 9-11). The amount of proteins used in each assay is noted above each lane. Lane 2 is equivalent to lane 3 except that the incubation included a 20-fold excess of unlabelled DR5 oligonucleotide as competitor. H: labled probe; C: unlabled probe. B) DR5 response assay. P19 cells were transiently transfected with a 3xβRARE reporter plasmid and either pSG5mRARα1 (1μg), dnRARα or dnRARα^{DBD} (0.5, 1 or 2μg for the RAR mutant expression vectors). Cells were subsequently treated with RA (1μM) or vehicle for 24 hours following which lysates were collected and assessed for luciferase activity. An expression vector encoding β-galactosidase (0.5μg) was included in all transfections and used to normalize for transfection efficiency. Results are expressed as fold induction by RA relative to vehicle and are the mean +/- S.D. of independent triplicate transfections.

A


RARα
dnRARα
dnRARα^DBD
Mock
H+C
0.5μg
1μg
2μg
0.5μg
1μg
2μg
0.5μg
1μg
2μg
1 2 3 4 5 6 7 8 9 10 11

B


RARE-luciferase activity
(fold induction)
200
150
100
50
0
dnRARα
dnRARα^DBD

Skin phenotypes elicited by dnRARα and dnRARα^{DBD}

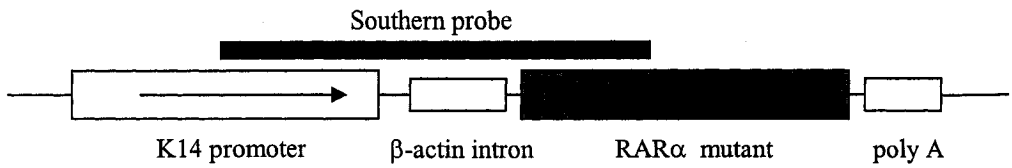
To target expression of the mutant receptors to basal keratinocytes, transgenes were constructed using the human K14 promoter (Fig. 4-2A) (32). Approximately 20% of the pups recovered at E18.5 were identified as transgenic for the dnRARα and dnRARα^{DBD}, respectively, as assessed by genomic Southern blot (Figure 4-2B). Several offspring generated from either construct exhibited severe skin abnormalities; in all such cases, affected pups expressed the mutant RARs in the epidermis, as assessed by transgene-specific PCR (Fig. 4-2C and data not shown). In all such offspring, the skin appeared shiny, sticky and was extremely thin and fragile (Fig. 4-3A; compare transgenics with littermate controls). Expression was never observed in unaffected littermates.

A minimum of two transgenic offspring was assessed for morphological and molecular anomalies. Histologically, the skin of affected transgenic offspring contained an intact basal layer. However, the suprabasal layer was greatly reduced, and there was a marked reduction in the number of hair follicles, which, when present, appeared to be arrested at early stages of development (Fig. 4-3B). These findings are similar to those made using a dn-hRARα transgene (21).

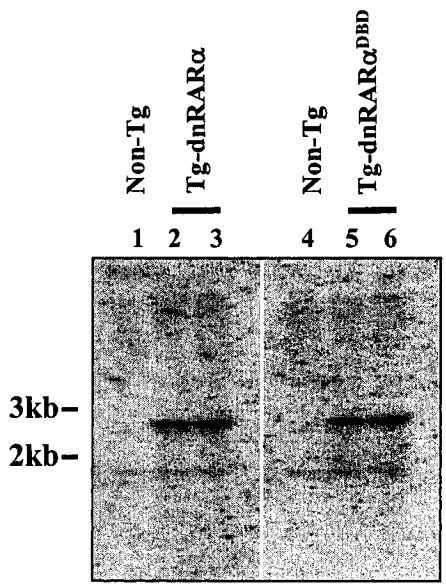
Analysis of a number of markers of skin differentiation revealed that, as for controls, both the dnRARα and dnRARα^{DBD} epidermis expressed K5 which was restricted to the basal keratinocytes. However, K5 expression was reduced in the transgenic offspring relative to controls

Figure 4-2. Generation of transgenic offspring. A) Schematic structure of the transgenic constructs and the sequences used for Southern blot analysis. Inserts comprising the K14 promoter, RAR open reading frames and poly adenylation signal, were released by digestion with Eco RI and Hind III and used for the generation of transgenics. B) Southern blot analysis of offspring. Genomic DNA (10μg) from tail biopsies was digested with EcoRV and assessed for transgene integration by Southern blot analysis using a probe derived from the sequences noted in A. C) RT-PCR analysis of epidermal transgene expression. Total RNA was extracted from the epidermis of offspring and used for RT-PCR. Amplifications were performed using primers specific either for the mutant RAR of interest or for GAPDH that was used as an internal control. Products were resolved by agarose electrophoresis.

A


Southern probe
K14 promoter
β-actin intron
RARα mutant
poly A

B


Non-Tg
Tg-dnRARα
Non-Tg
Tg-dnRARα^{DBD}
1 2 3 4 5 6
3kb–
2kb–

C

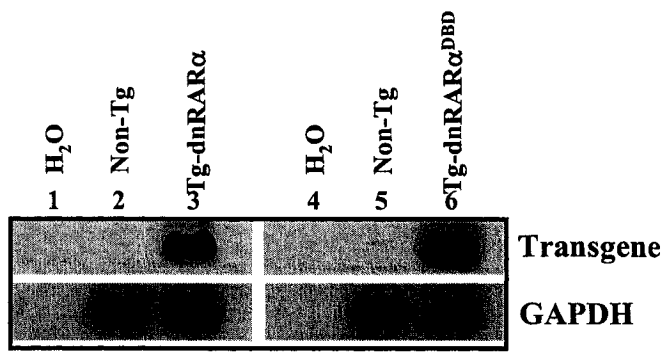
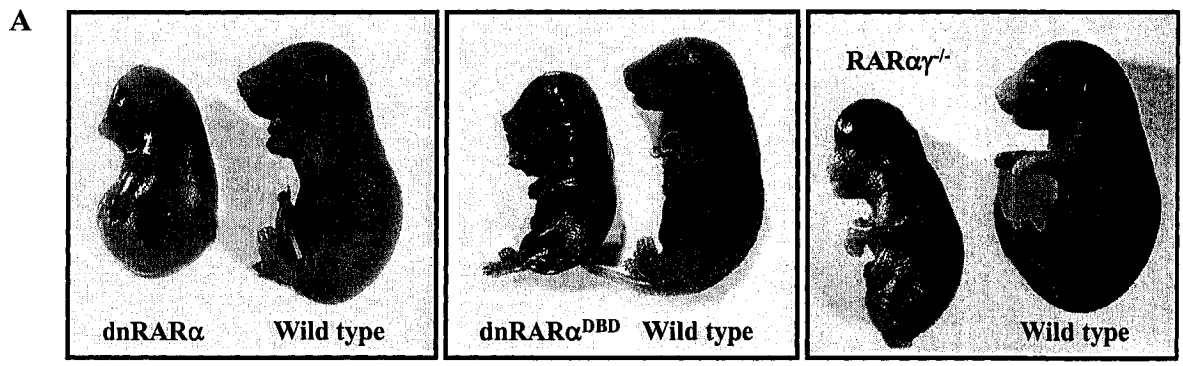
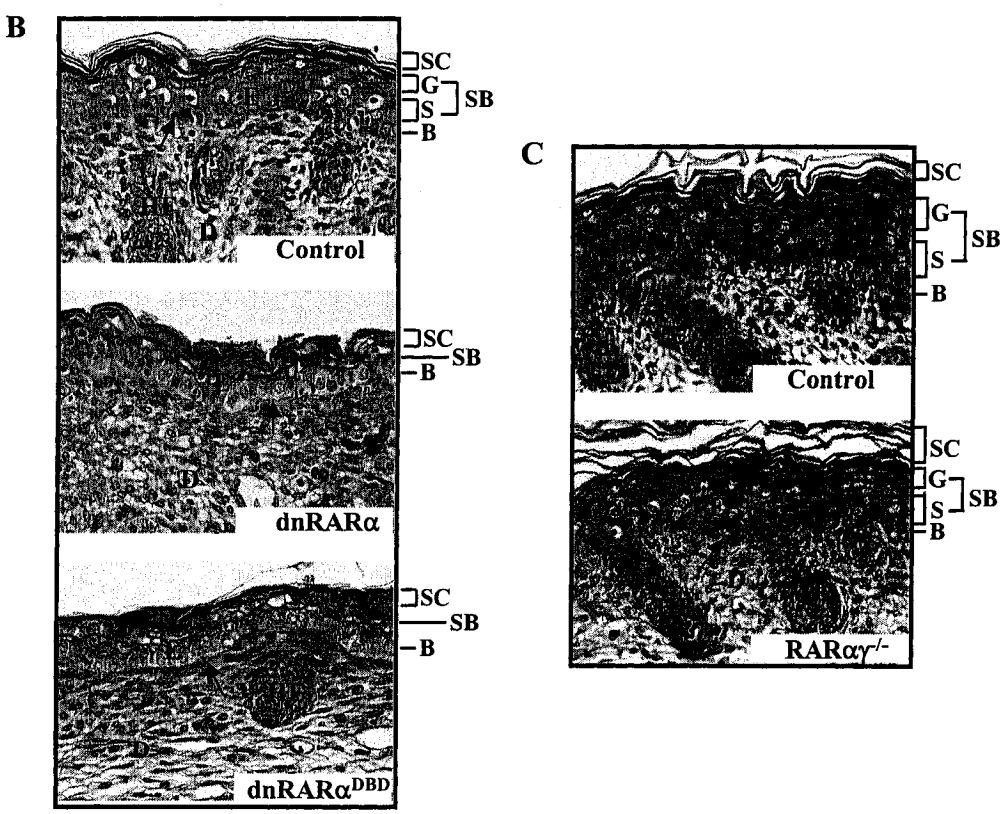

H$_2$O
Non-Tg
Tg-dnRARα
H$_2$O
Non-Tg
Tg-dnRARα^{DBD}
1 2 3 4 5 6
Transgene
GAPDH

Figure 4-3. Phenotype of dnRAR transgenic offspring. A) Transgenic offspring exhibit epidermal defects at term. Transgenic or RARαγ null mutant offspring were procured by Caesarean at term (E18.5) and photographed with non-mutant littermates for comparison. Note the overt epidermal defects elicited by both dnRARα and dnRARα^{DBD} and the lack of a comparable defect in RARαγ null mutant offspring. B) and C) Histological analysis of epidermal defects. Dorsal skin samples were sectioned and stained with haematoxylin and eosin. Relative to the non-transgenic offspring (B, upper panel), transgenic offspring exhibited poorly developed suprabasal (SB) layers and markedly fewer hair follicles (HF); note that similar defects were evident in offspring from both transgenes. C. RARαγ$^{-/-}$ offspring (lower panel) do not exhibit gross epidermal defects. Con, control; B, basal layer; S, spinous layer; G, granular layer; SC, stratum corneum; D, dermis; E, epidermis; arrows indicate the dermal/epidermal junctions.

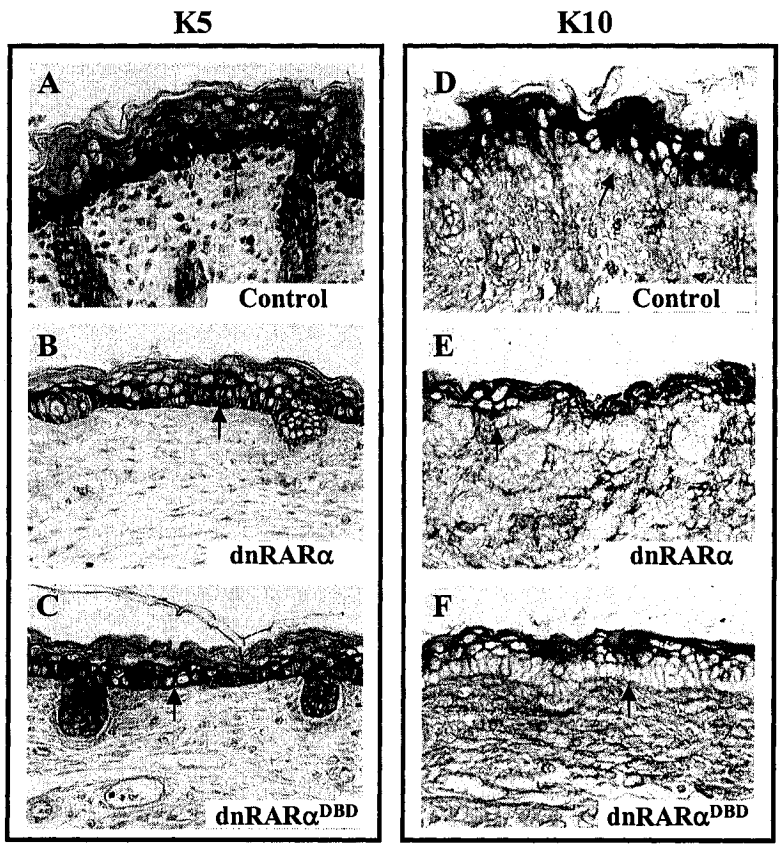

A
dnRARα
Wild type
dnRARα^DBD
Wild type
RARαγ-/-
Wild type


B
SC
G
S
SB
B
Control
dnRARα
dnRARα^DBD
C
Control
RARαγ-/-

(Figure 4-4B and -4C; compare to -4A). This differs somewhat from previous observations using the dn-hRARα transgene, which exhibited ectopic K5 staining which extended into the suprabasal layers (21). The basis for this discrepancy is presently unknown, but may be related to the different hybrid nature of the transgenic backgrounds between the two studies. Offspring from both transgenic backgrounds also exhibited a marked reduction in expression of K10, an early differentiation marker (Fig. 4-4E and -4F, compare to -4D).

Although the dnRARs used in both this study and in prior work elicit profound skin defects, similar outcomes are not observed in mice lacking the entire complement of RARs expressed in the epidermis. By contrast, RARαγ$^{-/-}$ pups appeared to exhibit essentially normal skin, with only a slight sheen relative to control littermates (Figure 4-3A). Although no overt histological abnormalities could be distinguished (Figure 4-3C), defects in the granular layer of RARγ$^{-/-}$ and RARαγ$^{-/-}$ have been described, although the molecular basis for this defect is unclear (7). Consistent with the apparent normal phenotype of the RARαγ$^{-/-}$ epidermis, expression of K5, K14, K1, K10, filaggrin, and involucrin was not affected, suggesting that the terminal differentiation program of keratinocytes is not perturbed in this mutant background (9).

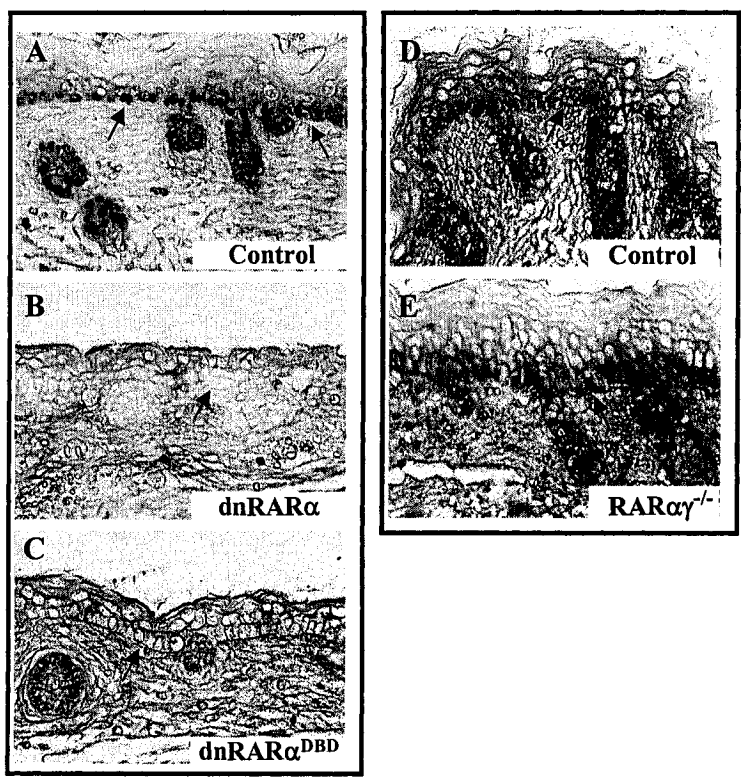
Figure 4-4. Keratin expression in transgenic offspring. Immunostaining for K5 (A) and K10 (B) expression was performed sections of dorsal skins from E18.5 offspring. Arrows indicate the dermal/epidermal junction.


K5
K10
A
Control
B
dnRARα
C
dnRARα^DBD
D
Control
E
dnRARα
F
dnRARα^DBD

p63 expression is lost in dnRARα epidermis

p63, a homolog of the tumor-suppressor p53, is essential for normal development and maintenance of a number of tissues including the epidermis (23-26;33). $p63^{-/-}$ offspring exhibit severe epidermal defects (23;24). Interestingly, a number of these defects, including blocked suprabasal differentiation, lack of hair follicles and loss of differentiation markers such as K10 (23;34), were recapitulated in the transgenic offspring in the present study. Consistent with this relationship, immunohistochemistry revealed that offspring from both transgenic backgrounds were essentially devoid of p63 expression which is normally evident in the nucleus in basal keratinocytes and the outer root sheath of hair follicles (Fig. 4-5B and -5C, compare to control in -5A). These findings are consistent with loss of p63 underlying, at least in part, the epidermal defects observed in the dnRAR transgenic offspring. However, similar effects were not observed in $RAR\alpha\gamma^{-/-}$ epidermis (Figure 4-5E, compare to -5D), suggesting that canonical RAR signalling is not required for p63 expression. This is also consistent with the effects on epidermal development, and p63 expression, elicited by the DNA-binding mutant $dnRAR\alpha^{DBD}$.

Figure 4-5. Dominant-negative RARs inhibit p63 expression. Immunostaining for p63 was performed using sections of dorsal skin samples from E18.5 offspring. Genotypes are noted in each panel. Note the near-complete loss of expression in the transgenic offspring.


A
Control
B
dnRARα
C
dnRARα^DBD
D
Control
E
RARαγ^-/-

Discussion

Dominant-negative RARs impact on epidermal development through a non-canonical pathway

The dominant-negative RARs used in the present study were created by modeling an inherited mutation (G347E) in the ligand binding domain (LBD) of the thyroid hormone receptor β (TRβ) seen in patients with generalized thyroid hormone resistance (31;35). It has been suggested that altered ligand binding and enhanced affinity to CoRs underlies the dominant-negative effects of such mutant receptors (36). Recently, comparable mutations in the LBD of RARα have also been found to modulate co-factor association. For example, RARα-A262 (K262A) has been found to block retinoid signalling in a manner consistent with enhanced affinity for CoRs (37). Thus, stable association of the dnRARα with CoRs, and resultant transcriptional repression, may underlie the potent dominant-negative effects of this mutant in transfection assays.

Although dnRARα evoked epidermal defects in both this and prior work (31), it is highly unlikely that these outcomes are the result of direct effects at retinoid target genes, since a similar phenotype was elicited by the dnRARα^{DBD} which cannot associate with a DR-5 element and which does not interfere with wild type RARs in transfection assays. Thus, it would appear that the effects of these mutant RARs on epidermal development operates through a non-canonical pathway(s).

The nature of this putative non-canonical pathway is presently unknown. It is conceivable that these receptor mutants could affect skin development by sequestration of co-factors necessary for the function of other signalling pathways. However, such ancillary factors would not likely be widely used among nuclear receptors, since expression of either a dominant negative TRβ (TRβ-E_{347}) or overexpression of wild type RARα in basal keratinocytes does not impact on epidermal development (21). Alternatively, such dominant-negative RARs could titrate out RXRs thus impacting on other nuclear receptor pathways involved in epidermal development such as the VDR or PPARs. In this regard, targeted expressed of RARα403, a dominant-negative RAR, to suprabasal keratinocytes results in abnormalities in the cornified layer and a loss of barrier function due to abnormal lipid incorporation. These defects have been proposed to result from interference with PPAR signalling (38;39). However, the mutant RARs used in the present study did not impact on PPAR signalling as assessed by transfection assays in keratinocyte cultures (data not shown), although interference with other RXR-dependent nuclear receptors cannot be excluded.

Loss of p63 expression and epidermal development

p63 is a homolog of the tumor suppressor p53, and is essential for development and maintenance of a number of tissues including stratified epithelia such as the epidermis (23-26;33). Loss of one p63 allele underlies

human EEC (ectrodactyly, ectodermal dysplasia, and facial clefts) syndrome, which also presents with skin defects (40;41).

p63 null mutant skin contains one single layer of basal cells, and all suprabasal layers, as well as hair follicles, are greatly reduced or absent. Consistent with this apparent block in epidermal differentiation, $p63^{-/-}$ mice do not express K1, filaggrin or loricrin. Expression of K14 is weak, while K6 is absent (23). Loss of p63 function is also influenced by genetic backgrounds, as mutants generated by another group (24) lack intact epidermis and hair follicles, with only sporadic regions of keratinocytes observed. These keratinocytes, however, do undergo terminal differential as evidenced by expression of loricrin, filaggrin and involucrin (24).

Both dnRARα and dnRARα^{DBD} offspring share phenotypic similarities with the $p63^{-/-}$ mice previously reported (23;24), although the defects exhibited by the transgenics appears less severe. Notably, transgenic skin contained an intact basal layer with greatly reduced suprabasal layers, and hair follicles as well as reduction K5 and K10 expression and absence of K6. Although the stratum corneum was formed, squames appeared fragmented. Transgenic offspring also exhibited a marked reduction in p63 immunoreactivity. In this regard, the less severe defects elicited by the dnRAR transgenic, relative to the phenotype of p63 null offspring, may be attributed either to residual expression of p63 or variation in genetic backgrounds between these studies.

A role for the RARs in epidermal development?

Exogenous RA can affect skin homeostasis under pharmacological conditions. Topical administration of RA to the skin causes epidermal hyperplasia (5;9;10), and impairs barrier function owing to reduced cohesiveness of stratum corneum (11). It has been showed that RARs, in particular RARγ, in the epidermis mediate the effects of topically administered RA (9;10).

In addition to pharmacological studies, deprivation of dietary vitamin A, the precursor of RA, leads to hyperkeratosis of the skin (12;13). However, despite these observations, targeted disruption of both RARα and RARγ, the totality of RAR types expressed in the epidermis (5;18), causes only mild skin defects (9;20). This observation suggests that RAR signalling is not essential for most epidermal development or homeostasis, a finding that is in marked contrast to the outcome elicited by dnRARs. In this regard, RARαγ$^{-/-}$ offspring exhibit normal expression of p63, consistent with the suggestion that p63 is not dependent on normal retinoid signalling in the epidermis. The ability of the dnRARs to cause loss of p63 expression, and associated epidermal defects, would therefore appear to be unrelated to normal RAR function. This supposition is further supported by the fact that this outcome appears unrelated to DNA binding by the mutant receptors. Although these finding may be unrelated to normal retinoid signalling, it will nevertheless be of interest to determine the molecular basis for this *trans*-repression. Moreover, it is conceivable that the effects provoked by retinoid antagonists may manifest, at least in part, through such cross-talk mechanisms.

Abbreviations

RA, all-trans retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; RARE, retinoic acid responsive element; Tg, transgenic.

Acknowledgements

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Chapter V

General discussion

A tumor suppressor function for RARs in the epidermis has been postulated, based on the fact that progressive RAR loss is associated with skin carcinogenesis (Darwiche et al., 1995; Darwiche et al., 1996; Tennenbaum et al., 1998; Xu et al., 2001). I investigated this using keratinocyte lines derived from various RAR null mice. These studies, described in Chapters II and III, revealed that RARγ is the major RAR subtype mediating RA-induced growth inhibition and cell death in keratinocyte culture. Loss of RARγ, but not RARα, also predisposed keratinocytes to tumor formation in a viral tumorigenesis model, suggesting that RARγ has tumor suppressive effects in the epidermis.

Pharmacological doses of RA affect epidermal homeostasis, suggesting that the RARs may play an important role in the epidermis (Fisher and Voorhees, 1996). This notion is further supported by the finding that transgenic mice expressing a dnRARα in the basal keratinocytes display severe epidermal defects (Saitou et al., 1995). By contrast, targeted deletion of RARs does not elicit any major epidermal abnormalities, arguing against any major role for the RARs in epidermal physiology (Chapellier et al., 2002; Ghyselinck et al., 2002; Lohnes et al., 1994). In searching for a molecular basis underlying the discrepancy between these two models, I made a direct phenotypic comparison of the RAR-null epidermis and the epidermis expressing dnRARα or a mutant dnRARα lacking DNA-binding ability. The experimental evidence presented in Chapter IV indicates that dnRARα causes epidermal defects through RAR DNA-binding-independent pathways.

1. RARγ is the major RAR subtype mediating RA epidermal function in vitro

Among RAR subtypes, the epidermis expresses RARα and RARγ, with RARγ as the prevalent receptor type (Darwiche et al., 1995; Fisher and Voorhees, 1996). Consistent with this, I found that RARγ is also the most abundant RAR type expressed in keratinocyte lines, while RARα is expressed at a much lower level. RARβ has never been detected in these lines irrespective of RAR status, indicating that disruption of RARα or RARγ does not cause a compensatory expression of the remaining receptor. This is in agreement with a recent study showing that RARβ expression is not detected in the RARαγ$^{-/-}$ epidermis *in vivo* (Chapellier et al., 2002).

RARαγ$^{-/-}$ keratinocytes lack all RAR expression, and display complete RA resistance with respect to all outcomes examined. Therefore, RARαγ$^{-/-}$ keratinocytes can be viewed as pan-RAR null material. This is of particular interest, since animals lacking all RARs are not available.

Ablation of RARα and/or RARγ does not affect keratinocyte growth or morphology in culture. This agrees well with gene knockout studies showing that RARαγ$^{-/-}$ epidermis is essentially normal (Chapellier et al., 2002; Lohnes et al., 1994; Ghyselinck et al., 2002).

Disruption of RARγ, but not RARα, attenuates most of the effects of RA on keratinocytes, including its inhibitory effect on cell growth, *trans*-repression of AP-1 and apoptosis, demonstrating that RARγ is the major RAR

subtype which mediates RA functions in this cell type. In line with this, a recent study suggests that the action of exogenous RA in the epidermis is also mediated mainly by RARγ (Chapellier et al., 2002).

RARs can regulate gene expression directly as DNA-binding transcription factors. RARs are also involved in gene regulation through crosstalk with other transcription factors, such as AP-1 (Altucci and Gronemeyer, 2001; Chambon, 1996; Wei, 2003). Several mechanisms have been suggested to explain RA-induced AP-1 repression (Caelles et al., 1997; Kamei et al., 1996; Lee et al., 1998a; Lee et al., 1999; Lee et al., 1998b; Pfahl, 1993b). In wild type keratinocytes, RA downregulates c-Fos and c-Jun expression, and transfection of c-Fos and c-Jun can partially restore AP-1 activity. Also, a similar partial "rescue" effect is observed after transfection with p300 or CBP. These observations suggest that RA induces AP-1 repression in multiple ways. Interestingly, RA does not seem to affect JNK activity in this system, in contrast to prior observations (Lee et al., 1998a; Lee et al., 1999).

Transrepression of AP-1 by RA may underlie the growth inhibitory effect of retinoids in many cell types (Karin et al., 1997; Pfahl, 1993a; Shaulian and Karin, 2002). My work also revealed a close parallel between RA-induced AP-1 repression and growth inhibition in keratinocyte cultures, suggesting that this mechanism is also important for RA function in this model system.

4-HPR, a synthetic retinoid, is a strong apoptogenic agent that shows anti-tumor effects in many models. It has affinity for RARγ and, to a lesser extent, RARβ (Altucci and Gronemeyer, 2001; Fontana and Rishi, 2002; Wu et al., 2001). Both RAR-dependent and RAR-independent mechanisms have been suggested to underlie the apoptogenic function of 4-HPR, and some functions of 4-HPR may be cell type-specific (Altucci and Gronemeyer, 2001; Fontana and Rishi, 2002; Wu et al., 2001). 4-HPR induced apoptosis of RARαγ$^{-/-}$ keratinocytes to an extent comparable to its impact on wild type keratinocytes, clearly demonstrating a RAR-independent function of 4-HPR (data not shown). Such an effect of 4-HPR is, however, independent of AP-1, as 4-HPR did not impact on AP-1 activity in keratinocytes used in my studies.

2. RARγ functions as a tumor suppressor in keratinocytes

Aberrant expression of RARs is associated with several human malignancies. For instance, RARβ silencing is frequently seen in diverse solid tumors (Altucci and Gronemeyer, 2001; Sun and Lotan, 2002). Expression of RARβ is able to inhibit tumorigenesis of a human lung cancer cell line in nude mice, and transgenic mice expressing antisense RARβ2 develop lung cancers spontaneously (Berard et al., 1996; Houle et al., 1993). These observations suggest that RARβ may function as a tumor suppressor in a wide range of tissues (Sun and Lotan, 2002). However, the epidermis does not express RARβ (Darwiche et al., 1995; Fisher and Voorhees, 1996), raising the

question whether other RAR subtypes in the epidermis can play a similar role in epidermal tumorigenesis.

Loss of RARα and RARγ is commonly associated with skin carcinogenesis (Darwiche et al., 1995; Xu et al., 2001). This outcome appears to be an epigenetic event, which is likely caused by tumor promoter, UV radiation, and/or oncogene activation (Darwiche et al., 1996; Kumar et al., 1994; Wang et al., 1999). To address the biological significance of RAR loss in skin tumorigenesis, the tumorigenicity of keratinocytes deficient in RARα, RARγ, or both, have been investigated using a well-established model system. These studies revealed that loss of RARγ, but not RARα, predisposed keratinocytes to tumor formation, suggesting that RARγ has tumor suppressive effects in this tissue. The SCC phenotype of tumors also indicates that loss of RARγ may promote malignant conversion. This is consistent with the suggestion that RA acts as an anti-tumorigenic agent that specifically targets events related to tumor promotion/progression during skin carcinogenesis (Chen et al., 1994; Chen et al., 1995; De Luca et al., 1993; De Luca et al., 1996).

Loss of RARα appears to affect tumor growth on a RARγ$^{-/-}$ background, as the RARαγ$^{-/-}$ tumors were larger than the RARγ$^{-/-}$ tumors. However, disruption of RARα alone did not enhance tumorigenic predisposition, possibly due to its relatively low levels of expression. In support of this, overexpression of RARα in RARαγ$^{-/-}$ keratinocytes suppressed their tumorigenic potential, and sensitized keratinocytes to RA in culture.

Functional redundancy between RARα and RARγ, which has been suggested in a number of studies (Ghyselinck et al., 1997; Lohnes et al., 1994; Lohnes et al., 1995; Lufkin et al., 1993; Mangelsdorf et al., 1995), may explain the ability of RARα to rescue the defects caused by loss of RARγ.

3. Trans-repression of AP-1 is important for the tumor suppressive effects of RAR

Transactivation through a canonical RARE by a pharmacological dose of RA (1μM) in the RARα$^{-/-}$ and RARγ$^{-/-}$ cells appeared comparable, although RARα$^{-/-}$ cells expressed 8-10 times more RAR relative to RARγ$^{-/-}$ cells. Therefore, RARγ may act to suppress tumorigenic potential through RARE-independent pathways.

In contrast to the differential effects of RARα and RARγ on transactivation, the potency of these receptors on AP-1 *trans*-repression appears identical (Lin et al., 2000). My work showed that overexpression of RARα in RARαγ$^{-/-}$ keratinocytes restored AP-1 *trans*-repression, and attenuated tumorigenic potential. Furthermore, there was a good correlation between the restoration of AP-1 *trans*-repression by RA and tumor inhibition. These findings suggest that RAR-mediated AP-1 transrepression is, at least in part, responsible for tumor suppression by RARs in keratinocytes. This is consistent with previous observations showing that RA suppresses tumorigenesis in chemically induced skin carcinogenesis through transrepression of AP-1 activity rather than transactivation (Huang et al.,

1997). Of note, AP-1 inhibition in wild type keratinocytes was seen even with physiological doses of RA (1-10nM).

4. Other possible factors underlying the tumor suppressive effect of RAR

My work demonstrated that loss of the major RAR, RARγ, predisposed keratinocytes to tumor formation. Although loss of AP-1 transrepression has been suggested to be important for tumor predisposition, loss of RARγ may affect other pathways which could contribute to tumorigenesis.

In the absence of RA, RARs bind to RAREs and are associated with co-repressor-HDAC containing complexes, and thereby repress gene expression (Altucci and Gronemeyer, 2001; Aranda and Pascual, 2001; Rosenfeld and Glass, 2001; Wei, 2003). RARγ is the major receptor type expressed in keratinocytes. Therefore, it is logical to speculate that loss of RARγ may relieve many target genes from repression. It will be interesting to investigate whether loss of RARγ causes constitutive de-repression of RAR-target genes and whether this contributes to tumorigenic predisposition of keratinocytes lacking RARγ.

RXRs act as dimeric partners for multiple nuclear receptors such as RAR, VDR, TR, and PPAR (Chambon, 1996; Ross et al., 2000). Loss of VDR sensitizes the skin to chemically induced tumorigenesis, and PPAR agonists can inhibit mouse skin tumor promotion (Thuillier et al., 2000; Zinser et al., 2002). Therefore, it will be interesting to determine whether loss of RARγ

causes changes in signaling mediated by these other RXR partners, and if so, what effect this may have on tumorigenic predisposition.

With time oncogenic Ras causes aneuploidy in keratinocytes, a typical characteristic of advanced SCCs (Yuspa, 1994; Yuspa, 1998). Additional genetic alterations, such as loss of TGFβ, can greatly accelerate this process and thereby enhance the malignant transformation of mouse keratinocytes (Glick et al., 1999). Similarly, RA-resistant papillomas, where RAR expression is greatly reduced, are at high risk to convert into SCCs (Tennenbaum et al., 1998). Consistent with this, v-Ha-ras-infected RARγ$^{-/-}$ and RARαγ$^{-/-}$ cells also formed malignant SCCs after sc injected into nude mice. Therefore, it will be of interest to determine whether loss of RARγ can affect Ras-induced aneuploidy.

5. The role of dnRARs in mouse epidermal development

RARα$^{-/-}$ mice do not exhibit any skin phenotype, while RARγ$^{-/-}$ mice display only minor abnormalities in the granular layer. Ablation of both RARα and RARγ does not cause any further epidermal defects than those seen in RARγ$^{-/-}$ offspring (Chapellier et al., 2002; Ghyselinck et al., 2002; Lohnes et al., 1994; Lufkin et al., 1993). These observations suggest that RARs are not a major regulator of epidermal development.

By contrast, expression of a dnRARα in the basal epidermis leads to profound defects. The suprabasal epidermal layers in these transgenics are greatly reduced, and terminal differentiation is impaired. These phenotypes

resemble those seen in p63 null mice, suggesting that p63 may be downregulated in the dnRARα transgenic epidermis (Mills et al., 1999; Yang et al., 1999). This is supported by my finding that expression of p63 is lost in the transgenic epidermis.

A recent study demonstrated that RA can inhibit the downregulation of ΔN-p63α, a dominant negative isoform of p63, during terminal differentiation of human keratinocytes (Bamberger et al., 2002). Despite this *in vitro* observation, immunohistochemistry studies using a pan-p63 antibody revealed that expression of p63 appeared unchanged in the RARαγ$^{-/-}$ epidermis, suggesting that p63 is not a classic RAR-target *in vivo*. Therefore, dnRARα may elicit epidermal defects, at least in part, through a non-canonical pathway. This is further supported by my finding that a mutant dnRARα (dnRARα^{DBD}), which lacks its DBD and thereby cannot transactivate RAR-target genes, recapitulated the skin defects observed in the dnRARα transgenic epidermis.

Other possible mechanisms may underlie the effect of the dnRARα in the epidermis. Expression of dnRARs may sequester RXRs from other nuclear receptors such as VDR, TR, or PPAR. For example, targeted expression of a dnRARα in the suprabasal epidermal layers using the K10 promoter causes a defective barrier function due to the absence of lipid multilamellar structures. This outcome has been suggested to be owing to abnormal PPAR signaling (Attar et al., 1997; Imakado et al., 1995). However, I have not detected abnormal PPAR signaling in our transgenic system (data

not shown). Further investigation is needed to explore whether signaling mediated by other receptors, such as VDRs or TRs, is affected by dnRARα.

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Chapter VI

Claims to original research

1) Chapter II of this thesis describes the establishment and characterization of keratinocyte lines deficient in RARα, and/or RARγ. This is the first description of RARγ as a major RAR subtype mediating RA-induced growth inhibition and AP-1 repression in keratinocyte culture.

2) Chapter III describes the roles of RARs in epidermal tumorigenesis. I provided the first evidence showing that loss of a specific RAR, RARγ, contributes to epidermal tumorigenesis.

3) Chapter IV describes a possible mechanism underlying the function of a dominant negative RARα in epidermal development. I showed, for the first time, that dnRARα can downregulate p63 in the epidermis, and that p63 is not a canonical RAR-target in the epidermis. Absence of epidermal p63 expression may explain, at least partially, the severe epidermal defects caused by dnRARα.

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) SUBSTANCES NUCLÉAIRES ET ÉQUIPEMENT AUTORISÉ

ARTICLE	*SUBSTANCE NUCLÉAIRE*	*SOURCE NON SCELLÉE QUANTITÉ MAXIMALE*	*ASSEMBLAGE DE LA SOURCE SCELLÉE QUANTITÉ MAXIMALE*	*ÉQUIPEMENT - FABRICANT ET MODÈLE*
1	Calcium 45	100 MBq	s/o	s/o
2	Cobalt 57	40 MBq	s/o	s/o
3	Cobalt 59	40 MBq	s/o	s/o
4	Chrome 51	400 MBq	s/o	s/o
5	Fer 59	2 GBq	s/o	s/o
6	Hydrogène 3	3 GBq	s/o	s/o
7	Iode 123	500 MBq	s/o	s/o
8	Iode 125	3 GBq	s/o	s/o
9	Iode 131	500 MBq	s/o	s/o
10	Phosphore 32	30 GBq	s/o	s/o
11	Phosphore 33	100 MBq	s/o	s/o
12	Rubidium 86	40 MBq	s/o	s/o
13	Soufre 35	3 GBq	s/o	s/o
14	Zinc 65	40 MBq	s/o	s/o
15	Césium 137	s/o	400 kBq	s/o
16	Radium 226	s/o	400 kBq	s/o
17	Césium 137	s/o	1110 kBq	Beckman LS series
18	Radium 226	s/o	370 kBq	Wallac Série 1200

La quantité totale d'une substance nucléaire non scellée possédée ne doit pas excéder la quantité maximale qui est indiquée pour une source non scellée correspondante. La quantité de substance nucléaire par source scellée ne doit pas excéder la quantité maximale indiquée par source scellée correspondante. Les sources scellées doivent être utilisées seulement dans l'équipement indiqué correspondant.

) ENDROIT(S) OÙ LES ACTIVITÉS AUTORISÉES PEUVENT ÊTRE EXERCÉES

utilisées ou entreposées à (aux)
endroit(s) suivant(s):

110, avenue des Pins Ouest