MOLECULAR AND CELLULAR MECHANISMS REGULATING CROSS-TALK BETWEEN CYCLOOXYGENASE AND LIPOXYGENASE BIOSYNTHETIC AXES

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ABSTRACT

Rheumatoid arthritis (RA) is characterized by chronic inflammation, synovial hyperplasia and joint destruction. The pathogenic mechanisms responsible for RA remain poorly understood both systemically and in the microenvironment of diarthrodial joint. Here we hypothesized, based on previous observations, that there is a positive interaction between resident mast cells and synovial fibroblasts (SF) within the rheumatoid synovial compartment. Our principal objectives were to define the cellular and molecular interactions between infiltrating mast cells and resident human synovial fibroblasts (HSF), and we focused our efforts on two genes, cyclooxygenase-2 (*COX-2*) and 5-lipoxygenase (*5-LO*), both implicated in the inflammatory response associated with RA. Furthermore, our research led to the discovery of novel epigenetic mechanisms governing the regulation of *5-LO* gene expression in RA-affected SF. Epigenetics refers to heritable changes in gene expression regulated by DNA methylation, histone modifications and RNA interference. Epigenetic regulation is critical for normal development and differentiation. But environmental factors can trigger epigenetic dysregulation, leading to the development of many diseases, including cancer and autoimmune diseases.

Using Western and Northern blot analyses in addition to reporter assays, we demonstrated that mast cell-derived leukotriene B_4 (LTB_4) contributes to the modulation of inflammatory response in part through stabilization of COX-2 mRNA and protein expression in SF, the key enzyme in prostaglandin biosynthesis and the target of all non-steroidal anti-inflammatory drugs (NSAIDs). Further transient and stable transfection experiments verified that LTB_4 exerted this COX-2 stabilization effect at the post-transcriptional level through Ras/c-Raf/MEK1/2/ERK1/2/p42 AUF1 signaling pathway.

Genomic bisulfite sequencing was used to detect DNA methylation profiles of *5-LO* gene (rate limiting in LTB_4 synthesis) in HSF, mast cells and other cell types. The *5-LO* gene promoter (DNA CpG islands) was heavily methylated in U937 cells (5-LO negative), but unmethylated in HL-60 cells (5-LO positive). Compared to the 5-LO-positive HMC-1 cells, the 5-LO-negative HMC-1 cells had much higher methylation levels of CpG islands in the promoter region. We found a strong correlation between *5-LO* gene expression and DNA methylation in HMC-1 cells. Dexamethasone (DEX) treatment of HMC-1 cells increased the expression of 5-LO, a process associated with reduced methylation of histone H3 on lysines 9 and 27. Interestingly, osteoarthritic (OA)/RA HSF are 5-LO negative; though the promoter region is CpG hypomethylated, histone H3 is hypermethylated at Lys-9 and -27 residues.

The research in this thesis provided innovative insights into the understanding of the pathophysiological mechanisms involved in inflammatory diseases like RA. The elucidation of the cellular and molecular mechanisms involved in these studies may help to establish new therapeutical targets in the treatment of RA patients. Moreover, the bisulfite sequencing and chromatin immunoprecipitation (ChIP) techniques used in this thesis provide reliable procedures for DNA methylation and histone modification studies in various inflammatory diseases and cancers.

RÉSUMÉ

L'arthrite rhumatoïde (AR) est caractérisée par une inflammation chronique, une hyperplasie de la membrane synoviale et la destruction des jointures. Les mécanismes pathogéniques responsable de l'AR sont peux compris à la fois systémiquement et dans le microenvironnement du joint diarthrodial. Notre hypothèse, basé sur des observations précédentes, qu'il y avait une interaction positive entre les cellules mastocytes résidentielles et les fibroblastes synoviaux dans le compartiment de la synovial rhumatoïde. Nos objectifs principaux étaient de définir les interactions cellulaires et moléculaires entre les cellules mastocytes infiltrantes et les fibroblastes synoviaux résidentielles, en particulier deux gènes, la cyclooxygénase-2 (COX-2) et la 5-lipoxygénase (5-LO), tous deux impliquées dans la réponse inflammatoire associée à l'arthrite rhumatoïde. De plus, notre recherche a abouti à la découverte de mécanismes épigénétiques gouvernant la régulation de l'expression du gène de la 5-LO chez les fibroblastes synoviales atteint d'arthrite rhumatoïde. L'épigénétique est l'ensemble des modifications de l'expression des gènes contrôlés par la méthylation de l'ADN, les modifications aux histones liées à l'ADN et par interférence ARN transmissibles d'une génération de cellule à l'autre. La régulation épigénétique est indispensable pour la différentiation et le développement normal. Cependant, des facteurs environnementaux peuvent entamer un désordre épigénétique causant ainsi plusieurs maladies, tels que le cancer et des maladies auto-immunes.

Avec les méthodes d'analyse par buvardage Western et Northern, en plus des essais rapporteurs, nous avons démontré que le leukotriène B_4 (LTB_4) produit par les cellules mastocytes contribues à la modulation de la réponse inflammatoire en parti par la stabilisation de l'ARN messager de la cyclooxygénase-2 (COX-2) et de l'expression de

la protéine chez les fibroblastes synoviaux, l'enzyme clé de la biosynthèse des prostaglandines et la cible de tous les drogues anti-inflammatoires non stéroïdiens. De plus, les expériences de transfections transitoires et stables ont démontré que le LTB_4 a exercé cet effet de stabilisation de la COX-2 au niveau post-transcriptionnelle par l'intermédiaire de la voie de signalisation de Ras/c-Raf/MEK1/2/ERK1/2/p42 AUF1.

Le séquençage génomique avec traitement au bisulfite a été utilisé pour déterminer le profile de méthylation de l'ADN du gène de la 5-LO (enzyme clé dans la synthèse du LTB_4) chez les fibroblastes synoviaux humains, les cellules mastocytes et d'autres types cellulaires. Le promoteur du gène 5-LO (îlots d'ADN CpG) était fortement méthylé chez les cellules U937 (5-LO négatif), cependant chez les cellules HL-60 le promoteur n'était pas méthylé (5-LO positif). En comparant les cellules HMC-1 qui expriment la 5-LO aux cellules HMC-1 qui n'expriment pas la 5-LO, ces derniers avaient de fort niveaux de méthylation des îlots CpG dans la région du promoteur. Nous avons trouvé une forte corrélation entre l'expression du gène 5-LO et la méthylation de l'ADN chez les cellules HMC-1. Le traitement des cellules HMC-1 avec la dexamethasone (DEX) augmente l'expression de la 5-LO, un processus associé à la réduction de la méthylation de l'histone H3 sur les lysines 9 et 27. Il est intéressant de voir que les fibroblastes synoviaux humains ostéoarthritique (OA)/RA sont 5-LO négatif; quoique la région du promoteur CpG est hypométhylé alors que l'histone H3 est hyperméthylé aux lysines 9 et 27.

La recherche de cette thèse propose un cheminement innovateur à la compréhension des mécanismes physiopathologique impliqué dans les maladies inflammatoires telle que l'arthrite rhumatoïde. L'élucidation des mécanismes cellulaires et moléculaires impliqués dans ses études peuvent aider à l'établissement de nouvelles

cibles thérapeutiques pour le traitement de l'arthrite rhumatoïde. De plus, la technique de séquençage de l'ADN avec le traitement au bisulfite utilisé dans cette thèse est fiable pour des études de méthylation de l'ADN et la modification des histones chez plusieurs maladies inflammatoires ainsi que les cancers.

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PREFACE

The research of my Ph.D. program is presented in this thesis. This thesis was written in accordance with McGill University guidelines (available at http://www.mcgill.ca/gps/students/thesis/programs/guidelines/preparation/#format). The format of this thesis consists of:

1) Abstract in both English and French
2) Acknowledgements
3) Comprehensive review of the literature
4) Project rationale and objectives
5) Experimental research
6) Conclusion
7) References

This thesis contains four chapters as follows: **Chapter 1** is a comprehensive literature review that is relevant to the work presented in this thesis; **Chapter 2** is adapted from a paper for project I of my research that is published in *The Journal of Biological Chemistry* [285 (31): 23568-80, 2010], and a reprint of this paper is attached to the end of this thesis; **Chapter 3** is adapted from the manuscript for project II of my research that is in preparation for submission to *Inflammation Research*; **Chapter 4** is a general discussion and conclusion for the whole research. The references for all of these four chapters are complied at the end of this thesis.

CONTRIBUTIONS OF AUTHORS

Most of the data presented in **Chapter 2** of this thesis was performed by me under the direct supervision of Dr. Di Battista. For **Chapter 3** of this thesis, I did all of the experiments myself with the direct guidance from my supervisor. Other individuals who have contributed to this research are listed below:

In **Chapter 2**:

- Dr. Huiqing Yang was a former postdoctoral fellow in Dr. Di Battista's laboratory. She performed the preliminary experiments in project I for this research.
- Dr. Qingwen He is a research associate in Dr. Di Battista's laboratory and provided her experimental expertise.
- Arturo Mancini is a Ph.D. candidate in Dr. Di Battista's laboratory and provided good suggestions and information for tristetraprolin (TTP) study in this research.
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In **Chapter 3**:

- Dr. Luigi Macchia is a professor of the department of Clinical Immunology and Allergology in university of Bari, Italy, and provided us with the genomic DNA of human mast cells (HMC-1) for the following DNA methylation study.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
AC	adenylate cyclase
ADP	adenosine diphosphate
ANOVA	analysis of variance
AP-1	activator protein-1
APCs	antigen presenting cells
ARDS	acute respiratory distress syndrome
ARE	AU-rich element
ASK1	apoptosis signal regulating kinase 1
ATCC	American Type Culture Collection
ATF-2	activating transcription factor-2
AU	adenylate/uridylate
AUBP	AU-binding protein
AUF1	ARE/poly(U)-binding/degradation factor 1 (hnRNP D)
AZA	5-aza-2'-deoxycytidine
Bcl-xL	B-cell lymphoma-extra large
bFGF	basic fibroblast growth factor
BLT	LTB_4 receptor
BRMs	biological response modifiers
CBF-A	CArG box-binding factor-A
CCCH	cysteine-cysteine-cysteine-histidine
CCPs	cyclic citrullinated peptides
C/EBP	CCAAT-enhancer-binding protein

CH_3	methyl
ChIP	chromatin immunoprecipitation
COPD	chronic obstructive pulmonary disease
COX	cyclooxygenase
COXib	COX-2 inhibitors
$cPLA_2$	cytosolic phospholipase A_2
CRE	cAMP-response element
CREB	cAMP response element binding
CRM1	chromosomal region maintenance protein 1
CTD	carboxy-terminal domain
DAG	diacylglycerol
DEX	dexamethasone
DIG	digoxigenin
DMEM	Dulbecco's modified Eagle's medium
DNMTs	DNA methyltransferases
dsRNA	double-stranded RNA
EGF	epidermal growth factor
ELAV	embryonic-lethal abnormal vision
Elk-1	Ets like gene-1
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinases
FAK	focal adhesion kinase-1
FBP	far upstream sequence element binding protein
FBS	fetal bovine serum

FLAP	5-LO-activating protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAPs	GTPase-activating proteins
GEFs	guanine-nucleotide exchange factors
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPCRs	G protein-coupled receptors
GPI	glucose-6-phosphate isomerase
G proteins	guanine nucleotide-binding proteins
HATs	histone acetyltransferases
HDACs	histone deacetylases
HLA	human leukocyte antigen
HL-60	human promyelocytic leukemia cells
HMC-1	immature human mast cells
HMCs	human mast cells
HSF	human synovial fibroblasts
Ier3	immediate early response 3
IFN	interferon
IL	interleukin
IL-1ra	IL-1 receptor antagonist
IMDM	Iscove's modified Dulbecco's medium
IP3	inositol (1,4,5)-trisphosphate
JMJC	Jumonji C
JNK	c-Jun NH_2-terminal kinases
LMB	leptomycin B

5-LO	5-lipoxygenase
LPS	lipopolysaccharide
LSD1	lysine-specific demethylase 1
LTA_4	leukotriene A_4
LTB_4	leukotriene B_4
LTC_4	leukotriene C_4
MAPK	mitogen-activated protein kinase
MAPKKs	MAPK kinases
MAPKKKs	MAPK kinase kinases
MAPKAPK-2/MK2	MAPK-activated protein kinase-2
MBDs	methyl-CpG binding domain proteins
MC/9	mouse mast cells
MCP-1	monocyte chemoattractant protein-1
MEK1/2	MAPK/ERK kinase 1/2
MHC-II	major histocompatibility complex class II
MIP-1α/β	macrophage inflammatory protein-1α/β
miRNA	microRNA
MLCK	myosin light chain kinase
MLK-3	mixed lineage kinase-3
MMP	matrix metalloproteinase
Mnk	MAPK interacting kinase
MTX	methotrexate
ncRNA	non-coding RNA
NF-κB	nuclear factor kappa B

NIH3T3	mouse embryonic fibroblasts
OA	osteoarthritic
ODF	osteoclast differentiating factor
PAF	platelet-activating factor
PDGF	platelet-derived growth factor
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
PGH_2	prostaglandin H_2
PI3K	phosphatidylinositol 3-kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PPAR-γ1	peroxisome proliferator-activated receptor-γ1
PTX	pertussis toxin
QKRAA	glutamine-leucine-arginine-alanine-alanine
RA	rheumatoid arthritic
RANK	receptor activator of nuclear factor-κB
RANKL	receptor activator of NF-κB ligand
RARs	retinoic acid receptors
RBD	RNA-binding domain
RF	rheumatoid factor
rhIL-1β	recombinant human interleukin-1β

RISC	RNA-induced silencing complex
rRNA	ribosomal RNA
RTKs	receptor tyrosine kinases
SAHA	suberoylanilide hydroxamic acid
SAM	S-adenosylmethionine
SAPK	stress-activated protein kinase
SCF	stem cell factor
SDS	sodium dodecyl sulfate
Ser/Thr	serine/threonine
SF	synovial fibroblasts
shRNA	short hairpin RNA
siRNA	small interfering RNA
snRNAs	small nuclear RNAs
SOCS	suppressor of cytokine signaling
STAT3	signal transducer and activator of transcription 3
TAK-1	TGF-β activated kinase-1
TEY	threonine-glutamate-tyrosine
TGF-β	transforming growth factor-β
TGY	threonine-glycine-tyrosine
TIA-1	T cell-restricted intracellular antigen-1
TIAR	TIA-related protein
TNF-α	tumor necrosis factor-α
TPL-2	tumor progression locus-2
TPY	threonine-proline-tyrosine

tRNA	transfer RNA
TSA	trichostatin A
TTBS	Tween/Tris-buffered saline
TTP	tristetraprolin
U937	human leukemic monocyte lymphoma cells
3’-UTR	3’-untranslated region
Xist	X-inactive specific transcript

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CHAPTER 1

GENERAL INTRODUCTION

1.1. EPIDEMIOLOGY OF RHEUMATOID ARTHRITIS (RA)

Rheumatoid arthritis (RA) is a chronic inflammatory disease of joints and usually affects joints symmetrically. It may initially begin in a couple of joints only, and most frequently attacks the wrists, hands, elbows, shoulders, knees and ankles. The major characteristic feature of RA is symmetric polyarticular arthritis with synovium hyperplasia, erosive synovitis, and abnormal cellular and humoral immune responses.

In epidemiological studies, the American College of Rheumatology criteria (Table 1) is widely used to define RA with 91-94% sensitivity and 89% specificity compared with control subjects (Sangha 2000). RA is prevalent throughout the world in all races with the incidence of about 1%, women three times more often than men. The risk factors for RA include family history and genetic factors, environmental exposures, such as smoking and alcohol intake, hormones, stress and socioeconomic status (Liao, Alfredsson et al. 2009). Since the 1950s, the mortality rate in RA patients has increased (1.5-1.6 fold higher than in the general population) because of cardiovascular disease, infection, renal and respiratory diseases (Sokka, Abelson et al. 2008). RA can cause marked functional impairment and disability in patients, with significant socioeconomic burden throughout the world because of both the therapeutic costs and the loss of productivity and decreased quality of life. A recent estimate of the indirect costs in RA is about $11 billion. As such, it is quite important to better understand the mechanisms of RA in order to provide more effective treatments.

Table 1: The revised criteria of 1987 for RA

Criterion	Comment
1. Morning stiffness	Duration >1 h lasting >6 weeks
2. Arthritis of at least 3 areas	Soft tissue swelling or exudation lasting >6 weeks
3. Arthritis of hand joints	Wrist, MCP, PIP joints lasting >6 weeks
4. Symmetrical arthritis	At least one area, lasting >6 weeks
5. Rheumatoid nodules	Observed by a physician
6. Serum rheumatoid factor	Assessed by a method positive <5% of control subjects
7. Radiographic changes	Seen on anteroposterior films of wrists and hands

MCP, metacarpophalangeal; PIP, proximal interphalangeal.

Reprinted from O. Sangha, Epidemiology of rheumatic diseases, *Rheumatology*, 2000, 39(Suppl. 2):3-12, by permission of Oxford University Press.

1.2. PATHOGENESIS OF RA

The aetiology of RA is still unclear. Current understandings from immunological studies and different animal models focus on autoantibodies, complement system, T cells, imbalanced cytokine networks and tumor-like behavior of synovial fibroblasts (SF).

1.2.1. Autoantibodies in the development of RA

RA was initially considered as an autoimmune disease because of the identification of the autoantibody — rheumatoid factor (RF) — in the sera of a number of patients. RF was originally characterized as an antibody that binds to the Fc portion of immunoglobulins to potentiate certain agglutination reactions (Kunkel, Franklin et al. 1959). Subsequently, Zvaifler *et al.* proposed the immunopathologic potential of RF in RA development. In their theory, immune complexes formed by RFs fixed complement

on the cartilage surface, leading to the production of chemotactic factors, like C5a inside the joints. A variety of immune cells, such as T cells, B cells, macrophages and neutrophils, were recruited into the joints along the chemotactic gradient. Then, these cells were activated and released a broad range of inflammatory mediators and proteolytic enzymes, resulting into the inflammation and destruction of the affected joints (Zvaifler 1973). In contrast to osteoarthritic (OA) synovium, RA synovium contained more complement factors and complement receptors using *in situ* hybridization and immunohistochemistry techniques, and ultrastructural studies also revealed immune complexes embedded in the superficial layers of cartilage (Neumann, Barnum et al. 2002). RF was initially considered to be a diagnostic marker and predictive for more aggressive RA, but further studies found that not all of the RA patients had RF in their serum. In some normal individuals and in patients with other chronic inflammatory diseases, RF could also be detected (Vossenaar, Zendman et al. 2004). Therefore, RF is not the only molecule that is responsible for RA development.

Using different mouse arthritis models, studies also discovered several other autoantibodies for RA initiation. For example, in the mouse model of collagen-induced arthritis, anti-type II collagen antibodies formed immune complexes with collagen on cartilage surface and provided a good substrate for complement fixation. The complexes used the same mechanism as RF to initiate RA (Stuart, Townes et al. 1984). Recently, significant interests have focused on the K/B×N mouse model with a spontaneously erosive arthritis similar to human RA. K/B×N mouse is a T-cell receptor transgenic mouse model. The arthritis in this model is triggered by the major histocompatibility complex class II (MHC-II)-specific recognition of the self-protein — glucose-6-phosphate isomerase (GPI) peptide (GPI 282-294) in T cells that have escaped negative

selection (Ditzel 2004). The GPI-anti-GPI complexes promote complement fixation on the articular surface, leading to immune cell activation and inflammatory cascades that orchestrate arthritis (Matsumoto, Maccioni et al. 2002). Passive transfer of the serum containing anti-GPI antibodies from K/B×N mice to the naive mice was sufficient to induce the erosive arthritis in the naive mice (Ditzel 2004). But in RA patients, anti-GPI antibody has a low prevalence (15%) and specificity. GPI protein does not appear to be a common target in human RA pathogenesis (Matsumoto, Lee et al. 2003).

In recent years, several newly characterized autoantibodies have become the promising candidates as diagnostic indicators for RA. The putative autoantigens include proteoglycan (Verheijden, Rijnders et al. 1997), aggrecan (Li, Zhang et al. 2000), heat shock proteins (Oda, Miyata et al. 1994), and cyclic citrullinated peptides (CCPs) (Steiner and Smolen 2002). CCPs are derived from post-translational modification of arginine by peptidylarginine deiminase. Study reported that anti-CCP antibodies were detected in the sera of 34% of individuals who subsequently developed RA. The combination of the presence of anti-CCP antibodies and RF showed a high specificity of 90%, providing a strong predictive value for RA development (Rantapaa-Dahlqvist, de Jong et al. 2003).

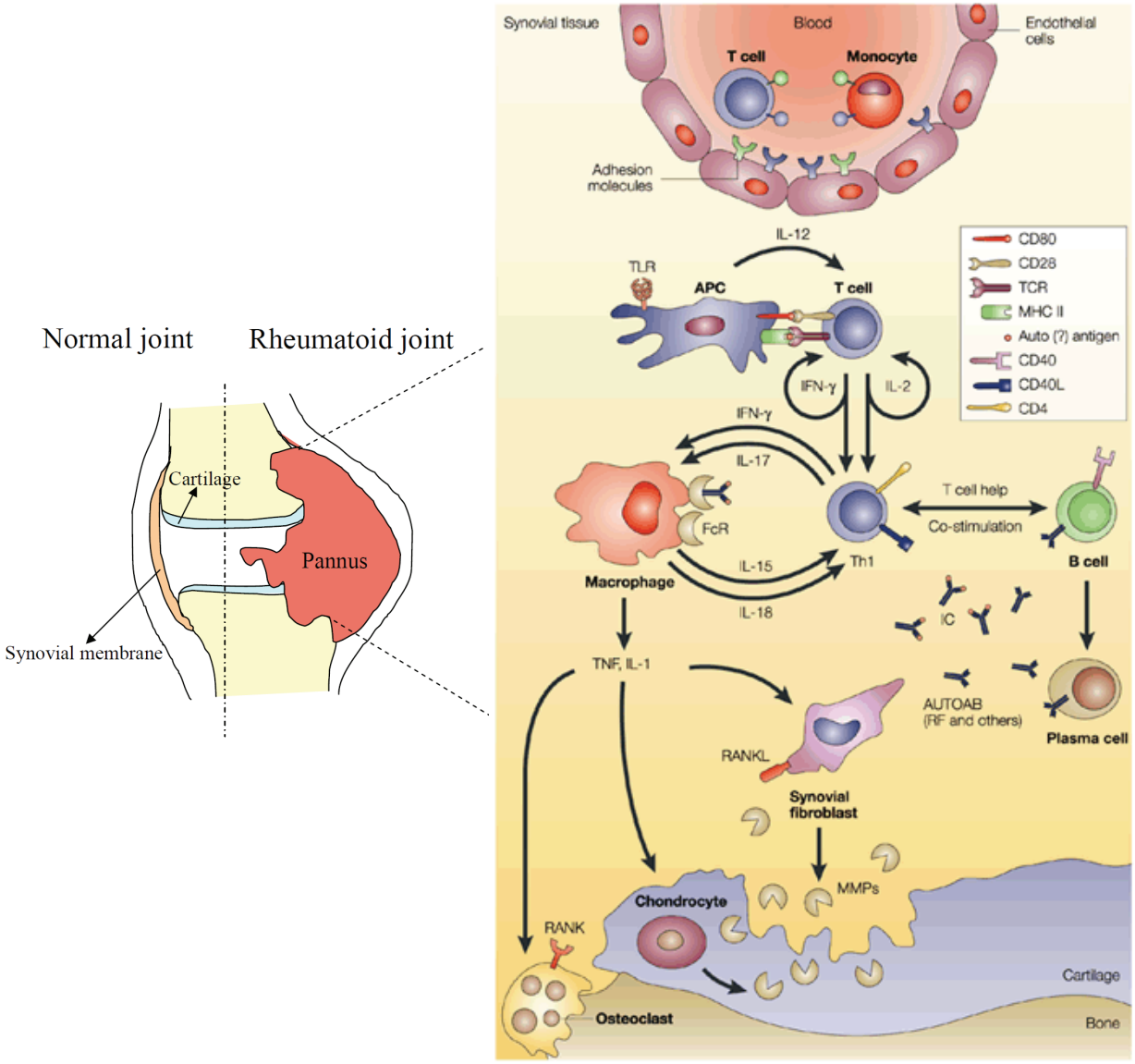
1.2.2. Immune cells and imbalanced cytokine networks in the development of RA

The immune-complex theory only explains part of the pathogenesis of RA because some RA patients don't have those autoantibodies in their sera. Compared to normal individuals, the synovium of RA patients are highly infiltrated with different immunological cells that produce a variety of inflammatory mediators in synovium and synovial fluid. The imbalanced cytokine networks between pro-inflammatory and anti-

inflammatory cytokines became the focus of RA pathogenesis.

In adaptive immune response, T-cell activation needs two pairs of signals. The first is antigen presentation through the MHC molecules on antigen presenting cells (APCs), such as macrophages and dendritic cells, to T-cell receptors; and the second signal is the ligation of the costimulation molecules expressed on APCs with its cognate receptors on T cells (Stuart and Racke 2002). Activated T cells can promote arthritis development through activation and interaction with other inflammatory cells, such as B cells, macrophages, mast cells and SF inside the synovium. These cells can produce autoantibodies, proteases or different cytokines and chemokines to accelerate inflammation and destruction inside the affected joints (Steiner, Tohidast-Akrad et al. 1999; Smolen and Steiner 2003) (Fig. 1). For example, activated T cells can produce low levels of interferon (IFN)-γ and interleukin (IL)-2 to differentiate to promote cellular immunity (enhance the killing efficacy of macrophages and the proliferation of cytotoxic CD8+ T cells) (Abbas, Murphy et al. 1996; Dolhain, van der Heiden et al. 1996; Schulze-Koops and Kalden 2001). T cell-derived IL-17 drives neutrophil activation and cytokine release. IL-17 can also stimulate macrophage and SF activation to produce a broad range of chemokines and pro-inflammatory cytokines, such as interleukins, tumor necrosis factor-α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF) and prostaglandins. IL-17 can also induce matrix metalloproteinase (MMP) production from SF and chondrocytes, and osteoclast activation for subsequent cartilage erosion and bone resorption in RA (Stamp, James et al. 2004; McInnes and Schett 2007).

Figure 1: Normal joint *versus* RA joint. The synovial joint is composed of two adjacent bony ends each covered with a layer of cartilage and surrounded by the synovial membrane. In normal joint, the synovial membrane is very thin and serves as an important source of nutrients and lubricants for cartilage. The synovial lining (facing the cartilage and bone) consists of a thin (1-3 cells) layer of SF. However, in RA joint, the lining layer of synovium becomes highly proliferative (it can have a thickness of > 20 cells) and forms a "pannus" at the junction of cartilage-bone-synovial membrane. The inflammatory response of synovial membrane (synovitis) locally activates a variety of infiltrated immune cells, such as T cells, macrophages, mast cells, B cells, plasma cells, dendritic cells, as well as the formation of new blood vessels. Angiogenesis is needed to sustain the hyperplasia of the synovial membrane. Through different cytokines and cell-cell interactions, these inflammatory cells constitute the pivotal events leading to the chronic inflammation and progressive erosions in cartilage and bone.



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On the other hand, anti-inflammatory cytokines, like transforming growth factor-β (TGF-β) and suppressive cytokine signaling mechanisms, such as suppressor of cytokine signaling (SOCS)-3, are also found in RA synovium, but the levels are insufficient compared to pro-inflammatory cytokines (Schulze-Koops and Kalden 2001; Shouda, Yoshida et al. 2001). Recently, treatments using biological response modifiers (BRMs), such as TNF-α blockers and IL-1 inhibitor, to target specific cytokines provided strong evidence that imbalanced cytokine networks are very important in RA development. For some RA patients, these BRMs not only relieve the clinical symptoms, but also slow the radiographical progression of the disease (Feldmann and Maini 2001; Bingham 2008). However, continuous anti-cytokine treatment is required for long-term control of the disease, suggesting additional mechanisms are involved in the development of RA.

Genetic factors have also been reported to be associated with RA. Human leukocyte antigen (*HLA*)-*DR* gene of the MHC is frequently found in RA patients with respect to non-RA controls, leading to a predisposition for RA (Stastny 1976). Further studies mapped the association with RA to the *HLA* gene locus encoding for the third hypervariable region of DRβ chain, especially the amino acids 70-74 with the sequences of glutamine-leucine-arginine-alanine-alanine (QKRAA) or QRRAA (Nepom, Byers et al. 1989). This epitope is thought to be closely related to disease susceptibility and severity. Arthritogenic peptides may bind to this epitope of MHC molecules to initiate T-cell activation, leading to inflammation and RA development in susceptible individuals (Weyand, Hicok et al. 1992).

1.2.3. Synovial fibroblasts (SF)

In normal synovium, the lining layer contains only one to three layers of cells, most of which are macrophage-like synoviocytes (Type A synoviocytes) and fibroblast-like synoviocytes (Type B synoviocytes) (Ritchlin 2000). However, in RA synovium, these SF proliferate greatly to more than ten layers of cells in the lining layer, and make up of a pannus in the area invading cartilage and bone. The sublining layer of RA synovium is heavily infiltrated with different inflammatory cells — macrophages, T/B lymphocytes, neutrophils and mast cells (Fig. 1). In recent years, a subpopulation of activated SF was identified in the lining layer of RA synovium that is different from the normal SF. In morphology, these activated RASF are rounder with large pale nucleus and prominent nucleoli inside. In culture, these SF show an aggressive behavior with loss of contact inhibition (Pap, Muller-Ladner et al. 2000). RASF co-implanted with normal human cartilage engrafted into SCID mice maintained their invasive and destructive behavior in the normal human cartilage, but SF from normal subjects did not (Muller-Ladner, Kriegsmann et al. 1996). This study suggests that the aggressive phenotype of RASF could be maintained over long period of time even in the absence of pro-inflammatory cytokines.

Synovial fibroblasts, as part of a complex cellular network, also play an important role in the pathogenesis of RA. RASF can be stimulated by macrophage-derived TNF-α and IL-1 to express increasing levels of adhesion molecules that mediate the attachment of these activated SF to the articular cartilage, facilitating the deep invasion of SF into the extracellular matrix of cartilage. Activated RASF also overexpress matrix-degrading enzymes for the progressive breakdown of the cartilage collagen in the affected joints (Pap, Muller-Ladner et al. 2000; McInnes and Schett 2007). Anti-TNF-α treatment not

only improved inflammation, but also reduced MMP levels in the sera of RA patients (Brennan, Browne et al. 1997). In adjuvant arthritic rats, the combined treatment with methotrexate (MTX) and IL-1 receptor antagonist (IL-1ra) also resulted in 84% inhibition of paw swelling and 97% inhibition of bone resorption (Bendele, Sennello et al. 1999). In addition, recent studies demonstrated that activated RASF expressed osteoclast differentiating factor (ODF) to induce macrophage differentiation into osteoclast-like bone resorbing cells. *In situ* hybridization was used to detect ODF mRNA in RA synovium, and results showed dominant levels of ODF mRNA in the lining layer and the sites where synovial tissue attached to the bone (Shigeyama, Pap et al. 2000). Therefore, SF contribute to joint destruction not only directly through the release of matrix degradative enzymes, but also indirectly through the activation of osteoclasts.

Furthermore, apoptosis of inflammatory cells and stromal cells plays a potential role in the resolution of inflammation. Insufficient apoptosis of SF, macrophages and T lymphocytes might contribute to their local accumulation and the persistence of RA (Pope 2002). Studies also demonstrated that because of the higher level of B-cell lymphoma-extra large (Bcl-xL) expression, T cells isolated from RA joint were more resistant to spontaneous apoptosis than T cells from gout patients (Salmon, Scheel-Toellner et al. 1997).

1.2.4. Role of mast cells in the pathogenesis of RA

1.2.4.1. Basic biology of mast cells

Mast cells are derived from bone marrow progenitor cells that migrate into tissues, where they undergo final maturation (Metcalfe, Baram et al. 1997). Mast cells are widely distributed throughout the body, especially in the epithelial surfaces contacting with the external environments, such as skin, airways and gastrointestinal tract. Mast cells usually cluster around blood vessels, nerves, smooth muscles and mucus-producing glands. Their distribution at the interface between host and environment reflects their role in early defence against invading pathogens (Marshall 2004).

Mast cells can be activated by a variety of stimuli through the following potential mechanisms: 1) cross-linking of FcεRI with IgE and its specific antigen (Kinet 1999); 2) Toll-like receptor-mediated activation by different pathogens, such as parasites, bacteria, viruses, as well as other microbial products (Marshall 2004); 3) indirect activation through the complement system (Marshall 2004); 4) activation by various cytokines, peptides (*e.g.* substance P and endothelins), and other inflammatory mediators (Galli, Nakae et al. 2005). Activated mast cells secrete a broad range of biologically active mediators for multiple critical functions, including innate immunity, host defense against infections, immunomodulation of the immune system, etc. (Metcalfe, Baram et al. 1997). These biological mediators can be classified into three groups as follows: 1) granule-associated mediators, such as histamine, proteases, proteoglycans and carboxypeptidase A (Boyce 2003); 2) growth factors, chemokines and cytokines; 3) lipid-derived substances, such as prostaglandin D_2 (PGD_2), leukotriene B_4 (LTB_4) and leukotriene C_4 (LTC_4) (Metcalfe, Baram et al. 1997).

1.2.4.2. Mast cells: a cellular link between different effector cells and RA

In RA synovium, mast cells exhibit a 6-25-fold increase in number compared with normal subjects and serve as an amplifier of inflammation either through soluble mediators or direct cell-cell interactions in RA (Fig. 2).

Activated mast cells recruit circulating neutrophils into RA synovium through different inflammatory mediators, such as histamine, LTB_4, TNF-α and IL-1 (Metcalfe, Baram et al. 1997). These mediators activate endothelial cells of nearby venules to produce adhesion molecules (selectins and integrins) and chemokines. Selectins and integrins mediate the rolling and adhesion of leukocytes on the endothelium; and chemokines induce leukocyte migration through the endothelium to the sites of inflammation (Kelly, Hwang et al. 2007). Mast cell-derived TNF-α, platelet-activating factor (PAF), LTB_4 and substance P can activate the arriving neutrophils to produce a broad range of cytokines and proteases to contribute to the ongoing joint injury in RA (Cassatella 1999). These neutrophil-derived mediators could in turn activate the resident mast cells to reinforce the inflammatory response (Befus, Mowat et al. 1999). In addition, mast cell-derived mediators, such as histamine, IL-1 and TNF-α, also promote the migration, maturation and function of dendritic cells in RA synovium (Galli, Nakae et al. 2005).

The hyperplastic RA synovium is highly infiltrated with T and B lymphocytes. Mast cells recruit T/B cells into the synovium by producing various chemoattractant mediators, such as LTB_4, IL-16, and macrophage inflammatory protein-1α/β (MIP-1α/β) (Rumsaeng, Cruikshank et al. 1997; Tedla, Wang et al. 1998; Tager, Bromley et al. 2003). These infiltrated T/B cells can become activated and display different effector functions in RA synovium. For example, mast cell-derived histamine and cytokines (*e.g.*

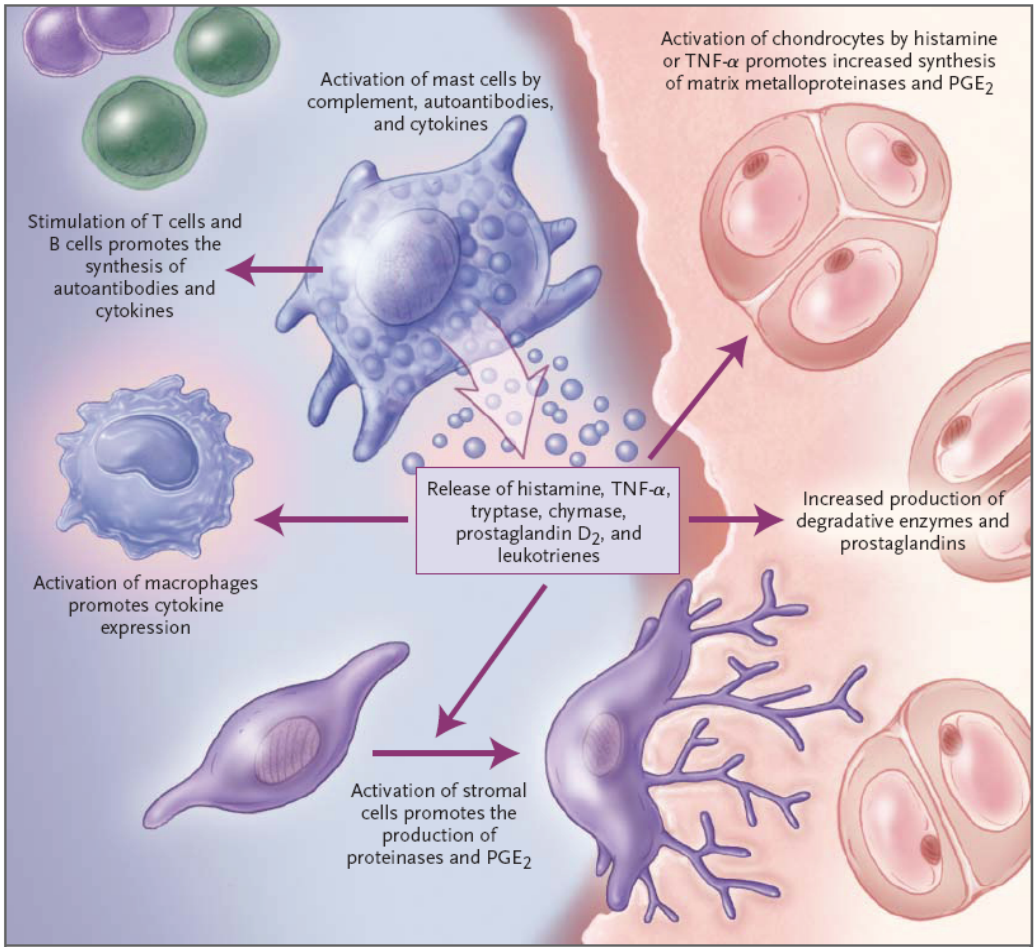
ILs and TNF-α) stimulate T/B cells to produce different autoantibodies and cytokines (Mekori and Metcalfe 1999; Jutel, Blaser et al. 2005). In turn, these activated T cells can induce mast cells to release more inflammatory mediators to augment the lymphocyte functions (Mekori 2004).

Normally, the macrophage is only a minority constituent of the synovial lining. However, in rheumatoid synovium, macrophages are prominently expanded to constitute a major component of the hyperplastic synovial lining as well as the sublining tissue. Mast cell-derived mediators, such as histamine, monocyte chemoattractant protein-1 (MCP-1) and IL-8, potently contribute to the recruitment of macrophages from circulating monocytes into the RA synovium. Mast cells can stimulate and activate these macrophages to produce a variety of cytokines (*e.g.* TNF-α and IL-1) to promote inflammation inside the affected joints (Metcalfe, Baram et al. 1997). In addition, macrophage-derived TNF-α promotes the expression of stem cell factor (SCF) on SF. SCF induces mast cell development, proliferation and chemotaxis, suggesting an important role of macrophages in the mast cell accumulation in RA synovium (Kiener, Hofbauer et al. 2000).

The expanded population of mast cells is also very important for tissue remodeling and joint destruction in RA. SF are highly proliferated in RA synovium, and mast cells promote this abnormal fibrosis by producing different fibroblast mitogens, such as histamine, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and TGF-β (Jordana, Befus et al. 1988; Li and Baek 2002). Many mast cell-derived mediators also exert angiogenic effects and contribute to angiogenesis in the formation and maintenance of pannus inside RA joints (Koch 2003). Proteases (*e.g.* tryptase and chymase) released directly from mast cells can activate MMP-1 for cartilage degradation.

Mast cells can also potentially stimulate the production of degradative enzymes, mainly MMPs, from local SF and chondrocytes (Tetlow, Adlam et al. 2001). In addition, mast cell-derived mediators, such as TNF-α, IL-1 and heparin, can induce receptor activator of nuclear factor-κB (RANK) expression on macrophages which, when interfering with RANK ligand (RANKL) on stromal cells (*e.g.* SF) or T cells, differentiate into osteoclasts for bone absorption (Scheven, Milne et al. 1999; Walton, Duncan et al. 2002; Smolen and Steiner 2003). This explains why patients treated with long-term heparin have decreased bone density and symptomatic fractures. Also, osteopenia is often found in systemic mastocytosis patients (Fallon, Whyte et al. 1981), suggesting the similar mechanism may be responsible for bone erosion in RA patients.

Figure 2: Mast cells and other cell types inside RA joint. The activation of mast cells by complement, autoantibodies and cytokines leads to the release of granule contents, as well as the production of cytokines and leukotrienes. All of these inflammatory mediators exert a variety of effects on neighboring cells to promote RA development.



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1.3. LEUKOTRIENE B_4 (LTB_4)

LTB_4 is a lipid inflammatory mediator derived from the cytosolic phospholipase A_2 ($cPLA_2$)-released arachidonic acid (AA) from membrane phospholipids in response to inflammatory stimuli. AA is converted to leukotriene A_4 (LTA_4) under the action of 5-lipoxygenase (5-LO), which is assembled with 5-LO-activating protein (FLAP) in the perinuclear region. LTA_4 is then enzymatically converted into either LTB_4 by LTA_4 hydrolase or LTC_4 by LTC_4 synthase (Fig. 3). The exact role of FLAP in LTB_4 biosynthesis has not been fully elucidated. One hypothesis is that FLAP might be a scaffolding protein that facilitates assembly of 5-LO and $cPLA_2$ in the perinuclear region during cellular activation (Byrum, Goulet et al. 1997). Another hypothesis is that FLAP may bind AA and present this substrate to 5-LO to promote leukotriene production (Abramovitz, Wong et al. 1993).

LTB_4 mediates its effects through two G protein-coupled receptors (GPCRs) — LTB_4 receptor (BLT)1 and -2. Genes for these two receptors are located very close together in both human and mouse genomes, and human BLT1 and BLT2 proteins show 45% homology at the amino acid level. Human BLT1 is primarily expressed in peripheral blood leukocytes; while human BLT2 protein is expressed more ubiquitously, especially in spleen, leukocytes, ovary and liver. BLT1 is a high-affinity receptor that is specific for LTB_4; whereas BLT2 is a low-affinity LTB_4 receptor that also binds other eicosanoids (Toda, Yokomizo et al. 2002).

LTB_4 is a potent leukocyte chemoattractant. LTB_4 can rapidly induce integrin expression and activation on neutrophils (van Pelt, de Jong et al. 1997). Integrins facilitate firm adhesion of neutrophils on the endothelium within the blood vessels, promoting neutrophil migration into the sites of inflammation (Kelly, Hwang et al. 2007).

Subcutaneous injection of LTB_4 induced neutrophil recruitment into the skin (Camp, Coutts et al. 1983), and neutrophils also infiltrated into airways following intratracheal instillation of LTB_4 (Martin, Pistorese et al. 1989). In the mouse model of inflammatory bowel disease, the LTB_4 receptor antagonist SC-53228 inhibited neutrophil infiltration into the colonic mucosa (Fretland, Anglin et al. 1995). LTB_4 has also been demonstrated to recruit monocytes and macrophages into inflammatory sites. In the mouse model of acute septic peritonitis, the LTB_4 receptor antagonist CP-105696 inhibited the recruitment of both neutrophils and macrophages into the peritoneum, leading to significantly increased mortality (Matsukawa, Hogaboam et al. 1999).

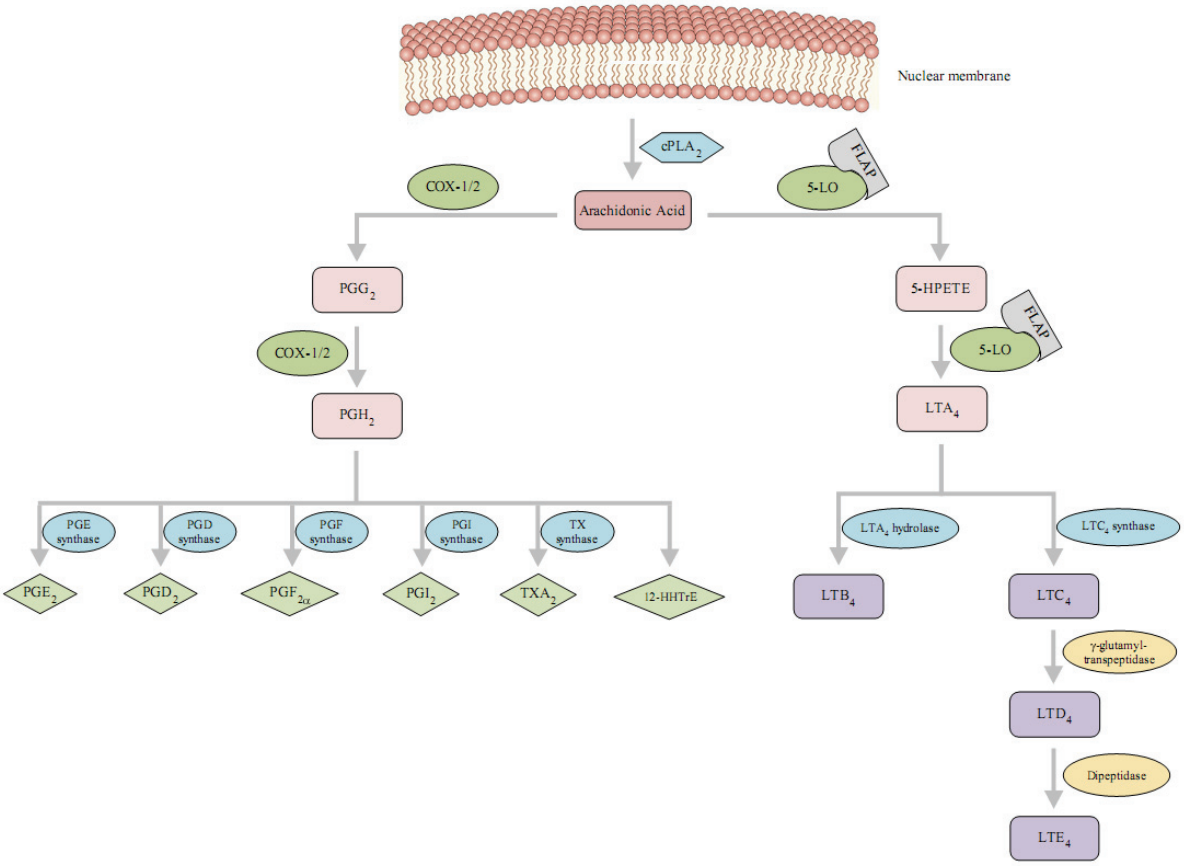
LTB_4 can activate leukocytes to produce reactive oxygen species (Jones 1993; Li, Ferrante et al. 1996) and release lysosome enzymes (Rae and Smith 1981). LTB_4 can also promote the phagocytic effects of neutrophils and macrophages in bacterial infections (Demitsu, Katayama et al. 1989). Neutrophils and macrophages from *5-LO*-knockout mice exhibited impairments in phagocytosis and killing of bacteria (Mancuso, Nana-Sinkam et al. 2001), and exogenous LTB_4 could restore the phagocytic effects of neutrophils and macrophages in leukotriene-deficient mice (Bailie, Standiford et al. 1996).

LTB_4 can also promote the effector functions of inflammatory cells. LTB_4 is involved in T-cell activation and proliferation. It enhances the production of IFN-γ, IL-4 and IL-10 in concanavalin A-treated T cells (Arcoleo, Milano et al. 1995), and an LTB_4 receptor antagonist inhibited T-cell proliferation and cytokine production (Morita, Takeda et al. 1999). LTB_4 preferentially stimulated IL-6 production in human peripheral blood monocytes (Poubelle, Stankova et al. 1991). In early host defense responses, LTB_4 augmented human natural killer cell activity against target cells in inflammatory and

infectious diseases (Rola-Pleszczynski, Gagnon et al. 1983). LTB_4 also activated isolated avian osteoclasts to form resorption lacunae and stimulated bone resorption (Flynn, Qiao et al. 1999). Moreover, LTB_4 can promote cell survival and proliferation through inhibition of apoptosis (Bortuzzo, Hanif et al. 1996; Hebert, Takano et al. 1996; Hennig, Ding et al. 2002).

Thus, LTB_4 participates in both the host defense responses to invading pathogens and the pathogenesis of many inflammatory diseases. Significantly elevated levels of LTB_4 were detected in the sputum, bronchoalveolar lavage or exhaled breath condensates from patients with cystic fibrosis (Konstan, Walenga et al. 1993), chronic obstructive pulmonary disease (COPD) (Crooks, Bayley et al. 2000), asthma (Montuschi and Barnes 2002) and acute respiratory distress syndrome (ARDS) (Antonelli, Bufi et al. 1989). Increased LTB_4 was also reported in the intestinal mucosa of patients with inflammatory bowel disease (Sharon and Stenson 1984), in the skin of psoriasis patients (Duell, Ellis et al. 1988), in the synovial fluid of RA patients (Ahmadzadeh, Shingu et al. 1991) and in the cerebrospinal fluid of patients with active multiple sclerosis (Neu, Mallinger et al. 1992). Further studies showed LTB_4 receptor antagonists can significantly ameliorate disease severity (Fink, O'Sullivan et al. 1993; Turner, Breslow et al. 1996), suggesting LTB_4 as a pivotal target for the treatment of inflammatory diseases.

Figure 3: Cyclooxygenase (COX)-1/2 and 5-LO pathways. Stimuli-activated $cPLA_2$ catalyzes AA to be released from membrane phospholipids. The 5-LO pathway leads to the formation of leukotrienes; whereas, the COX-1/2 pathway leads to the formation of prostaglandins and thromboxanes.



1.4. MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) SIGNALING PATHWAYS

MAPKs are a family of serine/threonine (Ser/Thr) protein kinases that are widely conserved among eukaryotes. MAPKs can be activated by a variety of stimuli including hormones, growth factors, cytokines and environmental stresses. These stimuli may act through different receptors, such as receptor tyrosine kinases (RTKs), GPCRs and cytokine receptors (Raman, Chen et al. 2007). MAPK signaling pathways constitute a large kinase network that regulates many cellular programs, such as cell proliferation, cell differentiation, cell movement, gene expression and apoptosis. Dysregulation of MAPK signaling pathways has been demonstrated in many pathological situations, such as inflammation, oncogenesis and metastasis (Orton, Sturm et al. 2005).

To date, three MAPK signaling pathways have been characterized in detail — extracellular signal-regulated kinases (ERK)1/2, c-Jun NH_2-terminal kinases (JNK)1/2/3/stress-activated protein kinase (SAPK) and p38 (p38 α/β/γ/δ). Other MAPK signaling pathways include ERK5, ERK7/8 and ERK3/4 cascades (Hayashi and Lee 2004), but less information is currently available. MAPK signaling pathways are organized hierarchically into modular cascades (Fig. 4). The upstream MAPK kinase kinases (MAPKKKs) are activated by different stimuli, leading to the sequential phosphorylation and activation of MAPK kinases (MAPKKs) and MAPKs (Raman, Chen et al. 2007). Activated MAPKs can regulate gene transcription through phosphorylation of target transcription factors. MAPKs can also regulate other cellular functions through phosphorylation of cytoplasmic proteins, such as cytoskeletal proteins (Zhang and Dong 2007).

1.4.1. ERK1/2 pathway

Ras → Raf → MAPK/ERK kinase 1/2 (MEK1/2) → ERK1/2 cascade can be activated strongly by mitogenic stimuli, such as growth factors, serum and cytokines (Goldsmith and Dhanasekaran 2007). Activation of cell surface receptors (*e.g.* RTKs and GPCRs) transduces signals to Ras, which is a membrane-bound guanosine triphosphatase (GTPase) cycling between inactive, GDP-bound and active, GTP-bound forms. The conversion from inactive to active state of Ras is mediated by guanine-nucleotide exchange factors (GEFs) that stimulate the exchange of GDP for GTP. Once bound to GTP, Ras is able to activate downstream effectors, resulting into the propagation of signals (McKay and Morrison 2007). In contrast, GTPase-activating proteins (GAPs) upregulate the GTPase activity of Ras, leading to decreased GTP-Ras and reduced activity. At the C-terminal end of Ras protein, there is a CAAX motif that is responsible for the membrane localization of Ras (Mor and Philips 2006). Ras can recruit Raf (A-Raf, B-Raf and C-Raf/Raf-1) to the plasma membrane for subsequent activation (Wellbrock, Karasarides et al. 2004). Raf then phosphorylates the downstream MEK1/2 on two serine residues. MEK1 and MEK2 are dual-specificity kinases and share 80% amino acid identity. Activated MEK1/2 phosphorylate and activate ERK1/2 within a conserved threonine-glutamate-tyrosine (TEY) motif (Zebisch, Czernilofsky et al. 2007) (Fig. 4). ERK1 and ERK2 share 83% amino acid identity and are expressed in a variety of tissues. Activated ERK1/2 can translocate to nucleus to phosphorylate many substrates, such as c-Fos, activating transcription factor-2 (ATF-2), Ets like gene-1 (Elk-1), c-Jun, c-myc and signal transducer and activator of transcription 3 (STAT3). Activated ERK1/2 can also phosphorylate cytoplasmic proteins (*e.g.* PLA_2, Syk and calnexin) and cytoskeletal proteins (*e.g.* neurofilaments and paxillin) (Yoon and Seger 2006).

ERK1/2 signaling pathway plays crucial roles in many cellular programs, such as cell proliferation, cell differentiation, cell migration and oncogenesis. Deficiency in ERK1 and ERK2 affected adipocyte differentiation and proliferation, respectively (Bost, Caron et al. 2002). *ERK*-knockout mice are deficient in thymocyte development (Pages, Lenormand et al. 1993). Also, approximately one third of human cancers show dysregulated ERK1/2 signaling. Many components are involved in these processes, including transcription factors [*e.g.* c-myc, c-Fos, cyclic adenosine monophosphate (cAMP) response element binding (CREB) and activator protein-1 (AP-1)] that control early response genes, elongation factor eIF4 and activator of RNA polymerase I that affect protein synthesis, and proteins involved in cell migration, such as myosin light chain kinase (MLCK), calpain, focal adhesion kinase-1 (FAK) and paxillin (Reddy, Nabha et al. 2003; Huang, Jacobson et al. 2004; Giehl 2005). ERK1/2 can also down-regulate some anti-proliferative genes, such as *Tob1* and *Ddit3* (Suzuki, J et al. 2002; Lawrence, McGlynn et al. 2007), further contributing to oncogenesis. Inhibition of ERK1/2 activation by a MEK inhibitor suppressed tumor growth in the mouse model of colon carcinoma (Sebolt-Leopold, Dudley et al. 1999). Other studies also demonstrated ERK1/2 signaling pathway as an important target in cancer treatment (Roberts and Der 2007).

1.4.2. JNK pathway

The JNK family is encoded by three genes — *Jnk1, Jnk2* and *Jnk3*. *Jnk1* and *Jnk2* genes are expressed ubiquitously, but *Jnk3* expression is largely restricted to brain, heart and testis. These genes are alternatively spliced into ten JNK isoforms that share 85% sequence identity (Gupta, Barrett et al. 1996). SAPK/JNK signaling pathway is potently

and preferentially activated in response to a variety of environmental factors, such as ultraviolet radiation, ceramides, DNA damaging agents, cytokines, and, to a lesser extent, some growth factors and GPCR agonists (Leppa and Bohmann 1999; Behrens, Jochum et al. 2000; Kyriakis and Avruch 2001). These stimuli activate SAPK/JNK signaling through several upstream MAPKKKs, such as apoptosis signal regulating kinase 1 (ASK1), mixed lineage kinase-3 (MLK-3), MEKK1-4, TGF-β activated kinase-1 (TAK-1) and tumor progression locus-2 (TPL-2), which then phosphorylate and activate MAPKKs (MKK4 and MKK7). MKK4 and MKK7 phosphorylate JNKs on the threonine-proline-tyrosine (TPY) site within the activation loop of the kinase (Weston and Davis 2007) (Fig. 4). Activated JNKs translocate into nucleus to regulate target gene transcription through phosphorylation of transcription factors, including c-Jun, Jun B, Jun D, ATF-2, c-Fos, etc. JNKs can also phosphorylate other substrates, such as nuclear hormone receptors [*e.g.* peroxisome proliferator-activated receptor-γ1 (PPAR-γ1) and glucocorticoid receptor] and retinoic acid receptors (RARs) (Bogoyevitch and Kobe 2006). Therefore, sustained JNK activity is necessary for cellular homeostasis involving many important processes, such as gene expression (Chaussepied, Lallemand et al. 1998), cell proliferation (Lan, Wang et al. 2004), and apoptosis (Liu and Lin 2005).

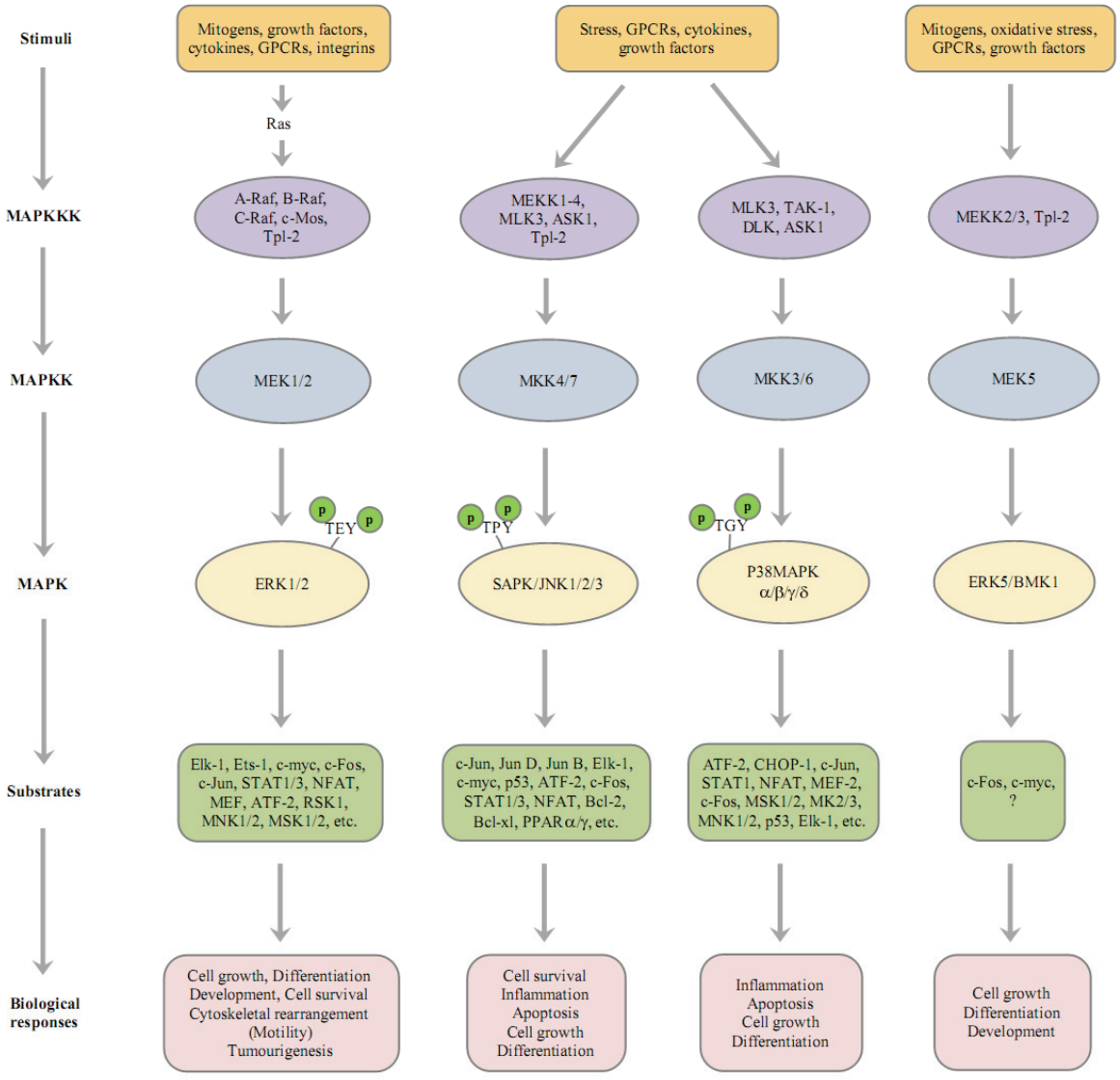
1.4.3. p38 pathway

Four isoforms of p38 MAPK have been identified — p38 α, β, γ (also known as ERK6/SAPK3) and δ (also known as SAPK4). p38 signaling pathway can be activated by a plethora of inflammatory cytokines, GPCR agonists and stresses, such as ultraviolet radiation, heat, hypoxia, and osmotic shock (Han, Lee et al. 1994; Rouse, Cohen et al. 1994). This signaling cascade can also be activated by some pathogens, insulin and

growth factors. The upstream MKK3 and MKK6 activate p38 isoforms through phosphorylation on the threonine-glycine-tyrosine (TGY) motif in the activation domain (Roux and Blenis 2004) (Fig. 4). Phosphorylated p38 kinases can activate an array of substrates, such as MAPK interacting kinase (Mnk)-1/2, MAPK-activated protein kinase-2 (MAPKAPK-2/MK2), and transcription factors (*e.g.* ATF-2, p53, c-Jun and c-Fos) (Zhao, New et al. 1999; Zarubin and Han 2005).

p38 signaling has been reported to regulate cell proliferation, angiogenesis and tumorigenesis. Compared with normal individuals, higher p38 kinase activity was detected in various cancers, and inhibition of p38 activity reduced cell proliferation and metastasis in several cancer cell lines (Johansson, Ala-aho et al. 2000; Greenberg, Basu et al. 2002; Gauthier, Pickering et al. 2005; Junttila, Ala-Aho et al. 2007). p38 pathway also plays essential roles in regulating production of inflammatory cytokines, mediating innate immune responses (Ashwell 2006) and apoptosis (Porras, Zuluaga et al. 2004; Grethe and Porn-Ares 2006).

Figure 4: Schematic representation of MAPK cascades. The activation of MAPK signaling pathways proceeds through the sequential activation of MAPKKK, MAPKK and MAPK.



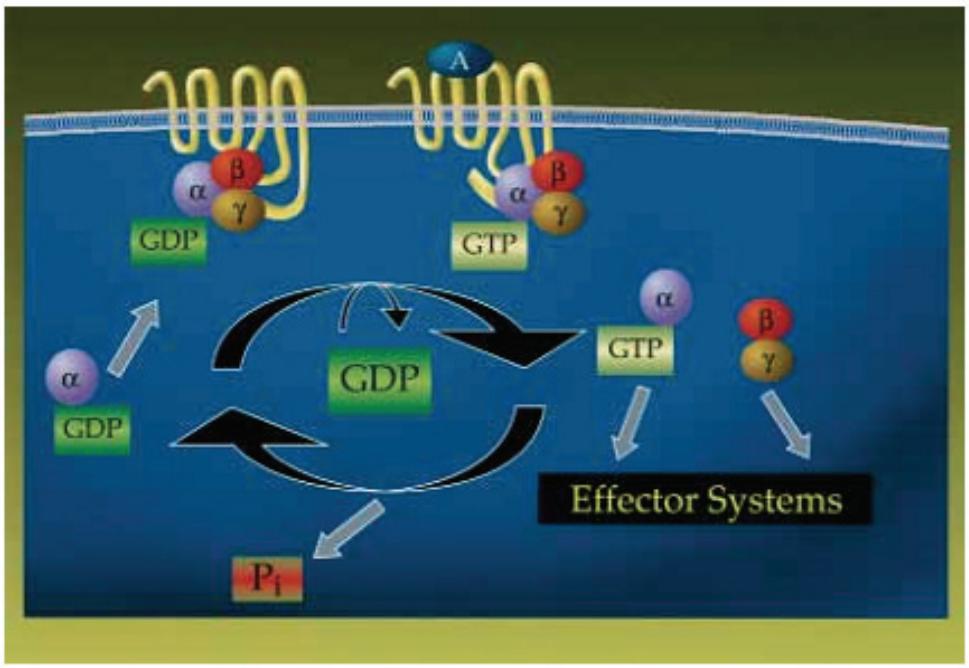
1.5. HETEROTRIMERIC GUANINE NUCLEOTIDE-BINDING PROTEINS (G PROTEINS)

Heterotrimeric G proteins, localized in the inner surface of plasma membrane, belong to a superfamily of GTPases and are key components in cell signal transmitting system. G proteins are activated by various GPCRs to mediate the effects of hormones, growth factors, neurotransmitters, chemokines and sensory stimuli (Forse 2000). Therefore, G protein-mediated signal transduction is very important for numerous cellular functions involving embryonic development, learning and memory, and organismal homeostasis (Neves, Ram et al. 2002). Alterations and mutations of G protein signaling have been linked to a variety of diseases, such as diabetes, cancer, cardiovascular disease, and allergy (Farfel, Bourne et al. 1999; Melien 2007). Heterotrimeric G proteins consist of G_α, G_β and G_γ subunits. To date, there are 20 known G_α subunits that have high affinity for guanine nucleotides, 6 G_β and 12 G_γ subunits (Neves, Ram et al. 2002). Based on the sequence similarities in G_α subunits, G proteins are classified into four families: $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$ and $G_{\alpha 12/13}$ (Offermanns 2001; Pierce, Premont et al. 2002).

Different subtypes of heterotrimeric G proteins are regulated through a common mechanism of GTPase cycle (Fig. 5). In the inactive state, the G_α subunit binds to GDP and exists as a heterotrimer with $G_{\beta\gamma}$ complex. The binding of ligand with GPCR induces a conformational change in the receptor to release GDP and replace with GTP on the G_α subunit. G_α-GTP causes destabilization of the heterotrimer of G protein and triggers the dissociation of G_α subunit from $G_{\beta\gamma}$ dimer and the receptor. Both G_α subunit and $G_{\beta\gamma}$ dimer are free to interact and activate effectors in various signaling cascades (Forse 2000; Neves, Ram et al. 2002). On the contrary, under the function of GAPs, the GTPase

activity of G_α subunit hydrolyzes G_α-GTP to G_α-GDP to terminate the effector functions. The resulting G_α-GDP reassociates with $G_{\beta\gamma}$ for another round of receptor activation to start a new cycle (Johnson and Druey 2002).

Figure 5: The G protein GTPase cycle. In the resting state, GPCR binds to G_α-GDP that is associated with $G_{\beta\gamma}$ subunits. Agonist binding to GPCR causes conformational change in GPCR and leads to the exchange of GTP for GDP on G_α subunit. G_α-GTP dissociates from $G_{\beta\gamma}$ complex, and both G_α and $G_{\beta\gamma}$ can activate downstream effectors. Eventually, GTP hydrolysis by the enzymatic activity of G_α subunit reverses to the GDP-bound G_α, increasing its binding affinity to $G_{\beta\gamma}$ dimer to close the cycle. A, agonist; P_i, inorganic phosphate.



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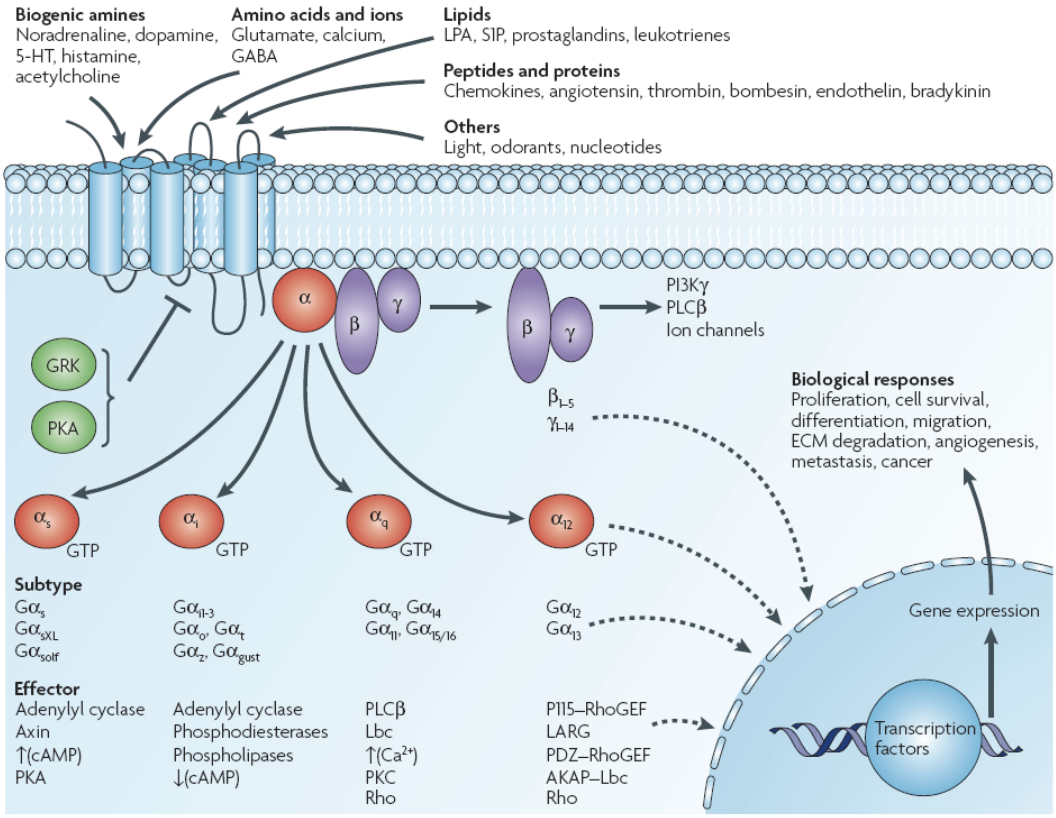
Various ligand-GPCR interactions activate distinct G_{α} subunits to exert different effects in cells (Fig. 6). Typically, $G_{\alpha s}$ activates adenylate cyclase (AC) to stimulate the production of cAMP from ATP. cAMP then acts as a second messenger to activate protein kinase A (PKA) for the phosphorylation of downstream metabolic enzymes, ion channels and transcription factors to regulate various metabolic and growth-related processes (Melien 2007). In contrast, activated $G_{\alpha i}$ protein causes inhibition of AC to reduce the cellular levels of cAMP. Pertussis toxin (PTX) can inactivate $G_{\alpha i}$ protein through adenosine diphosphate (ADP)-ribosylation to attenuate the inhibitory effect of $G_{\alpha i}$ on AC (Tsai, Adamik et al. 1984). All members of the $G_{\alpha i}$ family except G_z are sensitive to PTX (Murayama and Ui 1983). Members of the $G_{\alpha q}$ family bind to and activate phospholipase C (PLC) to catalyze the production of the second messengers inositol (1,4,5)-trisphosphate (IP3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP2). Subsequently, IP3 triggers the release of Ca^{2+} from endoplasmic reticulum (ER), which causes the cytosolic concentration of Ca^{2+} to increase, leading to a cascade of intracellular changes and activities. $G_{\alpha q}$-mediated liberation of DAG can also recruit and activate protein kinase C (PKC) to the cell membrane (Neves, Ram et al. 2002). G_{α} subunits, such as $G_{\alpha q}$ and $G_{\alpha 12/13}$, and $G_{\beta\gamma}$ are also involved in the activation of intracellular signaling cascades of MAPKs (Dorsam and Gutkind 2007) and the family of Rho GTPases for cytoskeletal rearrangement in cell migration (Johnson and Druey 2002; Sanders, Brian et al. 2008). Furthermore, $G_{\beta\gamma}$ can also directly activate PLC, K^+ channels and phosphatidylinositol 3-kinase (PI3K) (Neves, Ram et al. 2002).

Figure 6: G_{α} subtypes, their inducers and downstream effectors. Heterotrimeric G proteins are divided into four families by their G_{α} subunits: $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$ and $G_{\alpha 12/13}$. Various ligands, such as histamine, prostaglandins and leukotrienes, couple to their specific GPCRs to interact and activate one or more families of G proteins. Each G protein subtype can activate several downstream effectors for controlling many cellular functions. 5-HT, 5-hydroxytryptamine; ECM, extracellular matrix; GABA, gamma-aminobutyric acid; GRK, G protein receptor kinase; LPA, lysophosphatidic acid; S1P, phingosine-1-phosphate.



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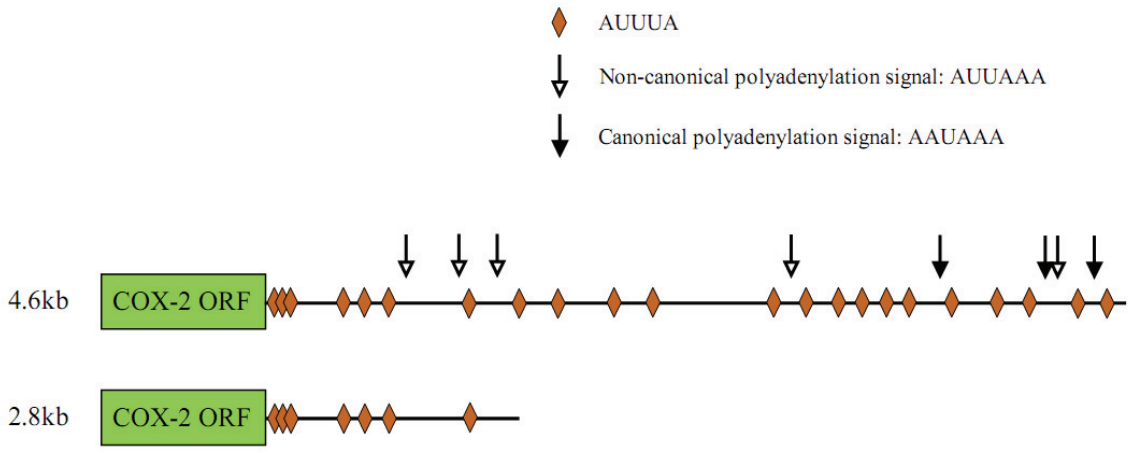
1.6. *COX-2* GENE

COX is the rate-limiting enzyme in the conversion from free AA to prostaglandin H_2 (PGH_2), which is further processed into different prostaglandins and thromboxanes by their respective synthases (Fig. 3). COX has two isoforms encoded by two related genes — *COX-1* and *COX-2*. These two isoforms are structurally similar, but are differentially expressed and regulated. Human COX-l and COX-2 proteins share 61% sequence identity. X-ray crystallographic analyses suggest that COX-1 and COX-2 proteins are monotopic, ER-associated homodimeric enzymes (Otto and Smith 1994; Picot, Loll et al. 1994). COX-2 monomer has three domains: a N-terminal epidermal growth factor (EGF)-like domain, a membrane-binding domain (Spencer, Thuresson et al. 1999), and a long α-helical catalytic domain that possesses heme-dependent peroxidase and cyclooxygenase activities (Kulmacz, van der Donk et al. 2003).

The *COX-2* gene consists of ten exons and nine introns. Sequence analysis of *COX-2* shows a TATA box 25 bp upstream from the transcription start site. The 800-bp 5'-flanking region of *COX-2* contains several other transcriptional regulatory sequences — one CCAAT-enhancer-binding protein (C/EBP) motif, two AP-2 sites, three SP1 sites, two nuclear factor kappa B (NF-κB) sites, one cAMP-response element (CRE) motif and one Ets-1 site (Faour, He et al. 2001). Exon 10 of *COX-2* gene is very large and contains the final 410 bp of the coding region and the entire 2550-bp 3'-untranslated region (3'-UTR) of COX-2 mRNA. Inside the COX-2 3'-UTR, there are canonical polyadenylation sites that can be used to transcribe into the primary 4.6-kb COX-2 mRNA, and the non-canonical polyadenylation sites result in multiple smaller COX-2 mRNAs. The COX-2 3'-UTR contains 22 copies of Shaw-Kamen AUUUA motif [AU-rich element (ARE)]

(Fig. 7) that are associated with COX-2 mRNA instability and translational inhibition (Tanabe and Tohnai 2002).

Figure 7: 3'-UTR of human COX-2 mRNA. There are 22 copies of AUUUA motif inside the 3'-UTR of the 4.6-kb *COX-2* transcript.



The mRNAs of many proto-oncogenes and genes encoding cytokines and growth factors contain the *cis*-acting elements known as AREs (van Hoof and Parker 2002). AREs can be divided into three categories as follows: Class I AREs (*e.g.* c-Fos) have one to three copies of scattered AUUUA motif with nearby U-rich stretches; Class II AREs (*e.g.* GM-CSF, TNF-α and COX-2) have multiple AUUUA motifs with some overlapping; Class III AREs (*e.g.* c-Jun) are lack of AUUUA motif, but contain U-rich sequences. These AREs appear to associate with multiple *trans*-acting RNA-binding proteins to mediate target mRNA degradation and/or translation efficiency (Piecyk, Wax et al. 2000; Wilusz, Wormington et al. 2001).

In contrast to the constitutively expressed COX-1, COX-2 expression can be rapidly induced at high levels upon stimulation by tumor promoters, growth factors and cytokines in a variety of cell types (Smith 1992). Overexpression of COX-2 is a common mechanism for the development of different cancers, inflammatory and rheumatic disorders (Lipsky 1999). *COX-2* gene expression can be regulated at transcriptional, post-transcriptional and translational levels (Tanabe and Tohnai 2002; Espel 2005). For instance, IL-lβ induces *COX-2* expression through both transcriptional activation and mRNA stabilization in endothelial cells (Ristimaki, Garfinkel et al. 1994). Post-transcriptional regulation of *COX-2* was also demonstrated in IL-1β-treated human synovial fibroblasts (Faour, He et al. 2001).

1.7. ARE-BINDING PROTEINS

1.7.1. Tristetraprolin (TTP)

TTP belongs to the family of CCCH (cysteine-cysteine-cysteine-histidine) tandem zinc-finger protein and can be transiently induced by stimuli, such as serum and growth factors (Blackshear 2002). TTP is normally localized in both the nucleus and cytoplasm, but can rapidly shuttle into the cytoplasm in response to growth factors or mitogens (Taylor, Thompson et al. 1996; Phillips, Ramos et al. 2002).

The function of TTP in gene regulation is still not clear. Many studies reported TTP as an mRNA destabilization protein that post-transcriptionally regulates target gene expression. TTP binds to class II AREs through two CCCH zinc-finger domains, targeting inflammatory mRNAs for rapid decay through recruiting exosome to these unstable mRNAs (Chen, Gherzi et al. 2001). The exosome is a complex of exonucleases that degrades mRNAs in 3'→5' direction (Houseley, LaCava et al. 2006). TTP can also

promote the deadenylation of target mRNAs for degradation by poly(A) ribonuclease (Lai, Kennington et al. 2003). Genome-wide analysis identified 250 mRNAs that were stabilized in *TTP*-/- mouse embryonic fibroblasts (Lai, Parker et al. 2006). Compared with wild-type cells, GM-CSF mRNA was more stable and had a longer poly(A) tail in *TTP*-/- macrophages (Carballo, Lai et al. 2000). *TTP*-/- macrophages also had prolonged TNF-α mRNA half-life and higher levels of TNF-α protein than wild-type mice. Because of this overproduction of TNF-α, *TTP*-/- mice developed generalized inflammation characterized by inflammatory arthritis, inflammatory bowel disease, dermatitis and neutrophilia (Carballo, Lai et al. 1998). TTP can also down-regulate IL-2 (Ogilvie, Abelson et al. 2005), immediate early response 3 (Ier3) (Lai, Parker et al. 2006) and COX-2 (Phillips, Kedersha et al. 2004; Sully, Dean et al. 2004) mRNAs through ARE-mediated degradation. Decreased level of the decay factor TTP promoted COX-2 overexpression and could contribute to tumorigenesis (Zhang, Zhou et al. 1997; Sawaoka, Dixon et al. 2003; Young, Sanduja et al. 2009).

1.7.2. HuR (HuA)

HuR protein belongs to ELAV (embryonic-lethal abnormal vision)-like family of RNA-binding proteins (Ma, Cheng et al. 1996). In *Drosophila*, ELAV is essential for cell differentiation and cell development in neural system (Brennan and Steitz 2001). HuR protein is widely expressed in spleen, thymus, intestine and reproductive organs; whereas, the other members of ELAV family only expressed in terminally differentiated neurons (Peng, Chen et al. 1998; Lu and Schneider 2004).

HuR has a high specificity and affinity for AREs in the mRNAs of many proto-oncogenes and inflammatory cytokines (Fan and Steitz 1998; Peng, Chen et al. 1998).

The functions of HuR include stabilization of target mRNAs and promoting translation efficiency. Overexpression of HuR in different cell types stabilized GM-CSF (Fan and Steitz 1998), TNF-α (Dean, Wait et al. 2001), IL-3 (Ming, Stoecklin et al. 2001) or COX-2 (Dixon, Tolley et al. 2001) mRNAs. Consistently, *HuR*-knockout studies showed destabilization of the target mRNAs (Levy, Chung et al. 1998; Wang, Caldwell et al. 2000; Wang, Furneaux et al. 2000).

HuR is predominantly a nuclear protein; however, it can shuttle between nucleus and cytoplasm via a HuR nucleocytoplasmic shuttling sequence HNS (Fan and Steitz 1998), which may interact with the nuclear export factor CRM1 (chromosomal region maintenance protein 1) (Brennan, Gallouzi et al. 2000) to facilitate this process. The mRNA stabilization effect of HuR may require its translocation from the nucleus to the cytoplasm. HuR may bind to ARE-containing mRNAs in the nucleus and then escort them during and after export to the cytoplasm (Fan and Steitz 1998). In the cytoplasm, HuR may stabilize mRNAs either through inhibitory competition with other ARE-binding proteins, or by impeding the recruitment of exosome to the target mRNAs for degradation (Mukherjee, Gao et al. 2002). Leptomycin B (LMB), an antifungal and antitumor reagent, can inhibit CRM1-mediated nuclear export of HuR protein. LMB treatment resulted in nuclear retention of c-Fos mRNA (Brennan, Gallouzi et al. 2000) and inhibition of COX-2 mRNA stabilization in mammary cancer cells (Jang, Munoz-Najar et al. 2003).

1.7.3. ARE/poly(U)-binding/degradation factor 1 (AUF1)/hnRNP D and CArG box-binding factor-A (CBF-A)/AUF2

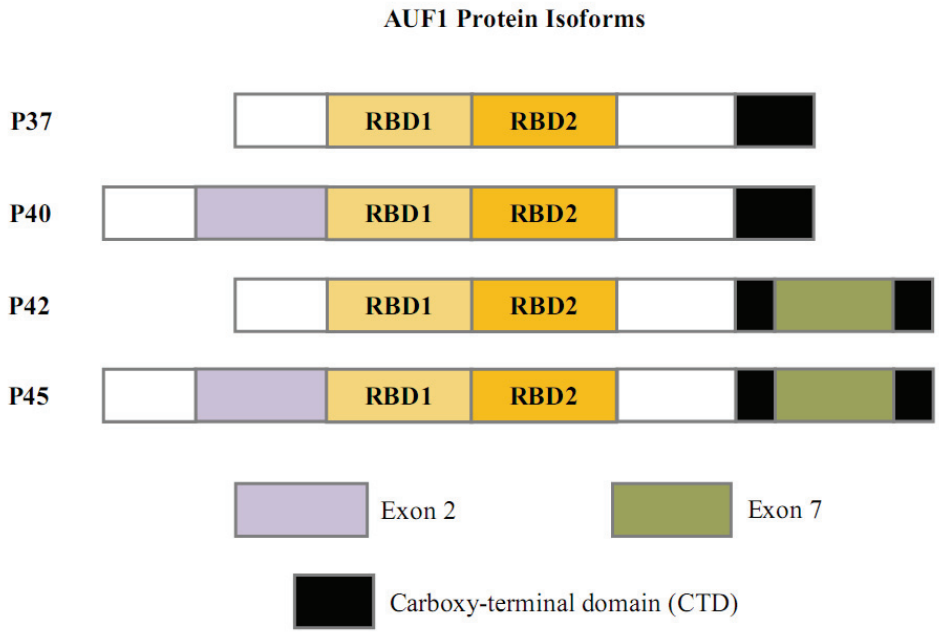
Due to alternative splicing from a single transcript, AUF1 consists of four isoforms — p37, p40, p42 and p45. All four isoforms contain the core protein that has two non-identical RNA-binding domains (RBD1 and RBD2), and an 8-amino acid glutamine-rich C-terminal region. In p40 and p45 isoforms, the 19-amino acid insert encoded by exon 2 is located in the N-terminal end of RBD1; while the 49-amino acid insert encoded by exon 7 is in the C-terminal domain of p42 and p45 isoforms (Wagner, DeMaria et al. 1998) (Fig. 8). These four AUF1 proteins are differentially expressed and distributed in a variety of cell types. For example, mononuclear cells from newborns express p37, p40 and p42 AUF1 isoforms; while cells from adults express p42 and p45 isoforms (Buzby, Lee et al. 1996). In the monocytic cell line THP-1, p37 and p40 AUF1 isoforms were almost exclusively found in the cytoplasm; whereas, p42 and p45 AUF1 proteins were recovered from the nuclear extract of these cells (Wilson, Lu et al. 2003).

All four isoforms of AUF1 can shuttle between nucleus and cytoplasm (Sarkar, Lu et al. 2003). Both destabilizing and stabilizing roles for AUF1 have been reported in different studies. p37 and p40 AUF1 were found to be partly responsible for the rapid decay of c-myc and GM-CSF mRNAs (Brewer 1991). In Chinese hamster ovary (CHO) cells, overexpression of p40, p42 and p45 AUF1 isoforms also strongly suppressed the levels of reporter mRNAs either containing or lacking AREs (Sarkar, Xi et al. 2003). The extent of destabilizing effect varies among these four isoforms, with p37 and p42 AUF1 displaying the most profound effects (Loflin, Chen et al. 1999). However, some studies also demonstrated mRNA stabilizing effect of AUF1 under some stresses and

differentiation signals in cells (Shyu and Wilkinson 2000). Overexpression of these four AUF1 isoforms in NIH3T3 cells differentially stabilized ARE reporter mRNAs, in particular, those carrying class II AREs, such as COX-2 (Xu, Chen et al. 2001). *In vivo*, overexpression of p37 AUF1 in transgenic mice resulted in increased or decreased expression of several target mRNAs (c-myc, c-Jun, c-Fos, GM-CSF and TNF-α), which played a key role in the development of sarcomas (Gouble, Grazide et al. 2002).

Recently, another ARE-binding protein AUF2 has been discovered that shares high sequence identity with AUF1. Alternative splicing of the *AUF2* transcript results in two isoforms — p37 and p42 that differ by a 47-amino acid insert close to the C-terminus (Dean, Sully et al. 2002). To date, the role of AUF2 in ARE-directed mRNA decay is unclear. AUF2 was found to be able to bind to the AREs of COX-2 and TNF-α mRNAs. In IL-1-stimulated HeLa cells, overexpression of AUF2 stabilized a reporter mRNA carrying the COX-2 ARE (Dean, Sully et al. 2002).

Figure 8: Schematic representation of AUF1 isoforms. RBD1 and RBD2 are in all isoforms. P40 and p45 AUF1 contain the fragment encoded by exon 2, and p42 and p45 AUF1 have the fragment encoded by exon 7. All isoforms have the C-terminal domain (CTD), which is interrupted by exon 7 in p42 and p45 AUF1.



Adapted from the research originally published in *The Journal of Biological Chemistry* (Bedabrata Sarkar, Jin-Yu Lu, and Robert J. Schneider. Nuclear import and export functions in the different isoforms of the AUF1/heterogeneous nuclear ribonucleoprotein protein family. *The Journal of Biological Chemistry*. 2003; 278(23):20700-20707. © the American Society for Biochemistry and Molecular Biology).

1.7.4. T cell intracellular antigen-1 (TIA-1), TIA-related protein (TIAR) and others

TIA-1 was originally found in a study to detect antigens in activated T cells and two isoforms (40 kD TIA-1 and 15 kD TIA-1) were identified. The 40 kD TIA-1 contains three RBDs and a glutamine-rich CTD; while the 15 kD TIA-1 appears to be derived from the CTD of the 40 kD TIA-1 through proteolytic processing (Tian, Streuli et al. 1991). Mutational analysis indicates that the second RBD mediates the specific binding of TIA-1 to uridylate-rich stretches of target RNAs (Dember, Kim et al. 1996). Although predominately localized in nucleus, TIA-1 can rapidly translocate to the cytoplasm in response to stresses and differentiation signals. TIA-1 colocalizes with untranslated mRNAs in the cytoplasm and plays a role as a translational silencer (Kedersha, Gupta et al. 1999). Lipopolysaccharide (LPS)-treated macrophages from *TIA-1*-deficient mice produced double the amount of TNF-α protein compared to wild-type mice, with no difference in TNF-α mRNA levels (Piecyk, Wax et al. 2000). Similar results were found in COX-2 regulation in embryonic fibroblasts from *TIA-1*-deficient mice (Dixon, Balch et al. 2003).

TIAR is a 42 kD protein that contains three RBDs and one CTD. TIAR shares 85% and 51% amino acid identity with TIA-1 in RBDs and CTD, respectively (Kawakami, Tian et al. 1992). TIAR also binds to the uridylate-rich stretches in target mRNAs with high affinity (Dember, Kim et al. 1996). Under normal conditions, TIAR is primarily localized in nucleus. In response to cellular stresses or Fas-mediated apoptosis, TIAR can rapidly translocate to the cytoplasm (Taupin, Tian et al. 1995; Kedersha, Gupta et al. 1999). However, the exact role of TIAR in gene regulation is not clear.

In addition, a number of other proteins have also been found to bind to the AREs of target mRNAs, such as hnRNP A0, CUGBP2, far upstream sequence element binding protein (FBP) family, and so on (Dean, Sully et al. 2004; Dixon 2004).

1.8. EPIGENETIC REGULATION OF GENE EXPRESSION

Epigenetics refers to the stable changes in gene expression that are not due to mutations or DNA base changes. These changes are heritable and may last for multiple generations (Holliday 2006). Several mechanisms are involved in the epigenetic regulation of genes — DNA methylation, histone modifications and RNA interference. Epigenetic regulation is critical for normal development and differentiation that involve many cellular programs, such as gene transcription, X chromosome inactivation, and genomic imprinting (Jaenisch and Bird 2003; Holliday 2006; Kouzarides 2007). Epigenetics also play important roles in maintaining chromosome stability through either defending against viral sequences or silencing of transposable elements (Yoder, Walsh et al. 1997).

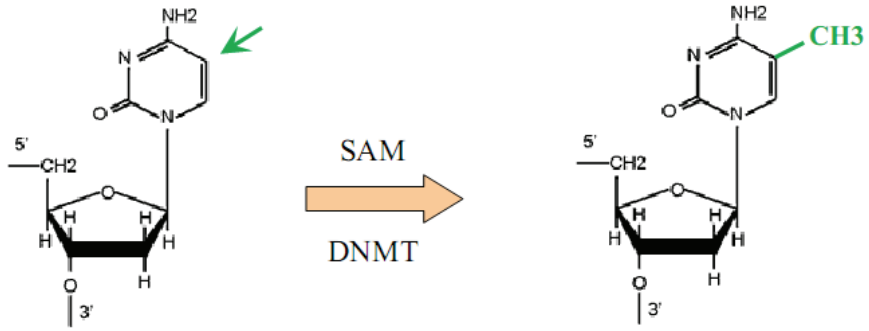
However, under the influence of environmental factors, epigenetic modifications can also contribute to the development of various pathological conditions, such as cancers (Ehrlich 2002; Esteller 2007), inflammatory and autoimmune diseases (*e.g.* RA and SLE) (Robertson 2005; Invernizzi 2007; Dieker and Muller 2009; Karouzakis, Gay et al. 2009), age-related diseases (Calvanese, Lara et al. 2009), and mental disorders (Iwamoto and Kato 2009; Kato 2009; Zawia, Lahiri et al. 2009). For example, aberrant DNA hypermethylation in the promoters of tumor suppressor genes can affect multiple cellular pathways involved in carcinogenesis, including cell invasion, cell cycle control, apoptosis, DNA damage repair, cytokine signaling, etc. (Esteller 2002; Laird 2003; Foley,

Craig et al. 2009). Meanwhile, global DNA hypomethylation is believed to cause chromosome instability and activate oncogenes to further contribute to cancer development (Ellis, Atadja et al. 2009). However, unlike genetic mutations, epigenetic alterations are reversible, offering the opportunities to use pharmacological approaches to reverse these changes and ameliorate the abnormal phenotypes (Ballestar, Esteller et al. 2006).

1.8.1. DNA methylation

A major epigenetic modification in eukaryotes is DNA methylation, which targets the 5'-cytosine in CpG dinucleotide by adding a methyl (CH_3) molecule to form methyl-CpG (Bird 2002). The source of CH_3 molecules is S-adenosylmethionine (SAM), which is produced through the folate and methionine pathways using methionine, choline, folic acid and vitamin B12 in our diet (Ulrey, Liu et al. 2005). DNA methylation is catalyzed by DNA methyltransferases (DNMTs) (Fig. 9). So far, three active DNMTs have been identified in mammals and the loss of any of which is lethal in mice (Li, Bestor et al. 1992). DNMT1 is the most abundant methyltransferase and has a higher preference for modifying hemimethylated CpG dinucleotides than unmethylated substrates. DNMT1 is essential for maintaining methylation patterns after DNA replication during cell division by copying the existing methylation profile of the old DNA strand onto the newly synthesized strand (Robertson and Wolffe 2000). In contrast, DNMT3a and DNMT3b are responsible for *de novo* DNA methylation to establish new somatic methylation patterns (Goll and Bestor 2005). Depending on the cell type and the stage of development, DNMT3a and DNMT3b target different genomic sites for methylation (Okano, Bell et al. 1999; Kaneda, Okano et al. 2004; Dodge, Okano et al. 2005).

Figure 9: DNA methylation is catalyzed by DNA methyltransferases (DNMTs).

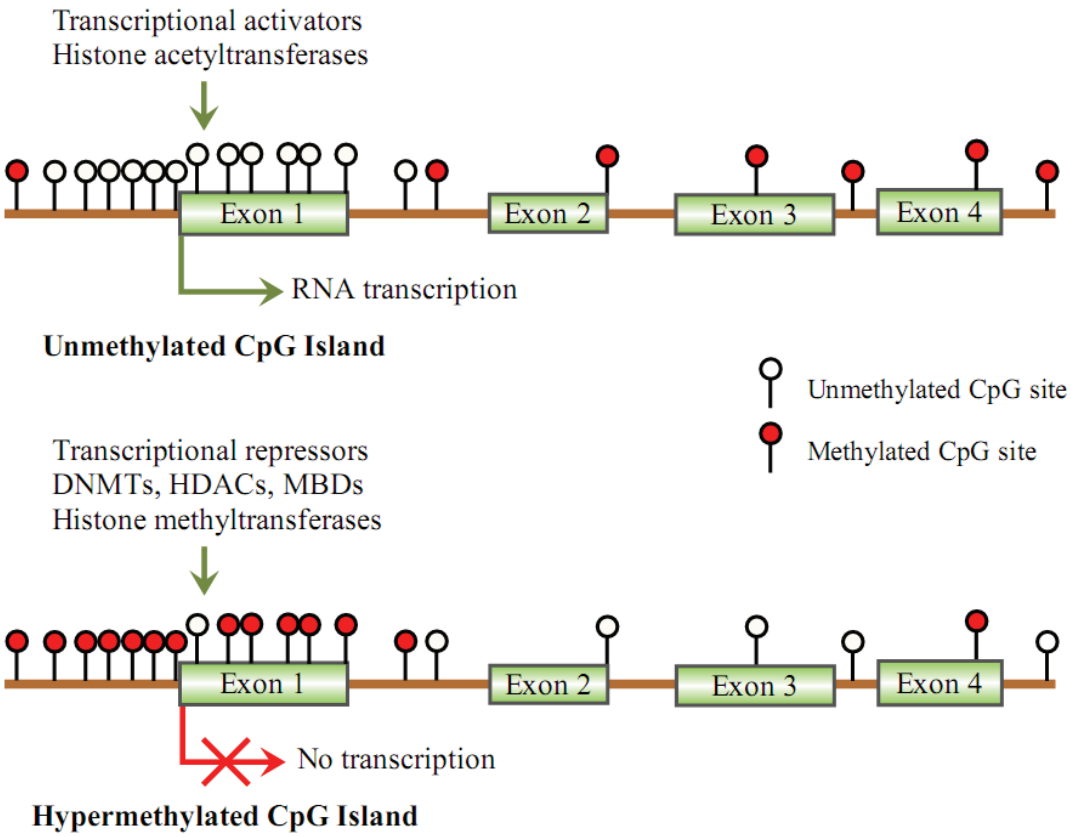


Deoxycytidine 5'-Methyl-cytidine

In normal cells, DNA methylation predominantly occurs in repetitive sequences, such as satellite DNA and parasitic elements (Yoder, Walsh et al. 1997), and helps to suppress the expression and mobility of transposable elements (Slotkin and Martienssen 2007). In humans, CpG dinucleotides only account for 1% of the whole genome and DNA methylation occurs at about 70-80% of all CpG dinucleotides. These CpGs are spread over the genome either as single dinucleotide sequences or grouped in clusters called "CpG islands". The definition of CpG island is a 0.5-4 kb long DNA with a G + C content ≥ 55% and an observation/expectation CpG ratio > 0.65 (Derks, Lentjes et al. 2004). CpG islands mostly located in the promoter regions of 50-60% of human genes, including most housekeeping genes (Bock, Paulsen et al. 2006). CpG islands are excellent markers for the beginning of genes. If the CpG island is in an unmethylated state and histones are in an acetylated and unmethylated state, the relaxed chromatin is accessible to transcription factors, leading to the transcription of that particular gene. However, if the CpG island is in a methylated state, gene expression will be suppressed through the following mechanisms (Illingworth and Bird 2009): (1) DNA methylation itself can block the binding of transcriptional activators to the CpGs-containing DNA-

binding elements; (2) Methylated DNA can bind to methyl-CpG binding domain proteins (MBDs) to either prevent the binding of transcriptional activators or recruit transcriptional repressors for gene silencing (Klose and Bird 2006); (3) Methylated DNA can also recruit histone deacetylases (HDACs) to form condensed chromatin structure that is inaccessible for gene transcription (Kimura and Shiota 2003) (Fig. 10).

Figure 10: Methylation status of CpG island controls target gene expression. Unmethylated DNA relaxes chromatin structure, allowing histone acetylation and the binding of transcriptional complexes for gene transcription; whereas, DNA methylation of the CpG island represses gene expression.



1.8.2. Histone modifications

Nucleosome consists of about 200-bp DNA wrapped around an octamer that contains two units of each histone H2A, H2B, H3 and H4 along with one linker histone H1 (Kornberg 1974). The N-terminal tails of histones protrude from the globular domains and are subject to various post-translational modifications on specific serine, lysine and arginine residues, such as acetylation, deacetylation, and methylation (Kouzarides 2007; Marmorstein and Trievel 2009). The multiple combinations of these modifications are called "histone codes". These modifications occur in response to environmental stimuli and directly affect chromatin structure for the accessibility of transcription factors for gene expression (Jaskelioff and Peterson 2003). Unlike DNA methylation, the effects of histone modifications are much more complicated. For example, same modification may have different effects on different amino acid residues or positions, and multiple modifications may occur at the same time.

So far, histone acetylation is the most studied modification. Histone acetyltransferases (HATs) catalyze the transfer of the acetyl group from acetyl-coenzyme A to the lysine residues on the N-terminal tails of histones (Yang 2004). HATs are divided into three main families — GNAT, MYST and CBP/p300 (Sterner and Berger 2000). Histone acetylation is generally associated with enhanced gene transcription. Acetylation brings in a negative charge to neutralize the positive charge of lysine residues on histone, reducing the affinity between histone and the negatively charged DNA. As a consequence, the condensed chromatin is transformed into an opening structure that is accessible to RNA polymerase and transcription factors for gene expression. Acetylation of lysine residues on histone H3 and H4 (*e.g.* H3K9, H3K14, H3K18, H3K23, H4K5, H4K8, H4K12 and H4K16) has been associated with

transcriptional activation (Dupont, Armant et al. 2009). Histone H2A and H2B are primarily acetylated at H2AK5, H2BK12 and H2BK15 residues (Kouzarides 2007).

In contrast, histone deacetylation promotes a closed chromatin structure and represses gene transcription. Histone deacetylation is catalyzed by HDACs to return the acetyl groups on lysine residues within histones and also non-histone proteins to coenzyme A. The resulting hypoacetylation of histone reduces the space between histone and the surrounding DNA, which blocks the binding of transcription factors to DNA and causes gene silence (Cress and Seto 2000). Mammalian HDACs can be divided into three classes based on their similarity to yeast deacetylases. Class I (HDACs 1, 2, 3 and 8) proteins are close to the yeast Rpd3-like proteins. Class II (HDACs 4, 5, 6, 7, 9 and 10) proteins are similar to the yeast Hda1-like proteins, and Class III HDACs are homologous to the yeast Sir2 protein. So far, there are approximately 17 HDACs in humans (Gray and Ekstrom 2001).

Histone methylation and demethylation further extend epigenetic modifications. A single lysine residue can be modified by mono-, di- and tri-methylation. Similarly, arginines on histone tails can also be mono- or di-methylated (Zhang and Reinberg 2001; Bannister, Schneider et al. 2002). Depending on the location and the extent (mono-, di- or tri-methylation), methylation of the lysine residues on histone H3 and H4 may be either associated with gene activation (*e.g.* H3K4, H3K36 and H3K79) or gene silence (*e.g.* H3K9, H3K27 and H4K20) (Dupont, Armant et al. 2009). Like lysine methylation, arginine methylation can be either active or repressive for gene transcription. At present, there is no enzyme that can reverse arginine methylation (Kouzarides 2007). Recently, many histone lysine demethylases have been identified. Lysine-specific demethylase 1 (LSD1) can demethylate the mono- and di-methylated lysine 9 residue on histone H3.

LSD1 can also demethylate H3K4 and repress gene transcription (Shi, Lan et al. 2004). Jumonji C (JMJC) domain-containing protein JMJD2C acts as a tridemethylase on H3K9. LSD1 and JMJD2C can both interact with androgen receptor and cooperatively demethylate H3K9 to stimulate androgen receptor-mediated gene transcription (Metzger, Wissmann et al. 2005; Wissmann, Yin et al. 2007). Methylation of H3K9 or H3K36 can also be reversed by other demethylases, such as JMJD2B (Kouzarides 2007).

In addition, other histone modifications include ubiquitination, phosphorylation, and sumoylation. Ubiquitination is a very large post-translational modification by the covalent attachment of ubiquitin and targets substrate proteins for proteasomal degradation (Ciechanover 1998; Hochstrasser 2000; Hochstrasser 2000). In humans, ubiquitination of H2AK119 is associated with transcriptional repression (Wang, Zhai et al. 2006), and H2BK120 ubiquitination is connected with transcriptional activation (Zhu, Zheng et al. 2005). Sumoylation, the covalent attachment of small ubiquitin-related modifier (SUMO) to target proteins, is also directed by an enzymatic cascade analogous to ubiquitination (Kim, Baek et al. 2002). Sumoylation has been shown to take place on histone H4, H2A and H2B (Nathan, Ingvarsdottir et al. 2006). Sumoylation antagonizes both acetylation and ubiquitination on the same lysine residue and mediates transcriptional repression in yeast (Kouzarides 2007). Sumoylation is involved in various cellular processes, such as gene expression, apoptosis and protein stability (Hay 2005). Furthermore, phosphorylation of serine (Ser/S) and threonine (Thr/T) residues on histone has also been reported. Phosphorylation at Ser-10, Ser-28 and Thr-11 sites of histone H3 is tightly related to chromosome condensation (Hendzel, Wei et al. 1997; Goto, Tomono et al. 1999; Preuss, Landsberg et al. 2003). Studies also verified that H3S10 phosphorylation is associated with the expression of NF-κB-regulated genes and

immediate early genes (*e.g.* c-Fos and c-Jun) (Kouzarides 2007).

1.8.3. Non-coding RNA (ncRNA)

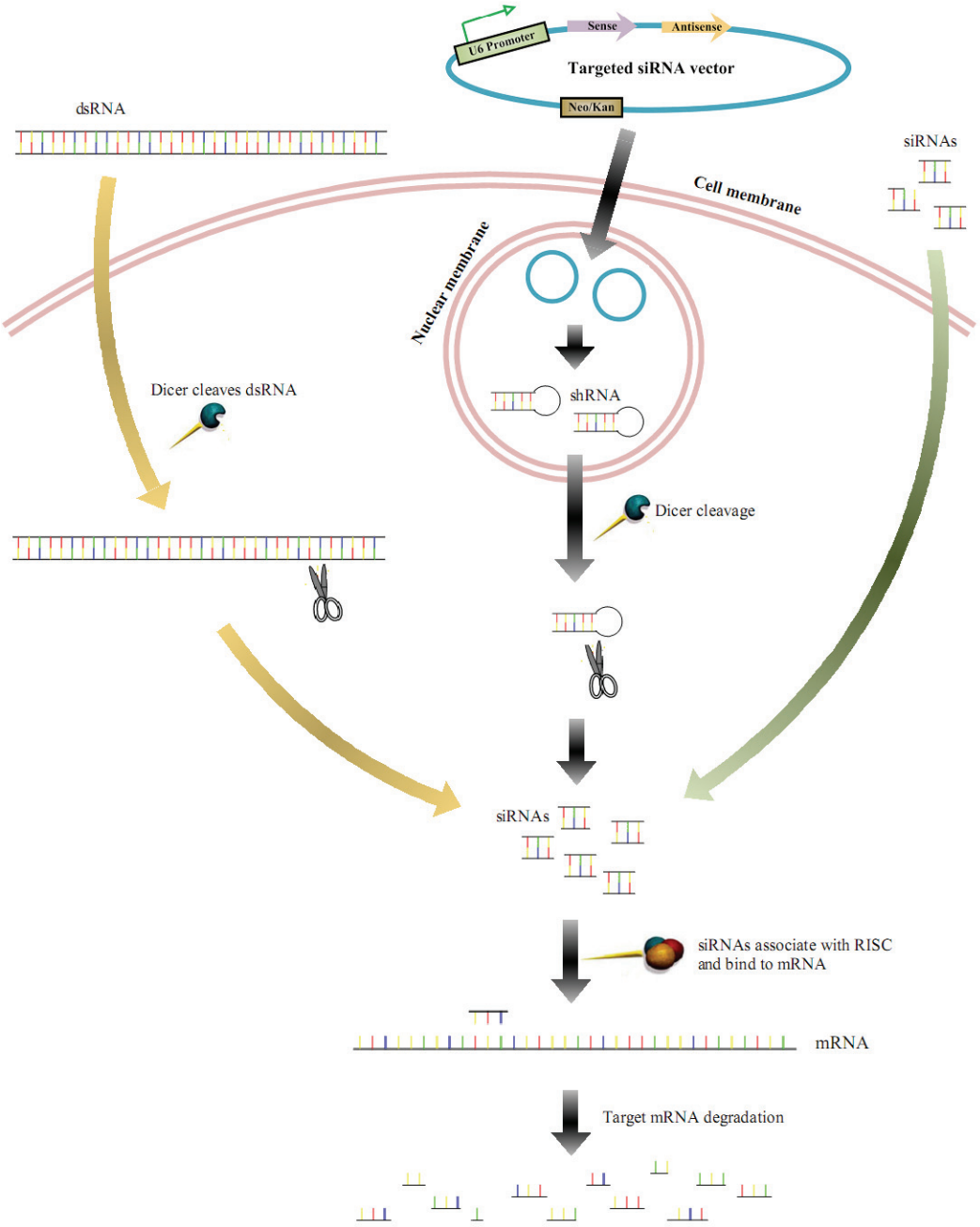
ncRNA are RNA molecules that are not translated into protein. ncRNA include many functionally important RNAs, such as transfer RNA (tRNA), ribosomal RNA (rRNA), and small nuclear RNA (snRNA). ncRNA play important roles in many cellular processes. For example, snRNAs are involved in RNA splicing, telomere maintenance and transcription factor regulation. X-inactive specific transcript (Xist) is another ncRNA that is responsible for the inactivation of one X chromosome in females (Barrandon, Spiluttini et al. 2008). Recently, researchers identified two other kinds of ncRNA — small interfering RNA (siRNA) and microRNA (miRNA) that can target gene silence (Kim 2005).

The applications of RNA interference can be mediated through two methods — the chemically synthesized double-stranded siRNA or the vector-based short hairpin RNA (shRNA). siRNA is 20-25-nucleotide in length, resulting from the processing by Dicer — an enzyme that converts either long double-stranded RNA (dsRNA) or shRNA into siRNA (Fig. 11). siRNA is an important tool for gene function studies because it can be artificially introduced into cells through transfection to knockdown the target gene expression (Rao, Vorhies et al. 2009). shRNA is a sequence of RNA with a hairpin turn that can also be used to silence gene expression. After delivery of the vector-based shRNAs into cells, shRNAs are synthesized in nucleus and are processed and transported to the cytoplasm. In the cytoplasm, the shRNA hairpin structure is cleaved by Dicer into siRNA, which is further incorporated into the RNA-induced silencing complex (RISC)

for RNA interference function either through mRNA cleavage and degradation, or through translational suppression by P-bodies (Cullen 2005).

However, too many dsRNAs, such as siRNAs, in a mammalian cell may be mistakenly considered as viral by-products to cause immune responses, and miRNA evolved to overcome this problem. miRNAs are 19-22-nucleotide single-stranded ncRNA and regulate about 30% of the human genes. miRNAs recognize the 3'-UTRs of their target mRNAs through complementary base pairing with the 6-8 nucleotides in their 5'-UTRs, leading to the target mRNA degradation (Pillai, Bhattacharyya et al. 2007). To date, miRNAs have been found to be involved in many physiological and pathological processes, such as cell differentiation and development (Song and Tuan 2006), metabolism (Krutzfeldt and Stoffel 2006), autoimmunity (Cobb, Hertweck et al. 2006; Rodriguez, Vigorito et al. 2007), apoptosis, and cancer (Dykxhoorn, Chowdhury et al. 2008).

Figure 11: Mechanisms of RNA interference. After the long dsRNAs or the vector-based shRNAs are delivered into cells, the cytoplasmic nuclease Dicer binds to the dsRNAs or shRNAs and cleaves them into short pieces of siRNAs. Synthetic siRNAs can also be introduced directly into cells. These siRNAs bind to the cellular complex RISC that uses one strand of siRNA to bind to the target mRNA. Then, the nuclease activity of RISC degrades the target mRNA, leading to the silence of the target gene.

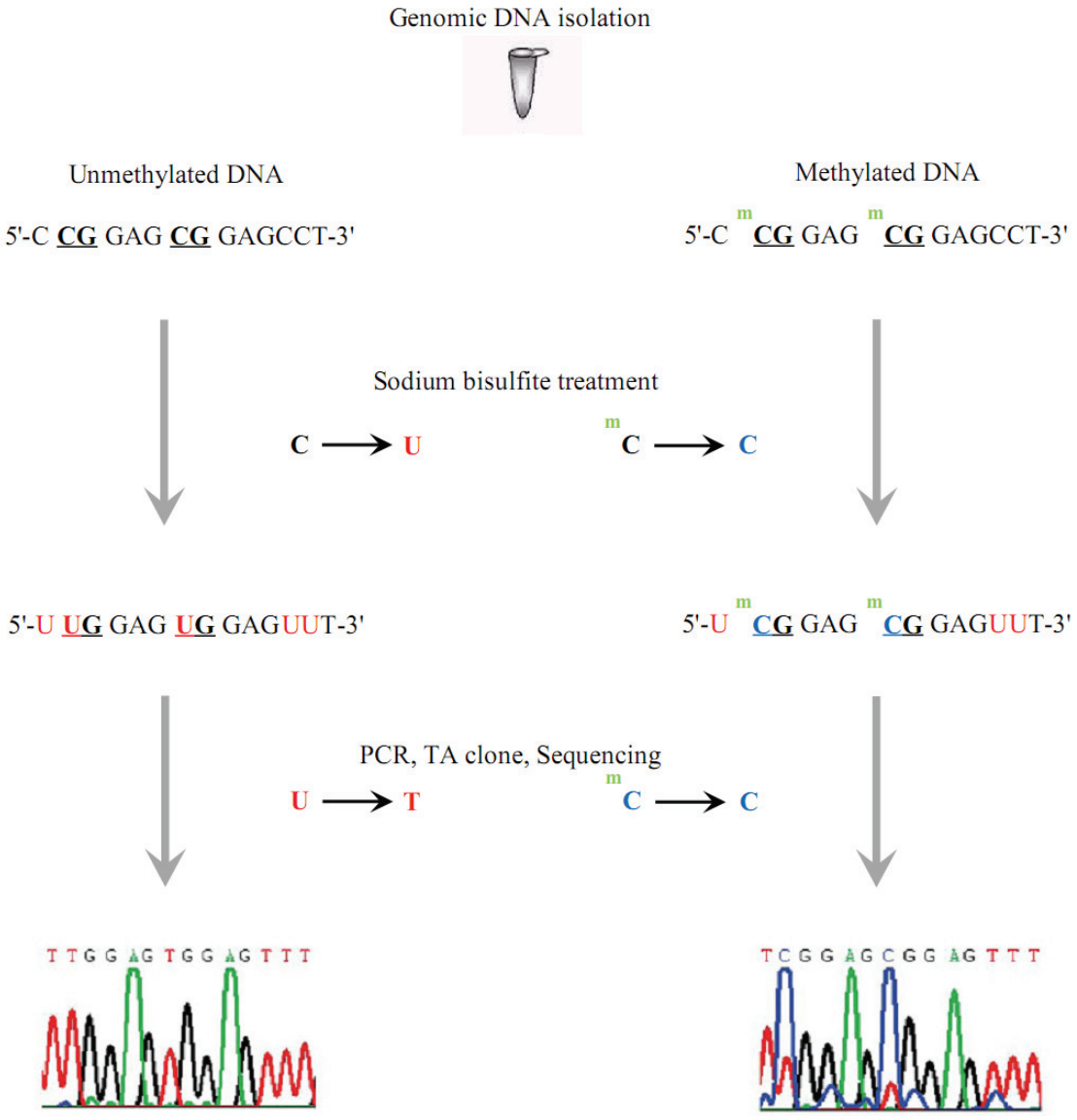


1.9. BISULFITE SEQUENCING — DNA METHYLATION DETECTION

To date, various methods have been developed to analyze DNA methylation by using very small amounts of genomic DNA obtained from tissue samples (Fraga and Esteller 2002; El-Maarri 2003; Wojdacz and Hansen 2006). The "gold standard" for DNA methylation analysis involves sodium bisulfite treatment of genomic DNA. Sodium bisulfite can selectively convert unmethylated cytosine residues to uracils, but leave 5'-methylcytosine residues unaffected. This modified genomic DNA can subsequently be assessed by DNA sequencing, PCR or mass spectroscopy (Hayatsu 2008; Wong, Morley et al. 2008; Kristensen and Hansen 2009).

The technique of bisulfite sequencing is used in this thesis to analyze the DNA methylation profile of *5-LO* gene promoter. After sodium bisulfite treatment, the modified genomic DNA is amplified by PCR with primers that can't discriminate between methylated and unmethylated sequences. Primers are designed to be strand-specific and bisulfite-specific (primers contain non-CpG cytosines), but not involve the methylation site of interest. These primers can amplify both methylated and unmethylated genomic sequences. In the resulting PCR product, the unmethylated cytosines in CpG sites are changed to thymines; whereas, the methylated cytosines will still be cytosines. The PCR product is then cloned into a vector for sequence analysis (Fig. 12). Alternatively, nested PCR can be used to enhance PCR product (Frommer, McDonald et al. 1992).

Figure 12: Detection of DNA methylation by bisulfite sequencing. Isolated genomic DNA is subjected to sodium bisulfite treatment. The modified DNA is amplified with the specific primers for the gene of interest. The methylation profile of the target gene is subsequently analyzed using cloning and gene sequencing techniques.



PREFACE TO CHAPTER 2

Recognizing the complexity of RA and the contribution of infiltrating neutrophils, T cells and macrophages, we put forth a decidedly bold hypothesis concerning a role for resident mast cells, based on a number of observations. In RA synovium, mast cells comprise up to 5% of the immune cells and many of the mast cells show a degranulated appearance. Activated mast cells recruit neutrophils, macrophages and T cells into the synovial membrane, and produce different cytokines and bioactive lipids (*e.g.* LTB_4). In addition to the induction of matrix metalloproteinases (MMPs) from SF that degrade extracellular matrix, mast cell-released proteinases also contribute to cartilage destruction. In turn, these activated SF can produce different cytokines to further stimulate the mast cells. Therefore, we recognized a bidirectional relationship between resident mast cells and SF that received scant attention in the relevant literature. Finally, evidence that mast cell-deficient mice (strains W/W^v and Sl/Sl^d) showed resistance to inflammatory and erosive arthritis induced by arthritogenic serum, added considerable support to our hypothesis (Lee, Friend et al. 2002). As such, our research program was designed to define the potential cellular and molecular interactions that occur between infiltrating mast cells, mast cell-derived inflammatory mediators and resident SF, contributing to disease pathophysiology.

CHAPTER 2

LEUKOTRIENE B_4 BLT RECEPTOR SIGNALING REGULATES THE LEVEL AND STABILITY OF CYCLOOXYGENASE-2 (COX-2) mRNA THROUGH RESTRICTED ACTIVATION OF RAS/RAF/ERK/P42 AUF1 PATHWAY

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2.1. ABSTRACT

Recent studies suggest that active resolution of the inflammatory response in animal models of arthritis may involve leukotriene B_4 (LTB_4)-dependent stimulation of "intermediate" prostaglandin production, which in turn favors the synthesis of "down-stream" anti-inflammatory and pro-resolving lipoxins, resolvins, and protectins. We explored a putative mechanism involving LTB_4-dependent control of cyclooxygenase-2 (COX-2) expression, the rate-limiting step in inflammatory prostaglandin biosynthesis. Indeed, LTB_4 potently up-regulated/stabilized interleukin-1β-induced COX-2 mRNA and protein expression under conditions of COX-2 inhibitor-dependent blockade of PGE_2 release in human synovial fibroblasts (EC_{50} = 16.5 ± 1.7 nM for mRNA; 19 ± 2.4 nM for protein, n = 4). The latter response was pertussis toxin-sensitive, and semi-quantitative reverse transcription-PCR confirmed the quantitative predominance of the BLT2 receptor. Transfection experiments, using human *COX-2* promoter plasmids and chimeric luciferase-COX-2 mRNA 3'-untranslated region (3'-UTR) reporter constructs, revealed that LTB_4 exerted its stabilizing effect at the post-transcriptional level through a 116-bp adenylate/uridylate-rich sequence in the proximal region of the COX-2 3'-UTR. Using luciferase-COX-2 mRNA 3'-UTR reporter constructs and Ras/c-Raf expression and mutant constructs, we showed that the Ras/c-Raf/MEK1/2/ERK1/2 signaling pathway mediated LTB_4-dependent COX-2 mRNA stabilization. Knockdown experiments with specific short hairpin RNAs confirmed that LTB_4 stabilization of COX-2 mRNA was apparently mediated through the RNA-binding protein, p42 AUF1. The nuclear export of p42 AUF1 was driven by c-Raf/MEK1/2/ERK1/2 signaling and sensitive to leptomycin B treatment, suggesting a CRM1-dependent mechanism. We conclude that LTB_4 may support the resolution phase of the inflammatory response by stabilizing COX-2,

ensuring a reservoir of ambient pro-resolution lipid mediators.

2.2. INTRODUCTION

Inflammation and the inflammatory response involve the concerted and exquisitely timed interactions of cytokines, chemokines, growth factors, and lipid-derived mediators with inflammatory cells (Luster 1998; Burg and Pillinger 2001; O'Garra and Vieira 2004; Metz, Grimbaldeston et al. 2007; Yoshimura, Naka et al. 2007; Viola and Luster 2008). Coordinated control of the inflammatory response involves tightly regulated gene expression at transcriptional, post-transcriptional, and translational levels, the latter two manifested in part by sequence-specific RNA-binding proteins that regulate mRNA stability and translation (Dreyfuss, Kim et al. 2002; Dean, Sully et al. 2004). mRNA turnover mediated by the adenylate/uridylate (AU)-rich elements (ARE, also known as Shaw-Kamen sequences) (Chen, Gherzi et al. 2001), which can act in *cis* or through binding of ARE-binding proteins (BP) in *trans*, consists of rapid shortening of the poly(A) tail (deadenylation) and 5'-decapping, followed by decay of the mRNA body (Wilusz, Wormington et al. 2001; Dean, Sully et al. 2004; Saklatvala 2004; Yamashita, Chang et al. 2005; Lunde, Moore et al. 2007; Sandler and Stoecklin 2008; Clark, Dean et al. 2009). In this way, inflammatory levels of mRNAs and their translation products can rapidly attain newly programmed steady-state levels following the appropriate homeostatic signal, which may form a critical mechanism in the active resolution of acute inflammation (Serhan, Chiang et al. 2008).

Leukotriene B_4 (LTB_4), a 5-lipoxygenase-derived metabolite of arachidonic acid, is a potent lipid mediator released from activated neutrophils, macrophages, mast cells, and arthritis-affected synovial fibroblasts (Serhan, Haeggstrom et al. 1996; Zhai, He et al.

2008). The bioactive lipid activates leukocyte chemotaxis, degranulation, and production of superoxide anions through cognate G protein-coupled receptors, BLT1 and -2 (Yokomizo, Izumi et al. 1997; Kato, Yokomizo et al. 2000; Yokomizo, Kato et al. 2000). Thus LTB_4 plays a defined and important role in host defense during the acute inflammatory phase, although recent studies have suggested that LTB_4 may also act in the transition to the resolution of inflammation, a return to homeostasis and preservation of tissue integrity (Serhan, Chiang et al. 2008). It has been hypothesized that the latter transition is made possible by the induction of pro-resolution bioactive lipids like prostaglandins (PGE_2, $-D_2$, and $-J_2$) and the directed downstream synthesis of lipoxins, resolvins, and protectins (Levy, Clish et al. 2001). Although the precise molecular and cellular events involved are not fully understood, it has been observed that prostaglandins, for example, stimulate the biosynthesis of pro-resolving mediators that favor local phagocytic activity and the removal of apoptotic cells and tissue debris from the site of inflammation (*e.g.* the synovium in acute inflammatory arthritis (Levy, Clish et al. 2001; Campbell, Louis et al. 2007)).

Our previous work demonstrated that PGE_2 has potent anti-cytokine and anti-catabolic activities in macrophages and synovial fibroblasts, and we recognize that its inflammomodulatory effects depend on the phase context (He, Pelletier et al. 2002; Faour, Alaaeddine et al. 2005; Faour, He et al. 2006). Given the putative role of LTB_4 in the transition phase and the fact that the latter may be proven to be a "tipping" point where acute inflammation becomes chronic (see (Lee, Friend et al. 2002) for mast cell, leukotriene, and inflammatory arthritis link), we hypothesized that LTB_4 controls the expression and synthesis of COX-2 in target cells at the site of inflammation (*e.g.* synovial fibroblasts). The latter enzyme forms the rate-limiting step in the synthesis of

eicosanoids/PGE_2 and would be the likely target for LTB_4 action, notwithstanding the prostaglandin synthases (Rocca and FitzGerald 2002; Smyth, Grosser et al. 2009). We have used COX-2 and cytokine expression as models for studying inflammatory gene expression in arthritis-affected synovial fibroblasts and have established feasibility for the proposed experiments (Faour, He et al. 2001; Faour, Alaaeddine et al. 2005).

In this study, we observed that signal activation through the leukotriene B_4 BLT receptors by LTB_4 and the BLT2-specific ligand (12*S*)-hydroxyheptadeca-5*Z*,8*E*,10*E*-trienoic acid (12-HHT, a COX-1 product) up-regulated the expression of COX-2 through a Ras/c-Raf/ERK1/2/p42 AUF1 cascade. This resulted in an increased nucleo-cytoplasmic shuttling of p42 AUF1, which, when bound to the proximal AU-rich region of the COX-2 3'-UTR, markedly increased the stability of COX-2 mRNA and COX-2 protein expression. The mechanism was predominantly post-transcriptional as the ligand had little or no direct effects on *COX-2* promoter/transcriptional activation.

2.3. EXPERIMENTAL PROCEDURES

2.3.1. Chemicals

Sodium fluoride, leupeptin, aprotinin, pepstatin, phenylmethylsulfonyl fluoride, actinomycin D, dithiothreitol, sodium orthovanadate, and bovine serum albumin were products of Sigma. Leukotriene B_4 (LTB_4) ((5*S*),(12*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid), 12-HHT ((12*S*)-hydroxyheptadeca-5*Z*,8*E*,10*E*-trienoic acid), cysteinyl leukotrienes LTC_4, LTD_4, LTE_4, NS-398 (*N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide), U-75302, and LY255283 were products of Cayman Chemical (Ann Arbor, MI). $_L$-NIL, Bay-11-7082, SB202190, U0126, staurosporine, Nonidet P-40, leptomycin B, and wortmannin were purchased from EMD/Calbiochem. SDS,

acrylamide, bisacrylamide, ammonium persulfate, and Bio-Rad protein reagent were from Bio-Rad. Tris-base, EDTA, $MgCl_2$, NaCl, $CaCl_2$, chloroform, DMSO, anhydrous ethanol (95%), methanol (99%), formaldehyde, and formamide were obtained from Fisher. Dulbecco's modified Eagle's medium (DMEM, Invitrogen), phosphate-free DMEM, TRIzol reagent, heat-inactivated fetal bovine serum (FBS), an antibiotic mixture (10,000 units of penicillin (base), 10,000 μg of streptomycin (base)), phosphate-buffered saline, TEMED, and *Taq* polymerase were products of Invitrogen. Puromycin was purchased from Cedarlane Laboratories (Hornby, Ontario, Canada), and human recombinant IL-1β (rhIL-1β) was from R&D Systems (Minneapolis, MN).

2.3.2. Specimen selection and cell culture

Synovial lining cells (human synovial fibroblasts (HSF)) were isolated from synovial membranes (synovia) obtained at necropsy from donors with no history of arthritic disease (mean age 30 ± 27). Additional experiments were conducted (where indicated) with HSF specimens obtained from osteoarthritic and rheumatoid arthritic (RA) patients undergoing arthroplasty who were diagnosed based on the criteria developed by the American College of Rheumatology Diagnostic Subcommittee for osteoarthritic/RA (mean age 67 ± 19) (Altman, Asch et al. 1986; Hochberg, Chang et al. 1992). Human synovial fibroblasts were released by sequential enzymatic digestion with 1 mg/ml pronase (Roche Applied Science) for 1 h, followed by 6 h with 2 mg/ml collagenase (type IA, Sigma) at 37 °C in DMEM supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin (Faour, Mancini et al. 2003). Released HSF were incubated for 1 h at 37 °C in tissue culture flasks (Primaria catalog no. 3824, Falcon, Lincoln Park, NJ), allowing the adherence of nonfibroblastic cells possibly

present in the synovial preparation, particularly from osteoarthritic and RA synovia. In addition, flow cytometric analysis (Epic II, Coulter, Miami, FL), using the anti-CD14 (fluorescein isothiocyanate) antibody, was conducted to confirm that no monocytes/macrophages were present in the synovial fibroblast preparation (Faour, He et al. 2001). The cells were seeded in tissue culture flasks and cultured until confluence in DMEM supplemented with 10% FBS and antibiotics at 37 °C in a humidified atmosphere of 5% CO_2, 95% air. The cells were incubated in fresh medium containing 0.5-1% FBS for 24 h before the experiments, and only second or third passaged HSF was used. HeLa cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and were grown in DMEM supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere with 5% CO_2, 95% air.

2.3.3. Preparation of cell extracts and Western blotting

Fifty-100 μg of cellular protein extracted in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of aprotinin, leupeptin, and pepstatin, 1% Nonidet P-40, 1 mM sodium orthovanadate, and 1 mM NaF) or hot SDS-PAGE loading buffer, from control and treated cells, were subjected to SDS-PAGE through 10% gels (16 × 20 cm, final concentration of acrylamide) under reducing conditions and transferred onto nitrocellulose membranes (GE Healthcare Amersham Biosciences). Following blocking with 5% BLOTTO for 2 h at room temperature and washing, the membranes were incubated overnight at 4 °C with polyclonal anti-human COX-2 (Cayman Chemical, 1:7500 dilution) in TTBS containing 0.25% BLOTTO. The second anti-rabbit antibody-horseradish peroxidase conjugate

(Cell Signaling, Danvers, MA; 1:10,000 dilution) was subsequently incubated with membranes for 1 h at room temperature and washed extensively for 30-40 min with TTBS and a final rinse with TTBS at room temperature. Following incubation with an ECL chemiluminescence reagent (Amersham Biosciences), membranes were prepared for autoradiography, exposed to Kodak X-Omat film, and subjected to digital imaging system (Alpha G-Imager 2000; Canberra Packard Canada, Mississauga, Ontario, Canada) for semi-quantitative measurements. In addition to the anti-COX-2 antisera (Cayman Chemical), the following polyclonal antibodies were used: pan-AUF1 antibody (Upstate Biotechnology, Inc., Lake Placid, NY); goat anti-actin, rabbit anti-GAPDH, and donkey anti-goat horseradish peroxidase conjugate (Santa Cruz Biotechnology, Santa Cruz, CA); anti-histone H3, anti-c-Raf, total (independent of phosphorylation state) and anti-phospho-p38 MAPK (Thr-180/Tyr-182), total and anti-phospho-c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (Thr-183/Tyr-185), total and anti-phospho-p44/42 (Thr-202/Tyr-204), and monoclonal anti-c-Jun (Cell Signaling, Danvers, MA).

2.3.4. Northern blot analysis of mRNA

Total cellular RNA was isolated (1×10^6 cells = 10-20 µg of RNA) using the TRIzol reagent (Invitrogen). Generally, 5 µg of total RNA were resolved on 1.2% agarose-formaldehyde gel and transferred electrophoretically (30 V overnight at 4 °C) to Hybond-N^{TM} nylon membranes (Amersham Biosciences) in 0.5× tris/acetate/EDTA (TAE) buffer, pH 7. After prehybridization for 24 h, hybridizations were carried out at 50-55 °C for 24-36 h, followed by high stringency washing at 68 °C in 0.1× SSC, 0.1% SDS. The following probes, labeled with digoxigenin (DIG)-dUTP by random priming, were used for hybridization: human COX-2 cDNA (1.8 kb, Cayman), initially cloned into

the EcoRV site of pcDNA1 (Invitrogen), was released by PstI and XhoI digestion, resulting in a 1.2-kb cDNA fragment; a 780-bp PstI/XbaI fragment from GAPDH cDNA (1.2 kb; American Type Culture Collection) that was initially cloned into a PstI site of pBR322 vector. This latter probe served as a control of RNA loading as GAPDH is constitutively expressed in cells used in these experiments. All blots were subjected to a digital imaging system (Alpha G-Imager 2000; Canberra Packard Canada) for semi-quantitative measurements, and changes in COX-2 expression were always considered as a ratio, COX-2:GAPDH mRNA.

2.3.5. Plasmids and transfection experiments

Transient transfection experiments were conducted in 4-, 6-, or 12-well cluster plates as described previously (Faour, He et al. 2001; Faour, Mancini et al. 2003). Transfections were conducted using FuGENE ^{TM} 6 (Roche Applied Science) or Lipofectamine 2000 (Invitrogen) reagents for 6 h according to the manufacturers' protocols with cells at around 30-40% confluence. Cells were re-exposed to a culture medium with 1% FBS for 2 h prior to the addition of the biological effectors. Transfection efficiencies were controlled in all experiments by co-transfection with 0.2-0.5 μg of pCMV-β-gal, a β-galactosidase reporter vector under the control of the CMV promoter (Stratagene, La Jolla, CA), or a pHSV-TK-driven *Renilla* luciferase construct (Promega Corp., Madison, WI). A Bsu36I *COX-2* promoter (-415 to +34)-LUC and the full-length *COX-2* promoter (-2390 to +34)-LUC plasmid were kindly provided by Dr. Stephen Prescott, University of Utah. Chimeric luciferase reporter plasmids fused with the human COX-2 mRNA 3'-UTR (1451 bp; 22 Shaw-Kamen sequences), AU-rich elements (429 bp, of which the first 116 bp contain a 6-AU cluster), the 3'-UTR minus

the AU-rich element cluster, or a construct completely devoid of the COX-2 3'-UTR but containing the SV40 poly(A) signal were employed (Dixon, Kaplan et al. 2000). The plasmids are designated LUC-3'-UTR, LUC-ARE, LUC-ΔARE, and LUC-Δ3'-UTR (pZEO-LUC3), respectively, and were a kind gift of Dr. D. Dixon, University of Utah.

Ha-Ras (p21) and c-Raf (Raf-1) expression construct sets were obtained from Clontech and were composed of pCMV-driven wild-type Ras, constitutively active RasV12 (G12V), dominant negative RasN17 (S17N), pCMV-driven wild-type c-Raf, constitutively active RafCAAX that localizes to the cell membrane when overexpressed, and the dominant negative RafS621A, which lacks the critical Ser-621 phosphorylation site. Expression plasmids for p37, p40, p42, and p45 AUF1 were generated by cloning AUF1 cDNAs into KpnI and NotI (p42 and p45) and KpnI and ApaI (p37 and p40) sites of pcDNA3. They were kindly provided by Dr. Gary Brewer (Department of Molecular Genetics, Microbiology, and Immunology, University of Medicine and Dentistry of New Jersey). The HuR and TTP expression plasmids (cloned into pcDNA3) were kindly provided by Dr. Imad Gallouzi (McGill University, Montreal, Canada) and Dr. William Rigby (Dartmouth Medical School, Hanover, NH), respectively.

RNA interference and gene knockdown were achieved through the use of short hairpin RNAs targeted to particular sequences of the *AUF1* gene, including all four isoforms, p37, p40, p42, and p45. The short hairpins were cloned into BamHI/HindIII sites and consisted of a 29-nucleotide target sequence, a 7-nucleotide loop, and the 29-nucleotide reverse complementary sequence followed by a termination sequence for RNA polymerase III. The expression was driven by a U6 small nuclear RNA gene promoter as part of the pRS (retroviral silencing) vector. Targeted sequences include the following: those from exon 9, shRNA-1; exon 1, shRNA-2; exon 3, shRNA-3; and exon

3/4, shRNA-4 (OriGene Technologies, Rockville, MD). The empty vector (pRS) served as a negative control. For stable transfectants, HeLa cells were grown to 50% confluence in DMEM supplemented with 10% FBS and transfected with AUF1-shRNAs using FuGENE 6 according to the manufacturer's instructions. After 48 h, cells were cultured in fresh medium containing puromycin (1 μg/ml), and the puromycin-containing medium was changed every 3-4 days. After 2-4 weeks, resistant isolated colonies appeared, subjected to limiting dilution procedures, and cultured further using standard culture conditions.

Luciferase values, expressed as enhanced relative light units, were measured in a Lumat LB 9507 luminometer (EG&G, Stuttgart, Germany) and normalized to the levels of β-galactosidase activity (absorbance at 450 nm after 24 h of incubation) and cellular protein (bicinchoninic acid procedure; Pierce).

2.3.6. RT-PCR

The oligonucleotide primers for the PCRs were prepared with the aid of a DNA synthesizer (Cyclone model, Biosearch Inc., Montreal, Quebec, Canada) and used at a final concentration of 200 nmol/liter. The sequences for the luciferase primers were as follows: 5'-ACGGATTACCAGGGATTTCAGTC-3' and 5'-AGGCTCCTCAGAAACAGCTCTTC-3' (antisense) for the luciferase fragment of 367 bp (Dixon, Kaplan et al. 2000; Faour, He et al. 2001). The sequences for the GAPDH (which served as a standard of quantitation) primers were 5'-CAGAACATCATCCCTGCCTCT-3', which corresponds to positions 604-624 bp of the published sequence, and 5'-GCTTGACAAAGTGGTCGTTGAG-3', from positions 901 to 922 bp, for an amplified product of 318 bp (Dixon, Kaplan et al. 2000; Faour, He et al.

2001). For BLT1, we used nested PCR as follows: nested-1 (sense) 5'-GCCCAAGGCACCTGGA-3' and (antisense) 5'-GCGGCGAAGGTCAGGATGAT-3' (460 bp: 6720-7180 bp); nested-2 (sense) 5'-GTCTGCGGAGTCAGCATGTA-3' and (antisense) 5'-TTCGTTTTCCAGGGCACTAC-3' (206 bp), corresponding to positions 6771-6976 bp of the published sequence (GenBank™ accession number AB008193). For the BLT2 receptor, standard RT-PCR was sufficient: (sense) 5'-AGACTCTGACCGCTTTCGTG-3' and (antisense) 5'-AAGGTTGACTGCGTGGTAGG-3' (182 bp), corresponding to positions 2059 to 2241 bp of the published sequence (Kato, Yokomizo et al. 2000; Yokomizo, Kato et al. 2000).

Two µg of total RNA, extracted with the TRIzol reagent, was reverse-transcribed and then subjected to PCR as described previously (Faour, He et al. 2001). RT and PCR assays were carried out with the enzymes and reagents of the GeneAmP RNA PCR kit manufactured by PerkinElmer Life Sciences. Both the RT and PCR reactions were done in a Gene ATAQ Controller (GE Healthcare). The amplification process was conducted over 10-30 cycles to define the linear range of product amplification as follows: the first cycle consisted of a denaturation step at 95 °C for 1 min, followed by annealing and elongation at 60 °C for 30 s and 72 °C for 1.5 min, respectively. All subsequent cycles were executed under the same conditions, with the exception of the last cycle, where the elongation step was extended to 7 min. We found a linear range (log luciferase/GAPDH *versus* log cycle number) between 10 and 17 cycles; as such, we chose 11-13 cycles depending on the type of experiment. In the case of nested PCR for BLT1, the conditions were essentially the same except cycle number was increased to 30-40 cycles.

The PCR products were analyzed and verified by electrophoresis on 1.15% agarose gels in a Tris borate/EDTA buffer system as described previously (Faour, He et al. 2001).

All gel photos were subjected to a digital imaging system (see above) for semi-quantitative measurements, and where indicated, results were expressed as a ratio of luciferase/GAPDH PCR fragments.

2.3.7. Statistical analysis

All results were expressed as the mean ± S.D. or mean and the coefficient of variation (*CV*) of 3-5 separate experiments as indicated. Transfection experiments were performed in triplicate. Statistical treatment of the data was performed parametrically (Student's *t* test) or by nonparametric (Mann-Whitney) analysis if Gaussian distribution of the data could not be confirmed. Where appropriate, ANOVA analysis was used to compare the statistical difference between multiple mean values. Significance was acknowledged when the probability that the Null Hypothesis was satisfied at < 5%.

2.4. RESULTS

2.4.1. LTB_4 up-regulated COX-2 mRNA and protein in HSF

Previous studies in our laboratory showed that COX-2 expression in HSF is controlled in part by a positive feedback loop involving PGE_2-mediated stabilization of COX-2 mRNA and that ambient PGE_2 may explain persistently high levels of COX-2 expression in RA-affected synovium (Faour, He et al. 2001). However, COX-2 mRNA and protein levels remained evident in explanted arthritis-derived synovial membranes exposed to COX-2 inhibitors (COXib) (He, Pelletier et al. 2002), suggesting that other COXib-insensitive inflammatory mediators may be responsible. Notwithstanding cytokine activity, and given the recent appreciation for the impact of neutrophils/mast cells and their cell-derived inflammatory mediators (*e.g.* LTB_4) in the biology of the

inflammatory response (Lee, Friend et al. 2002), we tested the hypothesis that activated neutrophil/mast cell products may play a role in the regulation of the prototypic inflammomodulatory gene *COX-2*. We examined a number of such products (LTC_4, LTD_4, and LTE_4, data not shown) and found that LTB_4, added in increasing concentrations, potently up-regulated/stabilized rhIL-1β-induced COX-2 mRNA and protein expression under conditions of COXib-dependent inhibition of PGE_2 release in HSF (Fig. 1, *A* and *B*, EC_{50} = 16.5 ± 1.7 nM for mRNA; 19 ± 2.4 nM for protein, mean ± S.D., n = 3-5). Because LTB_4 functions in target tissues through cognate G protein-coupled receptors BLT1 and -2, we performed semi-quantitative RT-PCR to determine, in a preliminary fashion, the expression profile in HSF. We observed that the apparent level of BLT2 mRNA was greater than BLT1 given that BLT1 mRNA detection required nested (two rounds of cycling) PCR procedures, whereas BLT2 mRNA PCR fragments could be clearly detected after 30 cycles of standard RT-PCR (data not shown).

To assess the contribution of transcriptional and post-transcriptional mechanisms to the LTB_4-dependent up-regulation of COX-2, we initially conducted experiments with *COX-2* promoter constructs in transfected HSF; the wild-type and Bsu36I constructs were used as a representative study (see Table 1). Recombinant human IL-1β induced a 1.65-2.78-fold increase in promoter activity as measured by the luciferase reporter activity, whereas LTB_4 co-incubated with rhIL-1β or alone was without effect. Judging by these observations, it seemed unlikely that LTB_4 modified COX-2 expression in HSF at the transcriptional level. As such, we examined post-transcriptional mechanisms involving strictly message stabilization and protein translation. As a first approach, we employed classical techniques involving measuring COX-2 mRNA in transcriptionally arrested cells (actinomycin D) in the absence or presence of LTB_4 (Faour, He et al. 2001;

Faour, Mancini et al. 2003). When HSF were activated with rhIL-1β for 2 h (steady state) followed by wash-out and a fresh change of medium, the elevated levels of COX-2 mRNA declined rapidly such that within 1-2 h the levels were similar to control unstimulated cells (Fig. 2*A*, *lanes 1*, *2*, and *7-10*). The inclusion/exclusion of actinomycin D (1 μg/ml) had no effect on the rate of COX-2 mRNA decay in these experiments (data not shown). However, if LTB_4 (50 nM, saturation kinetics) was added to fresh medium (in the presence of actinomycin D), COX-2 mRNA levels declined slowly such that COX-2 messages were discernible for up to 8 h (Fig. 2*A*, *lanes 1-6*). Parenthetically, these results could be reproduced in both isolated neutrophil and macrophage populations in short term culture conditions (data not shown).

Because the BLT receptors mediate both pertussis toxin-sensitive and -insensitive cellular responses to LTB_4 and likely associate with $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha 11/14}$ subunits (Okuno, Iizuka et al. 2008), we used, in a preliminary context, cell-permeable inhibitors of key kinase signaling cascades and found that U0126, a MEK1/2 inhibitor, abolished LTB_4-dependent up-regulation/stabilization of induced COX-2 mRNA (Fig. 2*B*); p38 MAPK, NF-κB, protein kinase C, phosphatidylinositol 3-kinase inhibitors were far less active in this regard. Indeed, the inclusion of cell-permeable chemical inhibitors into the basic protocol (*i.e.* Fig. 1) supported the previous observations regarding the role of ERK1/2 cascade, as U0126 substantially reversed the stabilization effects of LTB_4 on induced COX-2 mRNA and protein (Fig. 3, *A* and *B*). LTB_4 stabilized basal levels of COX-2 (Fig. 3*B*, *last 3 lanes*) but, in the absence of induced *COX-2* gene transcription, the levels are necessarily low. Finally, we used a Western blotting screening protocol of kinase activation using specific anti-phospho antibodies (data not shown) and verified the predominant activation of the ERK1/2 pathway by LTB_4 (Fig. 4, *A* and *B*). Interestingly,

the LTB_4-dependent activation of ERK1/2 phosphorylation was blocked by both a BLT1-specific antagonist (U75302) and the BLT2 antagonist (LY255283) (Fig. 4*C*). The specificity of the antagonists was verified with 12-HHT, a preferential, COX-1/thromboxane synthase-derived BLT2 receptor agonist; 12-HHT-dependent activation of ERK1/2 phosphorylation was reversed exclusively by the BLT2 antagonist (Fig. 4*C*).

2.4.2. LTB_4 stabilized COX-2 mRNA through the proximal 116-bp AU-rich region of the COX-2 3'-UTR

The COX-2 mRNA has multiple copies of the Shaw-Kamen AU-rich sequences that are believed to influence message stability (Appleby, Ristimaki et al. 1994; Dixon, Kaplan et al. 2000). Studies (Dixon, Kaplan et al. 2000; Sawaoka, Dixon et al. 2003) have provided evidence that the six AU-rich elements in the first 116 bp of the 3'-UTR may mediate COX-2 mRNA instability by a number of biological effectors, although PGE_2 functions via distal Shaw-Kamen sequences in the 3'-UTR (Faour, He et al. 2001). Furthermore, specific cytoplasmic AU-binding proteins (AUBPs) were identified that may initiate message degradation (Sawaoka, Dixon et al. 2003; Sengupta, Jang et al. 2003; Dean, Sully et al. 2004). To determine whether LTB_4/ERK1/2-dependent COX-2 mRNA stabilization was manifested through the 3'-UTR and AU-rich sequences, we transfected HSF with CMV-driven chimeric expression constructs containing luciferase cDNA (reporter) fused to the COX-2–3'-UTR (Luc+3'-UTR). Using an identical experimental design as in Fig. 1, we observed that rhIL-1β stimulated luciferase mRNA after 16 h of incubation by more than 3-fold using Luc+3'-UTR constructs in both HSF and HeLa cell cultures (Fig. 5, *A* and *B*). The addition of NS-398 COXib markedly reduced luciferase activity, whereas LTB_4 reversed NS-398-dependent inhibition and

stimulated reporter activity to levels similar to rhIL-1β; the LTB_4-dependent effect was blocked by the U0126 in both HSF and HeLa cultures (Fig. 5, *A* and *B*).

2.4.3. Role of Ras/c-Raf/MEK1/2/ERK1/2 signaling pathway in LTB_4-mediated COX-2 mRNA stabilization

Given the results with the MEK1/2 inhibitor, a role for the MAPK cascade in the LTB_4 stabilization of COX-2 mRNA was strongly suggested. In principle, signal transducers and kinases upstream of MEK1/2 and ERK1/2 should recapitulate the LTB_4-dependent effects. To this end, we used an overexpression strategy with c-Raf expression constructs together with their respective dominant negative mutants to substantiate our observations with the chemical inhibitor. Indeed, overexpression of the RafCAAX construct strongly stimulated ERK1/2 phosphorylation, whereas co-transfection with the RafS621A dominant negative mutant largely abrogated the effect (Fig. 6*A*), although it was not as efficient as U0126 (data not shown). We incorporated another dimension to this series of experiments by using deletion mutants of the 3'-UTR (see "Experimental Procedures") fused to the luciferase reporter to isolate the Shaw-Kamen sequences mediating the putative LTB_4-dependent stabilization effects. As shown in Fig. 6*B*, the overexpression of the constitutively active RafCAAX induced luciferase reporter activity in the full-length construct and the construct harboring the ARE in the first 116 bp; the distal sequences were largely refractive to the c-Raf-induced effect. Fig. 6*C* confirmed the expression patterns of RafCAAX and activation of ERK1/2 phosphorylation under the experimental conditions of Fig. 6*B*. The effect of 3'-UTR sequences on basal levels of luciferase activity and mRNA is highlighted in Fig. 6*B* (*1st column versus 10th column*) and Fig. 6*D* (*6th lane* from *left versus 9th lane*), respectively.

Our RafCAAX construct harbors a K-Ras C-terminal localization signal targeting the kinase to the plasma membrane where a Ras-independent mechanism completes the activation process through phosphorylation (Zimmermann and Moelling 1999). A number of receptor-associated kinases can phosphorylate c-Raf resulting in its interaction/activation of pathways not necessarily associated with the G protein-coupled receptors (BLT)/Ras pathway (Zimmermann and Moelling 1999; Yuryev and Wennogle 2003). As such, we investigated whether a constitutively activated form of p21 Ras (and a wild-type construct) could activate endogenous c-Raf (Raf-1) and propagate the same signal required to stabilize COX-2 mRNA. The intent was to validate our observations in Fig. 6 and link our findings to G_i- and/or G_q-coupled receptors. As shown in Fig. 7*A*, co-transfection of RasV12 with the luciferase-COX-2–3'-UTR fusion construct resulted in a greater than 3-fold (3.3 ± 0.9; n = 5) increase in reporter activity; the addition of RasN17 (*3rd column versus 5th column*) to the latter transfection mixture abrogated the inductive effect. Data in Fig. 7*B* confirmed the expression patterns of the plasmids RasV12 (Ha-Ras) and RasN17 and the activation/inhibition of ERK1/2 phosphorylation under the experimental conditions of Fig. 7*A*.

2.4.4. Role of AUF1 (heterogeneous nuclear ribonucleoprotein D) in the LTB_4/Ras/c-Raf/MEK1/2/ERK1/2 signaling pathway-mediated stabilization of COX-2 mRNA

Well-characterized AUBPs either stabilize target mRNA through as yet ill-defined mechanisms or enhance the degradation of the target through recruitment of the exosome, a scaffold containing a number of RNA-degrading enzymes (Chen, Gherzi et al. 2001). Both systems respond rapidly to changes in extracellular signaling patterns (*e.g.* as in

pathological states). The simplest interpretation of our results suggests that LTB_4 either suppresses the expression and/or activity of AUBPs that destabilize mRNA or the reverse. In this regard, we used our luciferase reporter screening assay (see above) to evaluate the behavior of TTP, HuR, and AUF1 under basal and MAPK signaling conditions. These AUBPs were chosen based on previous reports regarding COX-2 mRNA stability (Appleby, Ristimaki et al. 1994; Dixon, Kaplan et al. 2000; Saklatvala 2004; Clark, Dean et al. 2009). As shown in Table 2, ectopic expression of TTP precipitated a marked and specific (*i.e.* minus effects of pcDNA3 cloning vector) reduction in reporter activity under basal conditions; TTP was apparently refractive to MAPK signaling because the magnitude of reporter suppression was statistically similar in both basal (pCMV) and RafCAAX-transfected cells. Furthermore, when HuR (ELAVL1), a member of the embryonic lethal abnormal vision (ELAV)-like family of RNA-binding proteins (Sengupta, Jang et al. 2003; Dean, Sully et al. 2004), was overexpressed in the presence or absence of RafCAAX, a discernible diminution of reporter activity was observed under basal conditions, but a marked reduction was obtained in RafCAAX-transfected cells. In contrast, under similar experimental conditions, the p42 isoform of AUF1 increased basal but especially RafCAAX-induced reporter activity, whereas the p37, p40, and p45 isoforms were essentially without effect.

2.4.5. shRNA knockdown of p42 AUF1 and LTB_4/c-Raf-dependent COX-2 stabilization

The previous experiments suggested that the p42 AUF1 isoform mediated the LTB_4/c-Raf-controlled stabilization of COX-2 mRNA. To confirm this possibility, we chose a gene knockdown strategy involving the use of four different HuSH 29-mer

shRNAs targeting exons 1, 3, 4, and 9 of *AUF1* stably transfected in HeLa cells. As shown in Fig. 8*A*, only shRNA-4, spanning the junction of exon 3 and 4, reduced protein expression of AUF1 to a significant extent. Using a pan-AUF1 antibody, it was revealed that the expression levels of isoforms p40, p42, and p45 were reduced a minimum of 3-fold, whereas p37 remained stable (p42, 3.4 ± 0.7-fold *versus* pRS empty vector, mean ± S.D., n = 5 experiments). Parenthetically, exposure times for film development were increased because of relatively low levels of endogenous AUF1. Stable p42 AUF1 knockdown inhibited RafCAAX activation of luciferase reporter activity by 2.8 ± 0.31-fold (Table 3).

2.4.6. BLT/c-Raf/ERK1/2 signaling induced a leptomycin B-sensitive nuclear export of p42 AUF1

All AUF1 family members were detected in the nuclear compartment of HeLa cells and HSF under quiescent conditions, and small but discernible levels of p40 and p42 were observed in cytoplasmic extracts (data not shown). In principle, AUF1 proteins act on mRNA stability (and protein translation) in the cytoplasmic compartment and must undergo nucleo-cytoplasmic export through binding to an export receptor like chromosomal region maintenance 1 (CRM1); AUF1 proteins harbor nuclear export sequences in the C-terminal domain (exon 6/7) that promote binding to CRM1 (Fukuda, Asano et al. 1997; Meissner, Krause et al. 2004; Mazan-Mamczarz, Kuwano et al. 2009). In transiently transfected cell populations overexpressing p42 AUF1, the protein was predominantly nuclear. However, in co-transfections with RafCAAX, a 3.95 ± 0.45-fold increase (mean ± S.D., n = 5) in the cytoplasmic localization of p42 was observed in comparison with co-transfections with a pCMV vector (Fig. 8, *B*, compare *2nd versus*

3rd lanes from *left*, and *C*). This effect was mitigated by co-incubation of leptomycin B, a bacterial toxin that alkylates the Cys-528 residue of CRM1 and inhibits cognate protein binding (Fig. 8, *B*, compare *3rd versus 5th lanes*, and Fig. 8*C*) (Meissner, Krause et al. 2004). Nuclear fractions were further enriched with p42 AUF1 in the presence of leptomycin B as was the nuclear transcription factor c-Jun, further validating our assay procedure.

2.5. DISCUSSION

We previously proposed that PGE_2 was more akin to a modulator of inflammation as opposed to one of the many inflammatory mediators, based on our studies demonstrating the anti-cytokine and anti-catabolic effects of the most abundant of prostanoids (He, Pelletier et al. 2002; Faour, Alaaeddine et al. 2005; Faour, He et al. 2006). These observations were further supported by recent studies on PGE_2-dependent production of "downstream" eicosanoids such as lipoxins, resolvins, and protectins, which are believed to be involved in the transition and resolution phase of the acute inflammatory response in animal models (Serhan, Chiang et al. 2008). This study provided new data demonstrating that "early" mediators like LTB_4, synthesized and released by transmigrating neutrophils and resident mast cells, control PGE_2 synthesis through direct effects on COX-2 mRNA stability in the synovium. In addition, we provided a detailed mechanism involving BLT-dependent restricted activation of mitogenic pathways with the subsequent nucleo-cytoplasmic shuttling of p42 AUF1 and targeted message stabilization via 3'-UTR ARE sequences.

The BLT1 receptor expression is largely restricted to leukocytes (neutrophils, macrophages, and eosinophils) and functionally associates with pertussis-sensitive and -

insensitive G proteins that mediate calcium mobilization, accounting for the high affinity effects of LTB_4 (K_D ~ 1 nM) on target cell chemotaxis (Kato, Yokomizo et al. 2000). In contrast, BLT2 exhibits a wider tissue distribution (*e.g.* spleen, liver, and ovary) and associates with pertussis-sensitive G proteins (*e.g.* $G_{\alpha i}$) linked to strong MAPK/ERK activation (K_D ~ 50 nM) but weaker calcium mobilization effects (Yokomizo, Izumi et al. 1997; Kamohara, Takasaki et al. 2000; Yokomizo, Kato et al. 2000; Okuno, Yokomizo et al. 2005). We believe this study demonstrating BLT receptor expression and function in HSF suggests that the latter cell type is integrated into the inflammatory response partly through responses to LTB_4. Therefore, in acute inflammatory synovitis, the synovial fibroblast, the preponderant cell type, is the principle target of ambient mediators, and our work supports an important link of the LTB_4/receptor system to arthritis. Indeed, mast cell-derived LTB_4 may be the trigger for migration of $CD8^+$ effector T cells into the inflamed lesion/membrane (Lee, Friend et al. 2002; Campbell, Louis et al. 2007). Our RT-PCR data suggested that BLT2 mRNA was the predominant receptor species over BLT1, but functionally speaking, both activated mitogenic signaling. Others reported similar expression profiles in HSF but did not delineate functional activity (Hashimoto, Endo et al. 2003). Further studies using receptor knock-out mice and/or *in vitro* knockdown experiments would be required at this stage to resolve these uncertainties. *BTL1* is an inducible gene with restricted tissue expression, likely the result of promoter activity governed epigenetically through CpG island methylation (Kato, Yokomizo et al. 2000). Because the *BLT2* open reading frame encompasses all of the *BLT1* promoter and exon 1 on chromosome 14, it is difficult to assess how constitutive promoter methylation might have affected the BLT2 expression profile, particularly in pathological states where the methylation status of any given promoter may be altered.

Heterogeneous nuclear ribonucleoprotein D/AUF1 is transcribed from a single gene but, as a result of alternative splicing, is translated into four distinct isoforms, p37, p40, p42, and p45 AUF1 (pan-AUF1). AUF1 is a *bona fide* RNA-binding protein with high affinity for ARE sequences found in a variety of target mRNA-3'-UTR, and RNA electrophoretic mobility shift/supershift analysis strongly suggests that AUF1 binds to class II AREs like those found in COX-2 mRNA. There is, however, no consensus as to whether it functions to stabilize or destabilize mRNA (Sarkar, Lu et al. 2003; Wilson, Lu et al. 2003; Chen, Xu et al. 2004; Dean, Sully et al. 2004; Saklatvala 2004; Clark, Dean et al. 2009; Mazan-Mamczarz, Kuwano et al. 2009). In our hands, LTB_4/BLT markedly stabilized COX-2 mRNA as judged by Northern analysis and COX-2-ARE-luciferase reporter activity through an ERK1/2-dependent pathway with apparent recruitment of p42 AUF1 stabilizing capacity via a CRM1-dependent nuclear export. Furthermore, we incorporated RNA-binding proteins TTP and HuR into our assays (Table 2), and the results confirmed our previous observations that TTP strongly destabilizes COX-2 mRNA through a p38 MAPK-dependent process (Mancini and Di Battista 2008) and was not subject to ERK1/2 modulation as has been suggested by previous work (Saklatvala 2004; Clark, Dean et al. 2009). In our cell culture models (HSF and HeLa), HuR exhibited modest effects on COX-2 mRNA stability and on COX-2-ARE-luciferase reporter activity, but we were surprised to see how mitogenic activation by c-Raf/ERK1/2 strongly increased HuR-destabilizing effects as this was not evident from the literature (Saklatvala 2004; Clark, Dean et al. 2009). The congruity of our results using different experimental approaches and cell types strengthens the validity of our observations. Nevertheless, we used an overexpression protocol, and although pan-AUF1 (predominantly nuclear) and TTP (cytoplasmic) were modestly expressed in our cell

types, HuR is abundantly expressed and partitions between the nuclear and cytoplasmic compartments (Mancini and Di Battista 2007; Mancini and Di Battista 2008). The results may be subject to interpretation as it is unclear whether ectopically expressed HuR impacts on endogenous levels and whether c-Raf/ERK1/2 activation is targeting endogenous or ectopic HuR.

In a number of studies, p37 and p40 AUF1 isoforms have been ascribed predominant roles in the modulation of target mRNA stability via AU-rich sequences, largely because they contain putative nuclear localization sequences in the C-terminal domains, favoring nuclear "maturation" prior to cytoplasmic localization and polyribosome association (Chen, Xu et al. 2004). Furthermore, because of structural determinants, p42 and p45 AUF1 are predominantly nuclear, although a consensus is emerging that all isoforms can shuttle (Sarkar, Lu et al. 2003). In quiescent HSF and HeLa cells, the four isoforms were nuclear with roughly equal albeit low level expression, although some cytoplasmic p42 AUF1 staining was observed. A similar partitioning profile was observed when p42 AUF1 was overexpressed, although a marked cytoplasmic accumulation of p42 AUF1 was observed under strong c-Raf/ERK1/2 signaling suggesting that mitogenic signals support active nuclear export. c-Raf/ERK1/2-dependent phosphorylation of p42 AUF1 could increase its association with exportins thereby stimulating the overall shuttling rate. Interestingly, analysis of AUF1 from control and phorbol ester-treated THP-1 cells indicated that post-translational modifications of the major cytoplasmic isoform, p40 AUF1, are altered concomitant with changes in RNA binding activity and stabilization of ARE-containing mRNAs (Wilson, Lu et al. 2003). In particular, p40 AUF1 recovered from polysomes is phosphorylated on Ser-83 and Ser-87 in untreated cells but dephosphorylated following 12-*O*-

tetradecanoylphorbol-13-acetate treatment. In addition to post-translational modifications, another possibility may be that, in our cell cultures, CRM1 levels were up-regulated by mitogenic signaling with the accompanying increase in transcription of target genes (*e.g. CRM1*), an intriguing avenue for future research.

We believe this study to be the first report providing a plausible molecular mechanism whereby LTB_4, an early inflammatory mediator, supports the resolution phase of the inflammatory response by stabilizing COX-2 mRNA (protein), providing for an abundance of ambient pro-resolution lipid mediators (Serhan, Chiang et al. 2008). Furthermore, AUF1 function clearly varies with the target mRNAs, and one must consider that AREs may not be the only cognate binding sites and/or that the AUF1 exerts in effects by modulating mRNA transport processes (see above). Finally, AUF1 is evidently functionally integrated into the immune and inflammatory responses, and a better understanding of its role in this regard may lead to targeted therapeutic intervention.

In summary, we propose the following mechanism for the signaling cascade leading to the increase in COX-2 mRNA stability: LTB_4, from activated synovial neutrophil/macrophage/mast cell populations, binds BLT1/2:$G_{\alpha q}/G_{\alpha 11}$ on synovial fibroblasts resulting in increased $[Ca^{2+}]_i$ and downstream activation of the following intermediates: protein kinase C/CaMKii → Ras GRP/GRF → Ras → c-Raf → MEK1/2 → ERK1/2. Nuclear ERK1/2, acting through intermediates, stimulates p42 AUF1 nucleo-cytoplasmic translocation, which binds to COX-2 mRNA in the 3'-UTR region favoring stabilization. Increased levels of COX-2 mRNA/protein promote PGE_2 release with feedback suppression of macrophage-derived cytokines and metalloprotease production, principally from synovial fibroblasts and macrophages. In addition, we

propose a feedback switch to LXA_4 synthesis through PGE_2-dependent up-regulation of 15-lipoxygenase with changes in lipid mediator synthesis to anti-inflammatory resolvin/protectin synthesis, blocking further neutrophil recruitment and resolving the inflammatory response.

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Table 1: Transcriptional activation of the *COX-2* promoter; effect of LTB_4.

Condition	Luciferase activity (fold induction) wt *COX-2* promoter (-2390 bp)	Luciferase activity (fold induction) Bsu36I (-415 bp)
Control	1	1
rhIL-1β (100 pg/ml)	1.65±0.39 *	2.78±0.51 **
rhIL-1β + LTB_4 (1 μM)	1.71±0.48 $^{\&}$	2.61±0.53 $^{\&}$
LTB_4	1.03±0.22 $^{\#}$	0.95±0.17 $^{\#}$

HSF at 30-50% confluence were transiently transfected using FuGENE 6 for 6 h with 1μg/well of human *COX-2* promoter (wild type and Bsu36I)-luciferase constructs plus 200 ng of pCMV-β-gal expression vector (transfection efficiency control marker). Fold-induction values were expressed as mean ± S.D. from three to five determinations in duplicate. Typical values for promoter induction by rhIL-1β ranged from 2.45×10^4 to 5.6×10^4 relative light units. * $p < 0.025$; ** $p < 0.001$ *versus* controls; $^{\&}$ not significant (*NS*) *versus* rhIL-1β; $^{\#}$ not significant (*NS*) *versus* controls.

Table 2: Effect of RNA-binding protein overexpression on luciferase activity.

Luciferase-COX-2-ARE		
	Fold change *versus* empty vector (pcDNA3)	
	Basal (pCMV)	RafCAAX
TTP	↓ 2.44±0.4 *	↓ 2.78±0.37 *, ns
HuR	↓ 1.19±0.27	↓ 2.8±0.2 *, #
p37 AUF1	↓ 1.15±0.18	↓ 1.20±0.25
p40 AUF1	↓ 1.10±0.14	↓ 1.54±0.24 ns
p42 AUF1	↑ 1.58±0.48 ns	↑ 4.1±0.1 *, ##
p45 AUF1	↑ 1.04±0.12	↑ 1.24±0.16

HeLa cells at 30-50% confluence were transiently transfected using FuGENE 6 for 6 h with 10 ng per well of luciferase-COX-2-ARE fusion chimeric construct together with 500 ng of pcDNA3 or pcDNA3 harboring the TTP, HuR-FLAG, or p37, p40, p42, or p45 AUF1 cDNAs. In addition, 400 ng of pCMV or pRafCAAX constructs and 200 ng of pCMV-β-gal (transfection efficiency control marker) were also included in the mixture. Luciferase reporter activities were measured 24 (data not shown) and 72 h post-transfection. Fold-change values were expressed as mean ± S.D. from three to five determinations. Typical values for luciferase-COX-2-ARE reporter ranged from 7.3×10^3 to 1.29×10^5 relative light units corrected for β-galactosidase expression/activity. ns means not significant. *minimum $p < 0.01$ *versus* pcDNA3; basal (pCMV) *versus* pRafCAAX, #, $p < 0.007$, ##, $p < 0.005$.

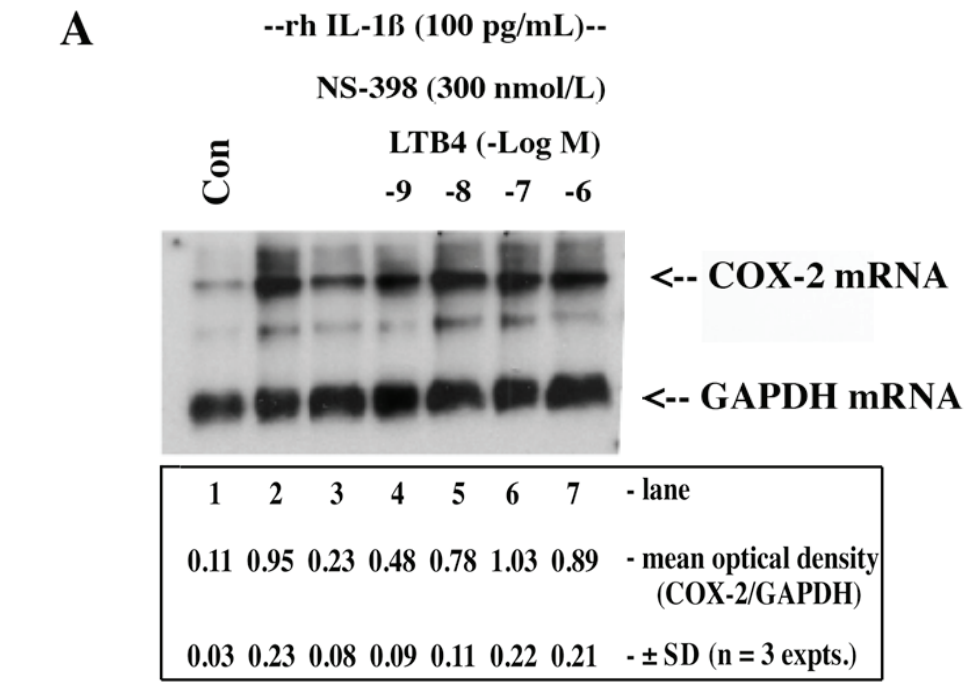
Table 3: AUF1 knockdown suppresses Raf-induced luciferase activity.

Luciferase-COX-2-ARE	
Fold-induction pRafCAAX *versus* pCMV	
pRS vector	24.07±2.41
AUF1-shRNA-4 with puromycin	9.3±1.75 *
AUF1-shRNA-4 without puromycin	9.63±0.76 *

HeLa cells (3×10^5 cells in 6-well plates), stably transfected with AUF1-shRNA-4, were transiently transfected with 10 ng/well luciferase-COX-2-ARE fusion chimeric construct, 400 ng of pCMV or pRafCAAX, and 200 ng of pCMV-β-gal. Luciferase reporter activities were measured 24 h post-trasfection. Fold-induction values were expressed as mean ± S.D. from four determinations. Typical values for luciferase reporter activity ranged from 1.2×10^4 (pCMV) to 4.58×10^5 relative light units corrected for β-galactosidase activity. * $p < 0.0008$ *versus* pRS vector.

Figure 1: LTB_4 up-regulated/stabilized COX-2 mRNA and protein in HSF. Cultured confluent HSF (1.2×10^6 cells in 6-well cluster plates) were preincubated overnight in DMEM supplemented with 1% FBS plus antibiotics at 37 ºC to ensure synchrony and quiescence. Cells were then treated with vehicle (*Con*) or with 5.7 pmol/liter (100 pg/ml) of rhIL-1β for 16 h in the presence or absence of the COX-2 inhibitor NS-398 and increasing concentrations of LTB_4 as indicated. Monolayers were extracted for RNA (*A*) and protein (*B*) as follows: 5 μg of total RNA was analyzed for COX-2 mRNA and GAPDH mRNA by Northern hybridization using specific DIG-labeled cDNA probes, and 50 μg of protein was analyzed for COX-2 and actin (loading control) protein by Western blotting using specific polyclonal antisera as described under "Experimental Procedures". ANOVA of means for densitometric analysis in *A* and *B* is as follows: IL-1β + NS-398 *versus* IL-1β + NS-398 + LTB_4 (-log M -9 to -6), $p < 0.007$; IL-1β *versus* IL-1β + NS-398 + LTB_4 (-log M -8 to -6), not significant (*NS*).

Fig. 1


A
--rh IL-1ß (100 pg/mL)--
NS-398 (300 nmol/L)
LTB4 (-Log M)
Con
-9 -8 -7 -6
<-- COX-2 mRNA
<-- GAPDH mRNA
1 2 3 4 5 6 7 - lane
0.11 0.95 0.23 0.48 0.78 1.03 0.89 - mean optical density (COX-2/GAPDH)
0.03 0.23 0.08 0.09 0.11 0.22 0.21 - ± SD (n = 3 expts.)

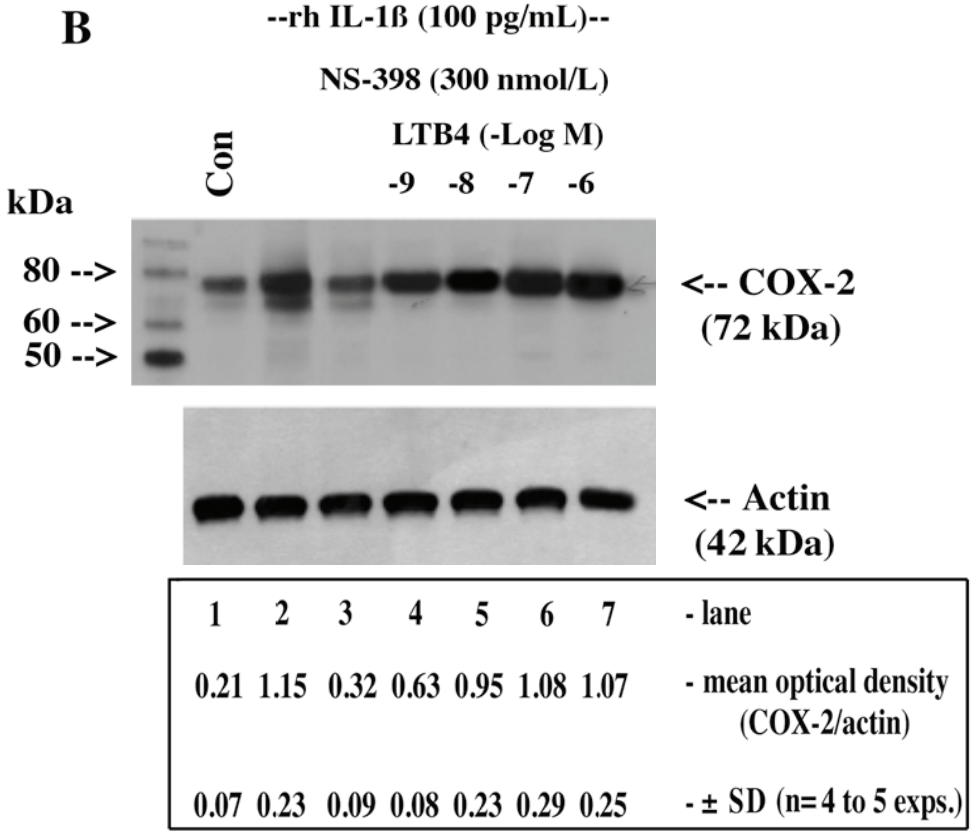
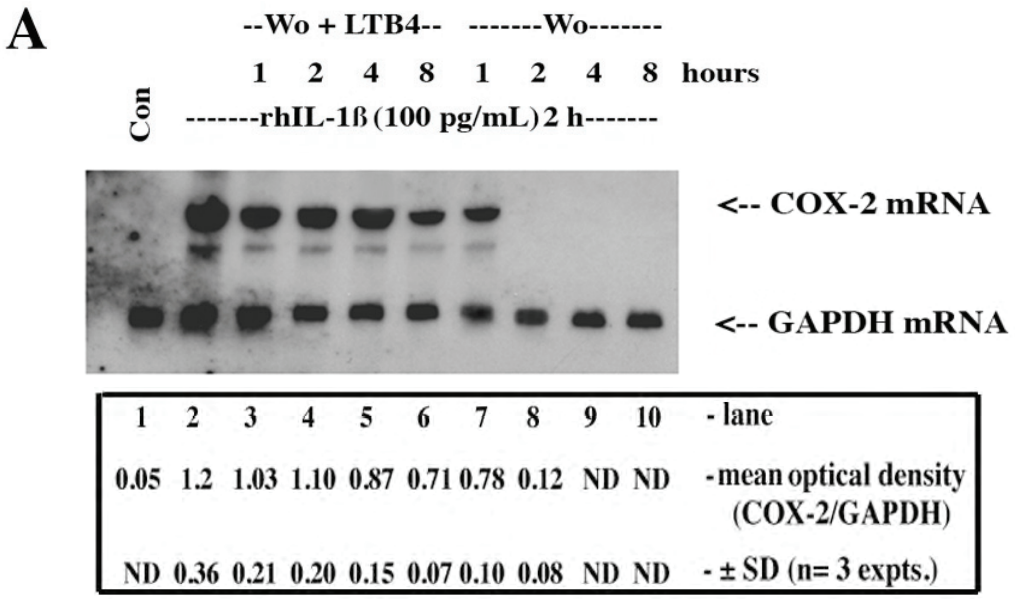

B
--rh IL-1ß (100 pg/mL)--
NS-398 (300 nmol/L)
LTB4 (-Log M)
Con
-9 -8 -7 -6
kDa
80 -->
60 -->
50 -->
<-- COX-2 (72 kDa)
<-- Actin (42 kDa)
1 2 3 4 5 6 7 - lane
0.21 1.15 0.32 0.63 0.95 1.08 1.07 - mean optical density (COX-2/actin)
0.07 0.23 0.09 0.08 0.23 0.29 0.25 - ± SD (n=4 to 5 exps.)

Figure 2: LTB_4-dependent stabilization of COX-2 mRNA; effects of chemical kinase inhibitors. *A*, quiescent HSF were treated with vehicle (*Con*) or with 100 pg/ml (5.7 pmol/liter) of rhIL-1β for 2 h (steady state), after which time cells were washed out (*Wo*) and treated with actinomycin D (1 μg/ml) for 30 min, and then fresh medium was added containing either vehicle (*Wo*) or LTB_4 (50 nmol/liter). After an additional 1, 2, 4, or 8 h of incubation, monolayers were extracted for RNA at each time point, and 5 μg of total RNA was analyzed for COX-2 mRNA and GAPDH mRNA by Northern hybridization using specific DIG-labeled cDNA probes. *B*, cells were treated with vehicle (*Con*) or with 100 pg/ml (5.7 pmol/liter) of rhIL-1β for 2 h, washed out (*Wo*), and treated with actinomycin D (1 μg/ml) for 30 min, and then fresh medium was added containing either vehicle (washed out 2 h) or LTB_4 (50 nmol/liter) alone or in the presence of $_L$-Nil (1 μmol/liter), SB202190 (*SB*, 1 μmol/liter), U0126 (2 μmol/liter), Bay-11-7082 (*BY*, 5 μmol/liter), wortmannin (*Wort*, 200 nmol/liter), or staurosporine (*ST*, 10 nmol/liter) for 2 more h. 5 μg of total RNA was analyzed for COX-2 mRNA and GAPDH mRNA by Northern hybridization using specific DIG-labeled cDNA probes. ANOVA of the means for densitometric analysis is as follows: *A*, IL-1β *versus* IL-1β + washed out (*Wo*) + LTB_4 (1-4 h), not significant (*NS*), and *ND*, not determined; IL-1β *versus* washed out (1, 2 h), $p < 0.001$; *B*, Student's *t* test; IL-1β *versus* IL-1β + washed out 2 h, $p < 0.003$; IL-1β *versus* IL-1β + washed out 2 h + LTB_4, not significant; IL-1β *versus* IL-1β + washed out 2 h + LTB_4 + U0126, $p < 0.006$; IL-1β *versus* IL-1β + washed out 2 h + LTB_4 + other kinase inhibitors, not significant.

Fig. 2


A
--Wo + LTB4--
-------Wo-------
1 2 4 8 1 2 4 8 hours
Con
-------rhIL-1ß (100 pg/mL) 2 h-------
<-- COX-2 mRNA
<-- GAPDH mRNA
1 2 3 4 5 6 7 8 9 10 - lane
0.05 1.2 1.03 1.10 0.87 0.71 0.78 0.12 ND ND - mean optical density (COX-2/GAPDH)
ND 0.36 0.21 0.20 0.15 0.07 0.10 0.08 ND ND - ± SD (n= 3 expts.)

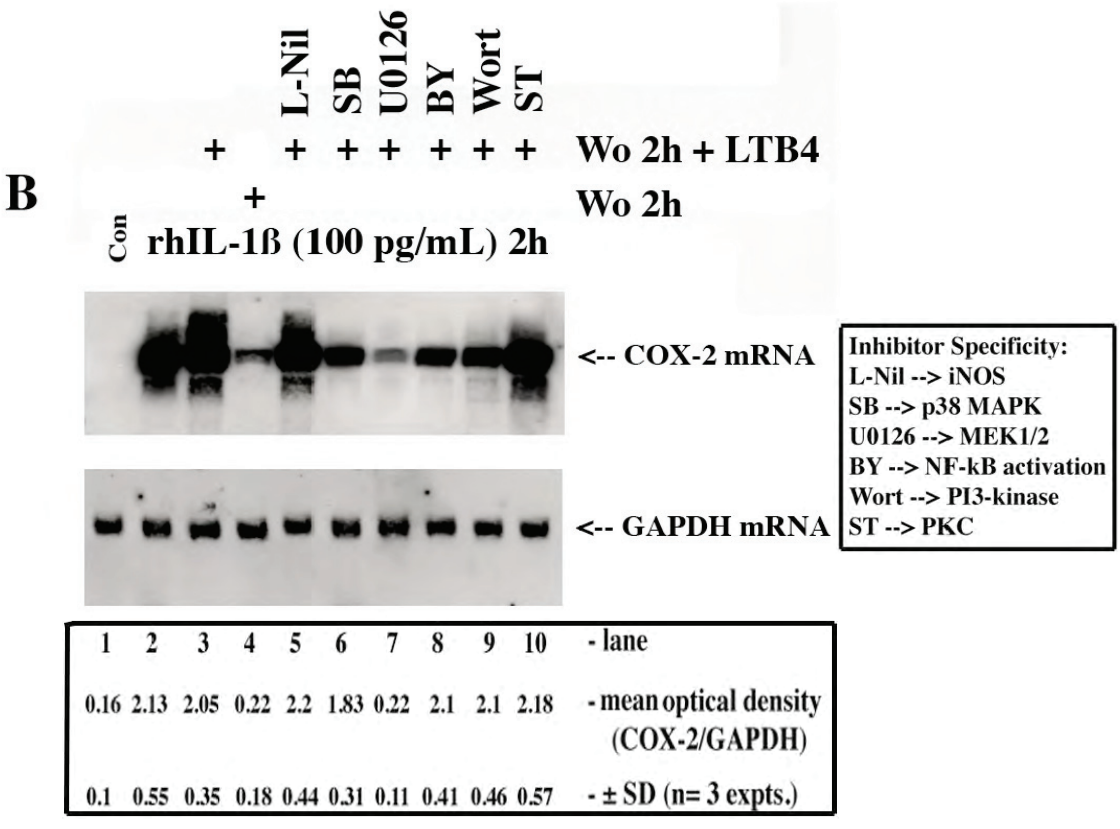
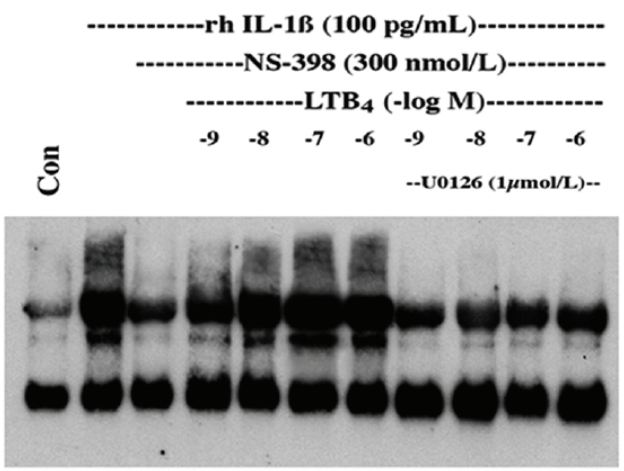

B
L-Nil
SB
U0126
BY
Wort
ST
+ + + + + + + Wo 2h + LTB4
+ Wo 2h
Con
rhIL-1ß (100 pg/mL) 2h
<-- COX-2 mRNA
<-- GAPDH mRNA
Inhibitor Specificity:
L-Nil --> iNOS
SB --> p38 MAPK
U0126 --> MEK1/2
BY --> NF-kB activation
Wort --> PI3-kinase
ST --> PKC
1 2 3 4 5 6 7 8 9 10 - lane
0.16 2.13 2.05 0.22 2.2 1.83 0.22 2.1 2.1 2.18 - mean optical density (COX-2/GAPDH)
0.1 0.55 0.35 0.18 0.44 0.31 0.11 0.41 0.46 0.57 - ± SD (n= 3 expts.)

Figure 3: LTB_4-dependent stabilization of COX-2 mRNA was mediated through MAPK signaling. Cells were treated with vehicle (*Con*) or with 5.7 pmol/liter (100 pg/ml) of rhIL-1β for 16 h in the presence or absence of the COX-2 inhibitor NS-398 and increasing concentrations of LTB_4 with or without U0126 (1 μmol/liter). Monolayers were extracted for RNA and protein as follows: *A*, 5 μg of total RNA was analyzed for COX-2 mRNA and GAPDH mRNA by Northern hybridization using specific DIG-labeled cDNA probes; *B*, 50 μg of protein was analyzed for COX-2 protein by Western blotting using specific polyclonal antisera as described under "Experimental Procedures". ANOVA of means for densitometric analysis in *A* and *B* is as follows: IL-1β + NS-398 *versus* IL-1β + NS-398 + LTB_4 (-log M -9 to -6), $p < 0.001$; IL-1β *versus* IL-1β + NS-398 + LTB_4 (-log M -8 to -6), not significant; IL-1β + NS-398 + LTB_4 (-log M -9 to -6) *versus* IL-1β + NS-398 + LTB_4 (-log M -9 to -6) + U0126, $p < 0.002$.

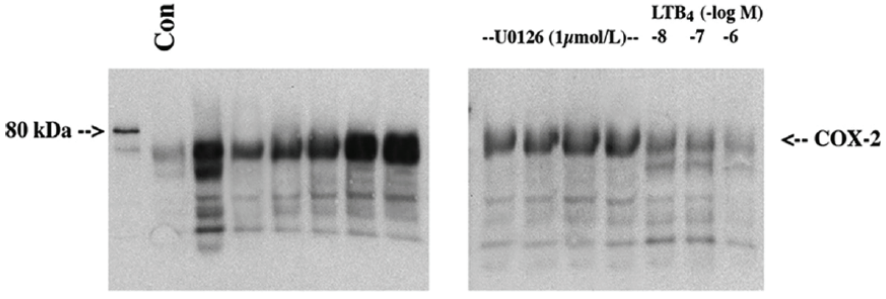
Fig. 3

A



1	2	3	4	5	6	7	8	9	10	11	- lane
0.12	1.66	0.55	0.92	1.43	1.81	1.78	0.6	0.55	0.56	0.57	- mean optical density (COX-2/GAPDH)
0.09	0.3	0.16	0.12	0.15	0.16	0.27	0.1	0.16	0.19	0.14	- ± SD (n = 4-5 expts.)

B

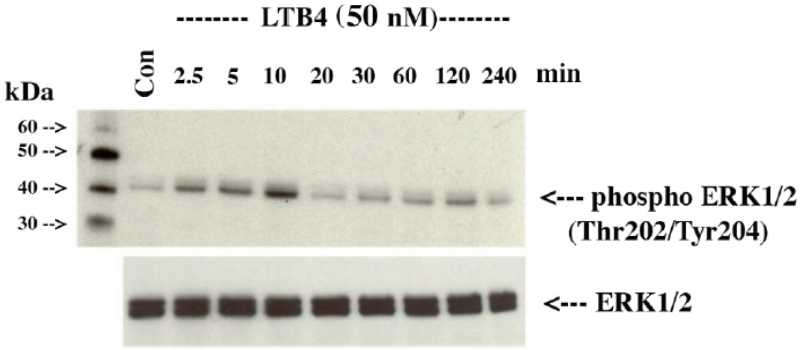


1	2	3	4	5	6	7	1	2	3	4	5	6	7	- lane
1	10.3	2.6	3.7	7.7	11.8	12.9	2.2	2.6	2.9	2.4	2.1	1.2	1.8	- mean optical density (Con = 1)
	2.1	0.54	0.61	1.08	1	2.1	0.52	0.66	0.43	0.63	0.36	0.50	0.7	- ± SD (n = 4-5 expts.)

Figure 4: LTB_4-dependent activation of ERK1/2 phosphorylation. *A*, cultured confluent serum-starved HSF (1.2×10^6 cells in 6-well cluster plates) were treated with vehicle (*Con*) or for varying time periods (2.5-240 min) with LTB_4 (50 nM). *B*, cells were treated with vehicle (*Con*) or with LTB_4 (50 nM) in the absence or presence of U0126 as indicated. *C*, cells were treated with vehicle (*Con*), LTB_4, or with 12-HHT in the absence or presence of the BLT1 antagonist U75302 or the BLT2 antagonist LY255283 for 10 min as indicated. Monolayers were extracted for protein, and 50 μg was analyzed for total and phospho-ERK1/2 by Western blotting using specific rabbit polyclonal antisera as described under "Experimental Procedures". ANOVA of means for densitometric analysis in *A*, control *versus* LTB_4 (2.5 to 10 min), $p < 0.009$; *B*, LTB_4 5-10 min *versus* LTB_4 5-10 min + U0126, $p < 0.0003$; *C*, Student's *t* test; LTB_4 *versus* LTB_4 + U75302, $p < 0.05$; LTB_4 *versus* LTB_4 + LY255283, $p < 0.02$; control *versus* 12-HHT, $p < 0.023$; 12-HHT *versus* 12-HHT + U75302, not significant; 12-HHT *versus* 12-HHT + LY255283, $p < 0.021$.

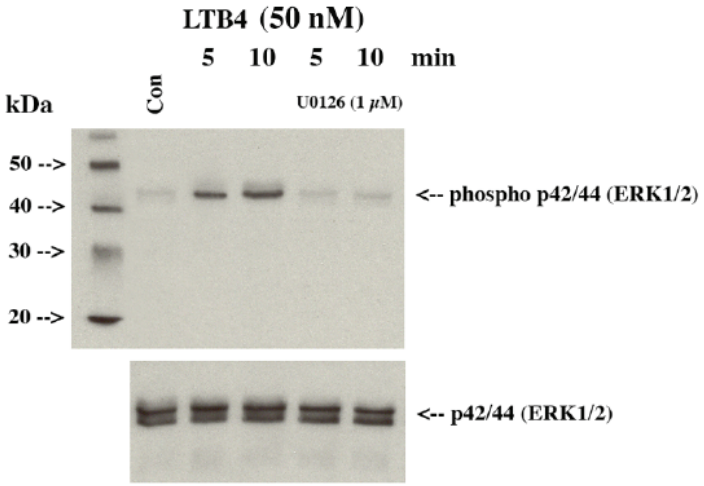
Fig. 4

A



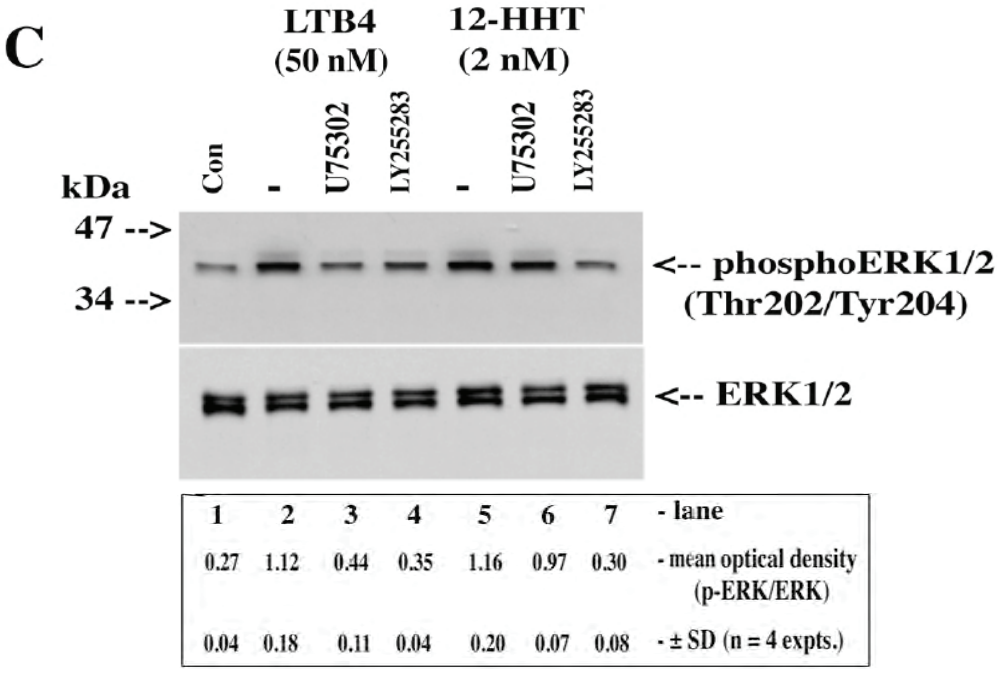
1	2	3	4	5	6	7	8	9	- lane
0.12	0.45	0.56	0.96	0.11	0.17	0.36	0.40	0.23	- mean optical density (p-ERK/ERK)
0.05	0.12	0.13	0.14	0.03	0.05	0.06	0.07	0.06	- ± SD (n = 4 expts.)

B



1	2	3	4	5	- lane
0.09	1.03	1.56	0.12	0.07	- mean optical density (p-ERK/ERK)
0.04	0.26	0.19	0.06	0.05	- ± SD (n = 3 expts.)

Fig. 4



1	2	3	4	5	6	7	- lane
0.27	1.12	0.44	0.35	1.16	0.97	0.30	- mean optical density (p-ERK/ERK)
0.04	0.18	0.11	0.04	0.20	0.07	0.08	- ± SD (n = 4 expts.)

Figure 5: LTB_4 stabilized luciferase activity expressed from a luciferase-COX-2−3'-UTR fusion chimeric construct; effect of the MEK1/2 inhibitor U0126. Both HSF (*A*) and HeLa cells (*B*) were plated at 40% confluence in DMEM supplemented with 10% heat-inactivated FBS and antibiotics. One μg of the luciferase-COX-2−3'-UTR chimeric fusion construct and 50 ng of a pHSV-TK-driven *Renilla* luciferase plasmid were co-transfected for 6 h using FuGENE 6 according to the manufacturer's instructions. Cells were incubated overnight in complete medium; following a change to medium containing 1% FBS (2 h), transfected cells were incubated for 16 h with vehicle (*Con*) or with rhIL-1β (5.7 pmol/liter), NS-398 (300 nmol/liter), LTB_4 (1-1000 nmol/liter), and U0126 (1 μmol/liter) as per the figure. Cells were lysed, and the luciferase activity and protein content were determined as described under "Experimental Procedures". Values were expressed as the mean of the ratio of RLU1 (Firefly)/RLU2 (*Renilla*) normalized to protein content.

Fig. 5

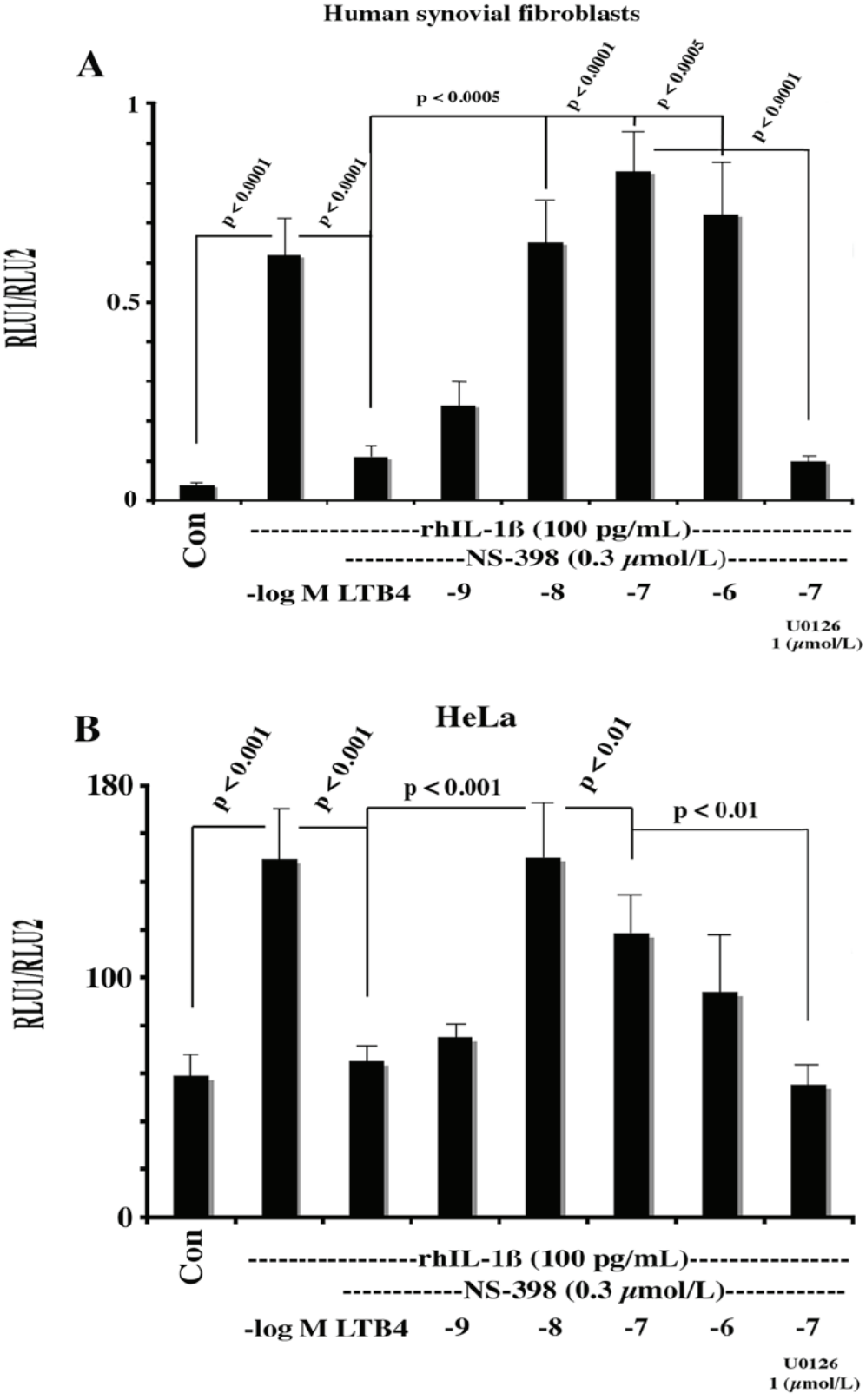
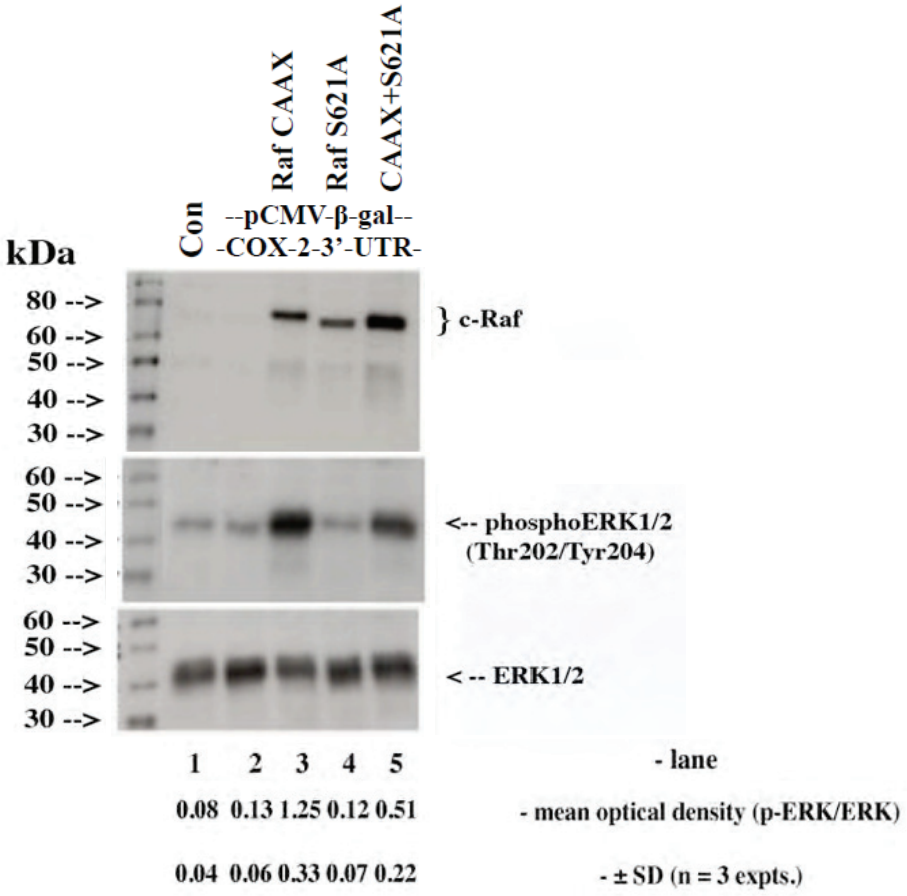

Human synovial fibroblasts
A
RLU1/RLU2
1
0.5
0
p < 0.0001
p < 0.0001
p < 0.0005
p < 0.0001
p < 0.0005
Con
----------rhIL-1ß (100 pg/mL)----------
----------NS-398 (0.3 µmol/L)----------
-log M LTB4 -9 -8 -7 -6 -7
U0126
1 (µmol/L)
B
HeLa
RLU1/RLU2
180
100
0
p < 0.001
p < 0.001
p < 0.01
p < 0.01
Con
----------rhIL-1ß (100 pg/mL)----------
----------NS-398 (0.3 µmol/L)----------
-log M LTB4 -9 -8 -7 -6 -7
U0126
1 (µmol/L)

Figure 6: Role of Ras/c-Raf/MEK1/2/ERK1/2 signaling pathway in the stabilization of luciferase activity expressed from a luciferase-COX-2−3'-UTR fusion chimeric construct. *A*, HeLa cells were plated at 40% confluence in DMEM supplemented with 10% heat-inactivated FBS and antibiotics. 200 ng of the luciferase-COX-2−3'-UTR chimeric fusion construct, 400 ng each of pCMV, pRafCAAX, or pRafS621A, and 200 ng of a pCMV-β-gal were co-transfected for 6 h using FuGENE 6 according to the manufacturer's instructions. Cells were incubated overnight in complete medium and following a change to medium containing 1% FBS (2 h), and monolayers were extracted for protein, and 50 μg was analyzed for c-Raf, total, and phospho-ERK1/2 by Western blotting using specific rabbit polyclonal antisera as described under "Experimental Procedures". Untransfected cells were cultured and extracted in the same way (*Con*). *B*, cells were transfected with 10 ng of pCMV-luciferase-Δ3'-UTR (pZEO-LUC3), pCMV-luciferase-COX-2−3'-UTR, pCMV-luciferase-COX-2-ARE, or pCMV-luciferase-COX-2-ΔARE with or without 400 ng of pRafCAAX, 200 ng of pCMV-β-gal, and U0126 (1 μmol/liter) as indicated. Values were expressed as the mean ± S.D. of luciferase reporter activity normalized to β-galactosidase activity and protein content. Monolayers from tandem experiments, including untransfected cells (*Con*), were extracted for protein, and 50 μg was analyzed for c-Raf, total, and phospho-ERK1/2 by Western blotting (*C*). *D*, either HeLa cells or HSF were transfected with 1 μg of pCMV-luciferase-Δ3'-UTR (pZEO-LUC3), pCMV-luciferase-COX-2−3'-UTR, pCMV-luciferase-COX-2-ARE, or pCMV-luciferase-COX-2-ΔARE for 6 h using FuGENE 6. Cells were incubated overnight in complete medium and, following a change to medium containing 1% FBS (2 h), monolayers were extracted for total RNA and subjected to RT-PCR using specific primers for luciferase and GAPDH as per "Experimental Procedures". Densitometric

analysis in *A*, Student's *t* test; pCMV *versus* pRafCAAX, $p < 0.022$; pRafCAAX *versus* pRafS621A, $p < 0.05$; *C*, β-gal *versus* pRafCAAX, $p < 0.001$; pRafCAAX *versus* pRafCAAX + U0126, $p < 0.021$.

Fig. 6

A


Con
Raf CAAX
Raf S621A
CAAX+S621A
--pCMV-β-gal--
-COX-2-3'-UTR-
kDa
80 -->
60 -->
50 -->
40 -->
30 -->
} c-Raf
60 -->
50 -->
40 -->
30 -->
<-- phosphoERK1/2
(Thr202/Tyr204)
60 -->
50 -->
40 -->
30 -->
< -- ERK1/2
1 2 3 4 5
- lane
0.08 0.13 1.25 0.12 0.51
- mean optical density (p-ERK/ERK)
0.04 0.06 0.33 0.07 0.22
- ± SD (n = 3 expts.)

B

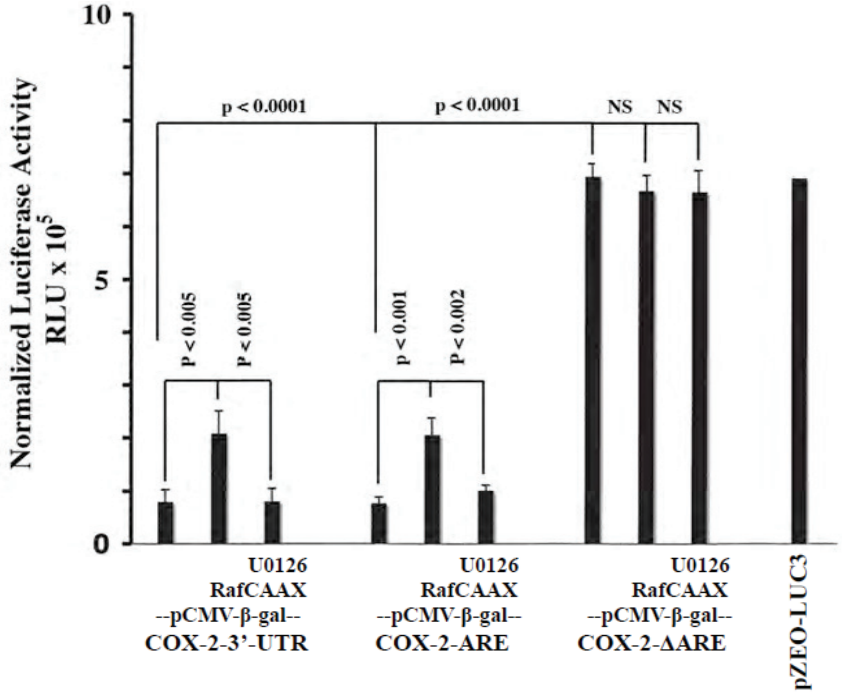
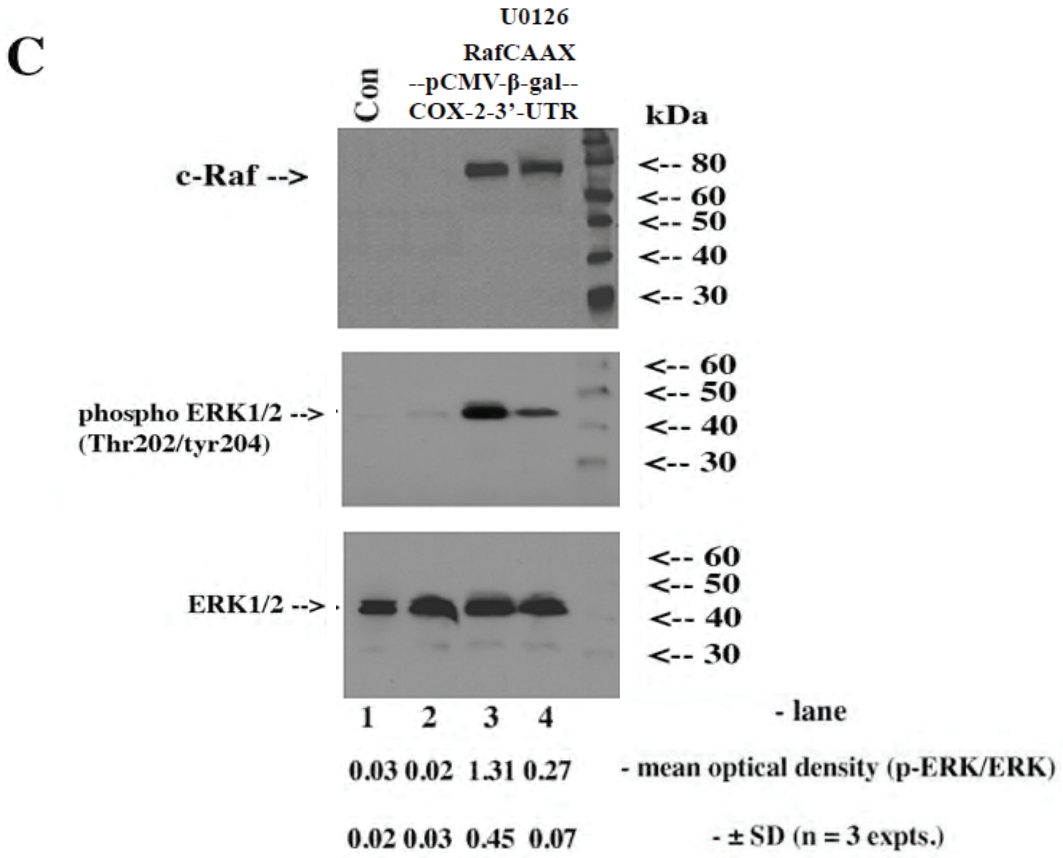

Normalized Luciferase Activity
RLU x 10^5
10
5
0
p < 0.0001
p < 0.0001
NS
NS
P < 0.005
P < 0.005
p < 0.001
P < 0.002
U0126
RafCAAX
--pCMV-β-gal--
COX-2-3'-UTR
U0126
RafCAAX
--pCMV-β-gal--
COX-2-ARE
U0126
RafCAAX
--pCMV-β-gal--
COX-2-ΔARE
pZEO-LUC3

Fig. 6


C
U0126
RafCAAX
--pCMV-β-gal--
COX-2-3'-UTR
Con
kDa
c-Raf -->
<-- 80
<-- 60
<-- 50
<-- 40
<-- 30
phospho ERK1/2 -->
(Thr202/tyr204)
<-- 60
<-- 50
<-- 40
<-- 30
ERK1/2 -->
<-- 60
<-- 50
<-- 40
<-- 30
1 2 3 4
- lane
0.03 0.02 1.31 0.27
- mean optical density (p-ERK/ERK)
0.02 0.03 0.45 0.07
- ± SD (n = 3 expts.)

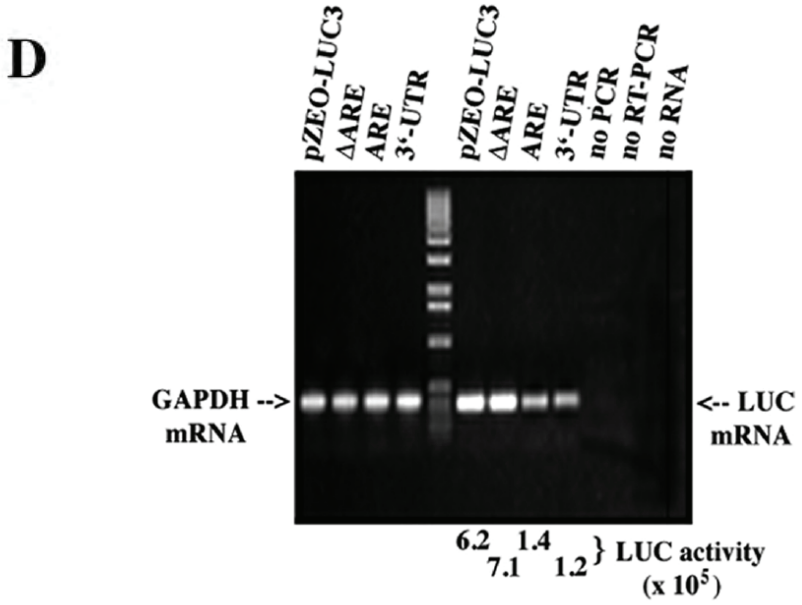
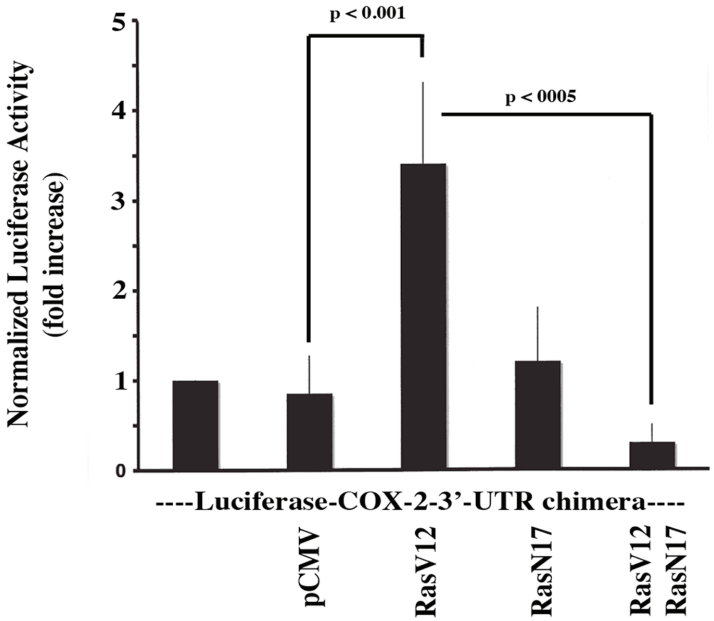

D
pZEO-LUC3
ΔARE
ARE
3'-UTR
pZEO-LUC3
ΔARE
ARE
3'-UTR
no PCR
no RT-PCR
no RNA
GAPDH -->
mRNA
<-- LUC
mRNA
6.2 1.4
7.1 1.2
} LUC activity
(x 10^5)

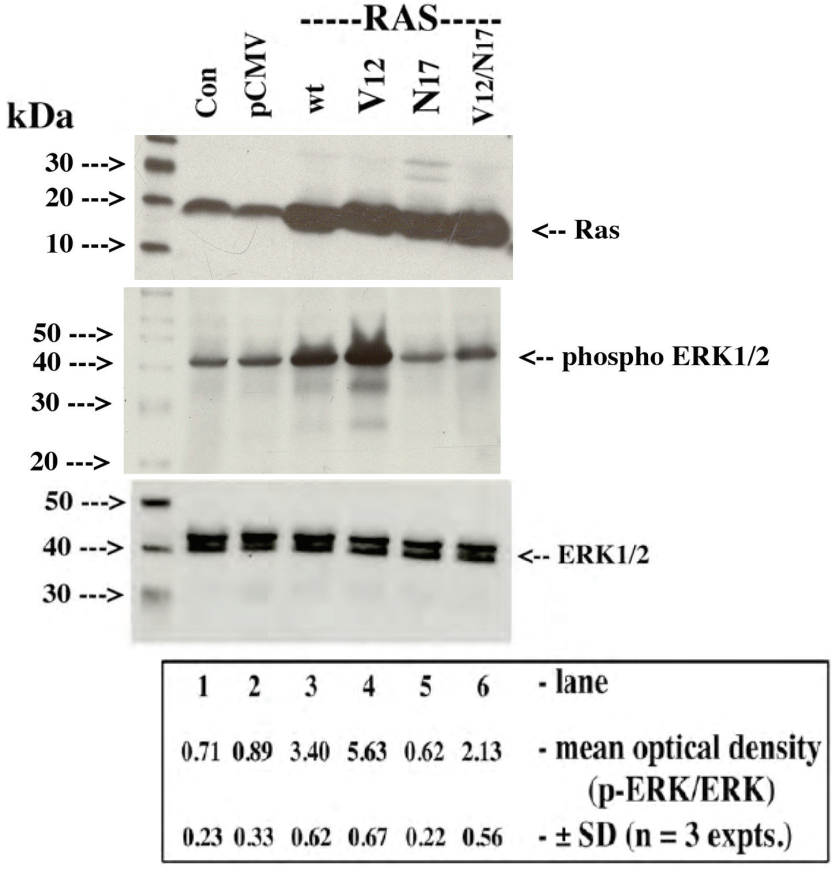
Figure 7: Ha-Ras (p21) stabilization of luciferase activity expressed from a luciferase-COX-2−3'-UTR fusion chimeric construct. *A*, HeLa cells were plated at 40% confluence in DMEM supplemented with 10% heat-inactivated FBS and antibiotics. 500 ng of the luciferase-COX-2−3'-UTR chimeric fusion construct, 500 ng each of pCMV, pRasV12, or pRasN17, and 50 ng of a pCMV-β-gal were co-transfected for 6 h using FuGENE 6 according to the manufacturer's instructions. Cells were incubated overnight in complete medium, and following a change to medium containing 1% FBS (2 h), the cells were lysed, and the luciferase activity, β-galactosidase activity, and protein content were determined as described under "Experimental Procedures". Values were expressed as the mean ± S.D. of fold induction of luciferase activity normalized to β-galactosidase activity and protein content *versus* the control (1% FBS for 2 h). *B*, tandem monolayers, including untransfected cells (*Con*), were extracted for protein, and 50 μg was analyzed for Ha-Ras and phospho-ERK1/2 by Western blotting using specific rabbit polyclonal antisera as described under "Experimental Procedures". Densitometric analysis in *B*, Student's *t* test; pCMV *versus* wtRas, $p < 0.01$; pCMV *versus* pV12Ras, $p < 0.004$; pV12Ras *versus* pV12Ras/RasN17, $p < 0.04$.

Fig. 7

A



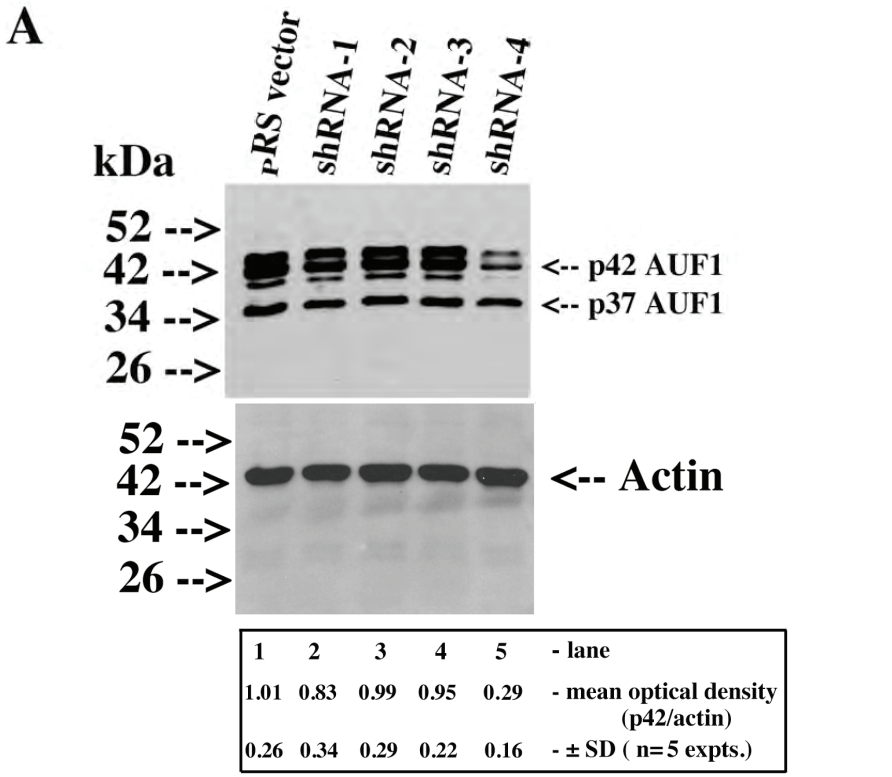
B



1	2	3	4	5	6	- lane
0.71	0.89	3.40	5.63	0.62	2.13	- mean optical density (p-ERK/ERK)
0.23	0.33	0.62	0.67	0.22	0.56	- ± SD (n = 3 expts.)

Figure 8: shRNA knockdown of p42 AUF1 and BLT/c-Raf/ERK1/2 signaling induced a leptomycin B-sensitive nuclear export of p42 AUF1. *A*, HeLa cells were stably transfected through puromycin selection with the pRS vector or four constructs containing shRNA gene-specific target sequences of *AUF1* as described under "Experimental Procedures". Monolayers were extracted for protein, and 50 μg was analyzed for AUF1 and actin by Western blotting using pan-AUF1 and actin antibodies. *B*, HeLa cells (3×10^5 in 6-well plates) were transiently co-transfected with 10 ng of luciferase-COX-2-ARE, 500 ng of pcDNA3, or pcDNA3-p42 AUF1, and 400 ng of pCMV or pRafCAAX as per the illustration. After 16 h, 3 nM leptomycin B or vehicle was added to the wells as indicated for an additional 16 h, after which time monolayers were extracted for nuclear and cytoplasmic protein and 30-50 μg was analyzed for AUF1, GAPDH (cytoplasm), histone H3 (nuclear), and c-Jun (nuclear) by Western blotting. Densitometric analysis in *A*, Student's *t* test; pRS *versus* pRSAUF1-shRNA-4, $p < 0.001$; pRS *versus* pRSAUF1-shRNA-1−3, *NS*, not significant. *C*, densitometric and statistical analysis of *B*.

Fig.8


A
pRS vector
shRNA-1
shRNA-2
shRNA-3
shRNA-4
kDa
52 -->
42 -->
34 -->
26 -->
<-- p42 AUF1
<-- p37 AUF1
52 -->
42 -->
34 -->
26 -->
<-- Actin
1 2 3 4 5 - lane
1.01 0.83 0.99 0.95 0.29 - mean optical density (p42/actin)
0.26 0.34 0.29 0.22 0.16 - ± SD (n=5 expts.)

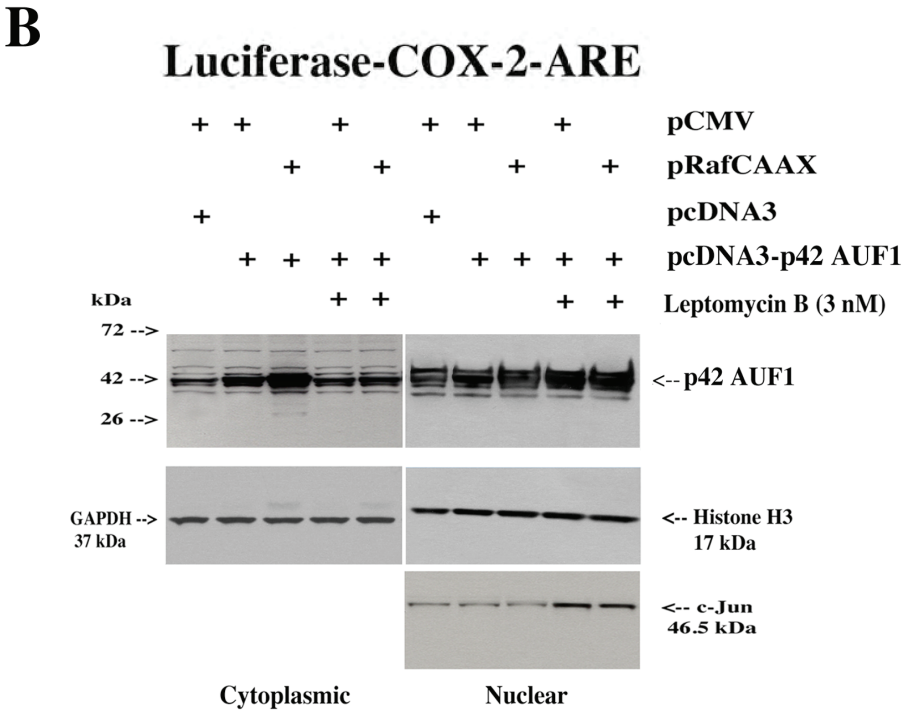
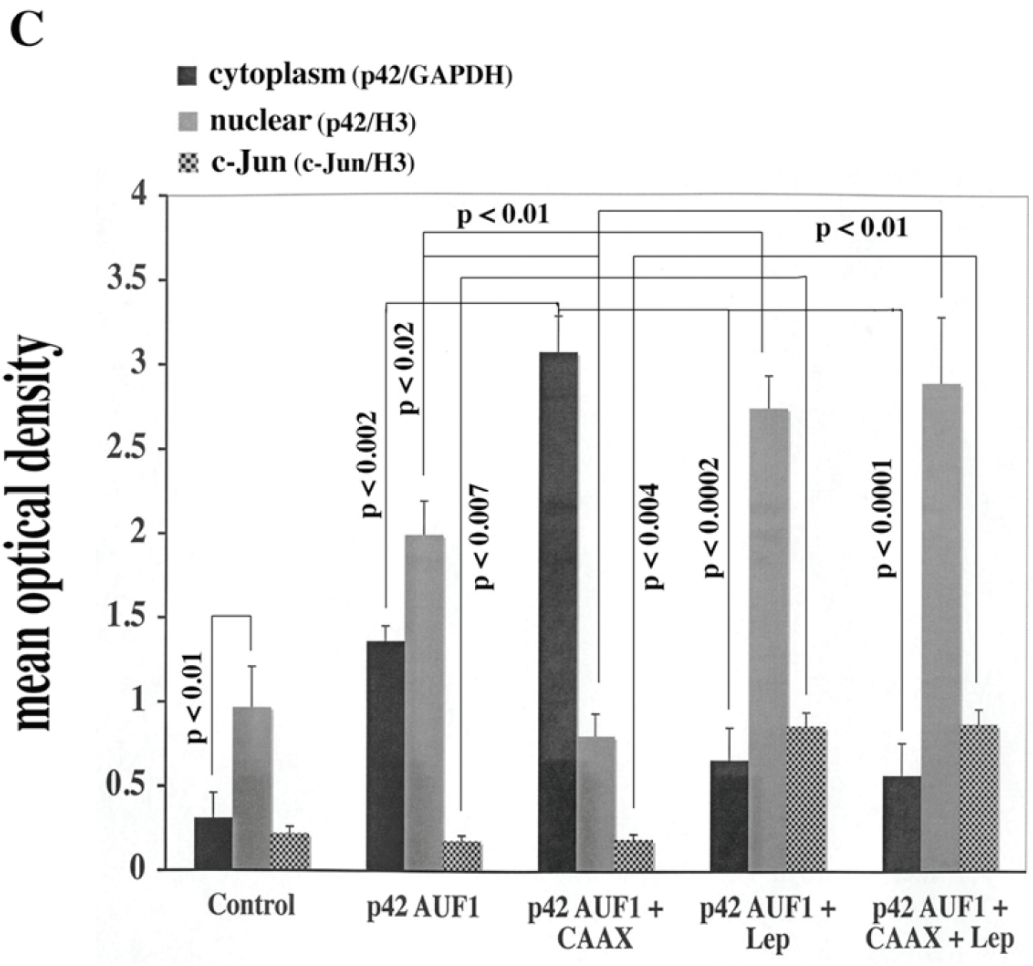

B
Luciferase-COX-2-ARE
pCMV
pRafCAAX
pcDNA3
pcDNA3-p42 AUF1
Leptomycin B (3 nM)
kDa
72 -->
42 -->
26 -->
<-- p42 AUF1
GAPDH -->
37 kDa
<-- Histone H3
17 kDa
<-- c-Jun
46.5 kDa
Cytoplasmic
Nuclear

Fig. 8


C
cytoplasm (p42/GAPDH)
nuclear (p42/H3)
c-Jun (c-Jun/H3)
mean optical density
4
3.5
3
2.5
2
1.5
1
0.5
0
p < 0.01
p < 0.01
p < 0.002
p < 0.02
p < 0.007
p < 0.004
p < 0.0002
p < 0.0001
Control
p42 AUF1
p42 AUF1 + CAAX
p42 AUF1 + Lep
p42 AUF1 + CAAX + Lep

PREFACE TO CHAPTER 3

Based on the increasing recognition that disruption of epigenetic processes can lead to various pathologies, we pursued this avenue of research in mast cells, where 5-LO, the key enzyme for the biosynthesis of LTB_4, is freely expressed and in human synovial fibroblasts (HSF), where the enzyme is undetectable. We hypothesized that epigenetic mechanisms, involving regulated methylation-demethylation of the *5-LO* gene promoter, are responsible for 5-LO expression and leukotriene production. In some RA patients, synovial fibroblasts (SF) can synthesize very small quantity of LTB_4, which may be due to the changes of DNA methylation or histone modifications in the regulation of *5-LO* gene, contributing to disease resolution or flare up in these RA patients. Our objective is to define the epigenetic mechanisms of *5-LO* gene expression in mast cells and HSF from arthritis patients, which could help us to further understand the pathophysiology of RA. We believe the results will elucidate more targets for the treatment of inflammatory diseases and/or find more uses for the established drugs.

CHAPTER 3

EPIGENETIC REGULATION OF 5-LIPOXYGENASE (*5-LO*) GENE

This chapter was adapted from a manuscript that is in preparation for submission to *Inflammation Research*. **Beibei Zhai**, Luigi Macchia, and John A. Di Battista, 2010.

3.1. ABSTRACT

OBJECTIVE: Leukotrienes (LTs) are potent bioactive lipids that play an important role in the initiation, progression, and resolution of the inflammatory response. We recently observed that 5-lipoxygenase (5-LO), a key rate-limiting step in the biosynthesis of LTs, may be subject to epigenetic regulation. In order to further characterize the molecular mechanism(s) involved, we investigated the relationship between 5-LO protein expression and the epigenetic regulations (DNA methylation and histone methylation) of *5-LO* gene in human mast cells (HMCs) and human synovial fibroblasts (HSF) from osteoarthritic (OA) and rheumatoid arthritic (RA) patients.

METHODS: *5-LO* gene expression and promoter methylation were analyzed by Western blotting and genomic DNA bisulfite sequencing, respectively. Histone H3 (lysines 9 and 27) methylation at the promoter, exonic, and intronic sequences of *5-LO* gene was detected using chromatin immunoprecipitation (ChIP) assay.

RESULTS: *5-LO* gene promoter (DNA CpG islands) was heavily methylated in U937 cells (5-LO negative), but unmethylated in HL-60 cells (5-LO positive). Compared to the 5-LO-positive HMC-1 cells, the 5-LO-negative HMC-1 cells had much higher methylation levels of CpG islands in the promoter region. We found a strong correlation between *5-LO* gene expression and DNA methylation in HMC-1 cells. Dexamethasone (DEX) treatment of HMC-1 cells increased the expression of 5-LO, a process associated with reduced methylation of histone H3 on lysines 9 and 27. Interestingly, OA/RA HSF are 5-LO negative; though the promoter region is CpG hypomethylated, histone H3 is hypermethylated at Lys-9 and -27 residues.

CONCLUSION: This is the first study investigating the association between epigenetic regulations (DNA methylation and histone methylation) and *5-LO* gene expression in

arthritis-affected HSF. Epigenetic dysregulation may also be involved in the pathogenesis of RA. It is important to study DNA methylation and histone post-translational modifications, such as methylation, acetylation and phosphorylation, in order to further specify the detailed molecular mechanisms in the regulation of inflammatory gene expression.

3.2. INTRODUCTION

Epigenetics refers to "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence" (Wu and Morris 2001). The most thoroughly studied epigenetic mechanisms are DNA methylation and post-translational modifications of histones, such as methylation, acetylation and phosphorylation (Kouzarides 2007). DNA methylation is the addition of methyl groups to the 5'-cytosines in CpG dinucleotides by DNA methyltransferases (DNMTs), resulting into repressed chromatin structure and gene silencing (Robertson 2005). Histone methylation is the addition of one, two or three methyl groups on some amino acids (*e.g.* lysine, arginine) in a histone protein. Usually, histone methylation is associated with condensed chromosome and suppresses gene transcription. But some histone methylation, such as methylation of lysine 4 residue on histone H3, and arginine methylation of histone H3 (Arg-2, -17 and -26) and histone H4 (Arg-3), results into transcriptional activation (Lee, Teyssier et al. 2005). Epigenetic regulation is essential for normal development and differentiation. It is associated with many cellular processes, including embryonic development, gene transcription, X chromosome inactivation, genomic imprinting and chromosome stability (Robertson 2005; Kouzarides 2007).

However, environmental factors, such as stress, diet, pollution and infections, can

cause epigenetic changes that contribute to the development of many diseases (Barros and Offenbacher 2009; Suter and Aagaard-Tillery 2009). Aberrant DNA hypermethylation of tumor suppressor genes are detected in numerous cancers. Meanwhile, global DNA hypomethylation causes chromosome instability and activation of oncogenes, further contributing to the carcinogenesis (Gopalakrishnan, Van Emburgh et al. 2008; Ellis, Atadja et al. 2009). Epigenetic regulation is also involved in inflammatory and autoimmune diseases. For example, reduced histone deacetylase (HDAC) activity was reported in biopsies from patients with chronic obstructive pulmonary disease (COPD) and severe asthma (Adcock, Tsaprouni et al. 2007). T cells from patients with active lupus had hypomethylated DNA, due to decreased DNMT1 level and activity (Hewagama and Richardson 2009). Furthermore, epigenetic abnormalities were also reported in some mental disorders (Tsankova, Renthal et al. 2007) and age-related diseases (Calvanese, Lara et al. 2009). An interesting study found DNA methylation and histone acetylation profiles were remarkably different in the older monozygous twins than the younger ones due to the various environments they were exposed to during aging (Fraga, Ballestar et al. 2005). Since aging is one of the risk factors for the development of osteoarthritis, the age-related epigenetic changes on target genes may play a role in the pathogenesis of this disease in some individuals.

Studies also reported the association of epigenetic changes with rheumatoid arthritis (Karouzakis, Gay et al. 2009). In RA synovium, the infiltrating mast cells and neutrophils produce large amounts of inflammatory mediators, such as prostaglandins and LTs. However, only low levels of leukotriene B_4 (LTB_4) (23.13 ± 8.67 pg/ml) were detected in the culture medium of HSF from RA patients (Xu, Lu et al. 2010). Since 5-LO is the key enzyme in the biosynthesis of LTB_4 and the promoter region of *5-LO* gene

also contains multiple CpG dinucleotides (Funk, Hoshiko et al. 1989), we examined the molecular mechanisms involved in the epigenetic regulation of *5-LO* gene expression in HMCs and HSF from OA and RA patients.

3.3. MATERIALS AND METHODS

3.3.1. Reagents

Acrylamide and Bio-Rad protein reagent originated from Bio-Rad Laboratories (Hercules, CA). Methanol (99%), heat-inactivated fetal bovine serum (FBS) and iron supplemented bovine calf serum were obtained from Fisher Scientific (Ottawa, Ontario, Canada). T-STIM with ConA (IL-2 culture supplement) was purchased from BD company (Franklin Lakes, NJ). Prostaglandin E_2 (PGE_2) was a product of Cayman Chemical (Ann Arbor, MI). Phenylmethylsulfonyl fluoride, 2-mercaptoethanol, monothioglycerol, phorbol 12-myristate 13-acetate (PMA), A23187 and dexamethasone (DEX) were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Sodium dodecyl sulfate (SDS), Tris-base, LB broth base, select agar, L-glutamine, *Taq* DNA polymerase, Dulbecco's modified Eagle's medium (DMEM), Iscove's modified Dulbecco's medium (IMDM) and antibiotic mixture [10,000 units of penicillin (base), 10,000 μg of streptomycin (base)] were products of Invitrogen™ (Burlington, Ontario, Canada).

3.3.2. Cell culture

HL-60 cells (human promyelocytic leukemia cells) and U937 cells (human leukemic monocyte lymphoma cells) were purchased from American Type Culture Collection (ATCC, Rockville, MD) and maintained in RPMI-1640 medium

supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2, 95% air. MC/9 cells (mouse mast cells) and NIH3T3 cells (mouse embryonic fibroblasts) were also obtained from ATCC. MC/9 cells were grown in DMEM with 4 mM L-glutamine, which is adjusted to contain 4.5 g/liter glucose and 1.5 g/liter sodium bicarbonate and supplemented with 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 10% rat T-STIM and 10% heat-inactivated FBS at 37 °C in a humidified atmosphere of 5% CO_2, 95% air. NIH3T3 cells were grown in DMEM supplemented with 10% FBS and antibiotics at 37 °C in a humidified atmosphere of 5% CO_2, 95% air. HMC-1 (immature human mast cells) was a kind gift from Dr. Joseph H. Butterfield (Mayo Clinic, Rochester, MN) and cultured in IMDM supplemented with 1.2 mM monothioglycerol, 10% heat-inactivated iron supplemented bovine calf serum and antibiotics at 37 °C in a humidified atmosphere of 5% CO_2, 95% air.

Human synovial fibroblasts were isolated from specimens obtained from OA and RA patients undergoing arthroplasty who were diagnosed based on the criteria developed by the American College of Rheumatology Diagnostic Subcommittee for OA/RA (mean age 67 ± 19) (Altman, Asch et al. 1986; Hochberg, Chang et al. 1992). Human synovial fibroblasts were released by sequential enzymatic digestion with 1 mg/ml pronase (Boehringer Mannheim, Laval, Quebec, Canada) for 1 h, followed by 6 h with 2 mg/ml collagenase (type IA, Sigma-Aldrich, Oakville, Ontario, Canada) at 37 °C in DMEM supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin and 100 µg/ml streptomycin (Faour, Mancini et al. 2003). Released HSF were incubated for 1 h at 37 °C in tissue culture flasks (Primaria catalog no. 3824, Falcon, Lincoln Park, NJ), allowing the adherence of nonfibroblastic cells possibly present in the synovial preparation. In

addition, flow cytometric analysis (Epic II, Coulter, Miami, FL), using the anti-CD14 (fluorescein isothiocyanate, FITC) antibody, was conducted to confirm that no monocytes/macrophages were present in the HSF preparation (Faour, He et al. 2001). The cells were seeded in tissue culture flasks, and cultured until confluence in DMEM supplemented with 10% FBS and antibiotics at 37 °C in a humidified atmosphere of 5% CO_2, 95% air. The cells were incubated in fresh medium containing 0.5-1% FBS for 24 h before the experiments and only second or third passaged HSF were used.

3.3.3. Western blotting

Forty-70 µg of cellular protein extracted in hot SDS-PAGE loading buffer, from control and treated cells, were subjected to SDS-polyacrylamide gel electrophoresis through 10% gels (final concentration of acrylamide) under reducing conditions, and transferred onto nitrocellulose membranes (GE Healthcare Amersham Biosciences, Piscataway, NJ). Following blocking with 5% BLOTTO for 2 h at room temperature and washing, the membranes were incubated overnight at 4 °C with polyclonal anti-5-LO antibody (Abcam, Cambridge, MA; 1:1000 dilution) in Tween/Tris-buffered saline (TTBS) containing 0.25% BLOTTO. The secondary anti-rabbit antibody-horseradish peroxidase conjugate (Cell Signaling Technology, Danvers, MA; 1:2000 dilution) was subsequently incubated with the membranes for 1 h at room temperature and washed extensively for 30 min with TTBS. Following incubation with an ECL chemiluminescence reagent (Cell Signaling Technology, Danvers, MA), membranes were prepared for autoradiography, exposed to Kodak X-Omat film, and subjected to digital imaging system (Alpha G-Imager 2000; Canberra Packard Canada, Mississauga, Ontario, Canada) for semi-quantitative measurements. In addition to anti-5-LO antibody, the

following polyclonal antibodies were used: anti-5-lipoxygenase activating protein (FLAP) antibody (1:1000 dilution), goat anti-actin (1:500 dilution), and donkey anti-goat antibody-horseradish peroxidase conjugate (1:5000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA).

3.3.4. Bisulfite sequencing

Genomic DNA was isolated using DNeasy Blood & Tissue Kit (QIAGEN, Mississauga, Ontario, Canada). The genomic DNA of HMC-1 was kindly provided by Dr. Macchia (University of Bari, Bari, Italy). Bisulfite treatment of genomic DNA (1 μg) was performed using EpiTect Bisulfite Kit (QIAGEN, Mississauga, Ontario, Canada) following the manufacturer's instruction. The modified DNA that corresponds to the promoter region of *5-LO* gene (GenBank™ accession number M38191) was amplified by nested PCR with 35 cycles for each amplification using primers specific for the non-coding strand of *5-LO* gene. The primers for the distal promoter of *5-LO* gene were (R = reverse primer, F = forward primer): 5'-TCAAACATCTAAATATACATCTCTCC-3' (R-1207/-1182) and 5'-TGTAGAGGAAGAGYGATTTATGAGAG-3' (F-528/-553) for nested-1; 5'-AACTCTACTACTTCACAAACATAAAACRT-3' (R-1087/-1059) and 5'-GAGGAAGAGYGATTTATGAGAGAAT-3' (F-532/-556) for nested-2, with the annealing temperatures of 55 °C and 58 °C for nested-1 and nested-2 PCRs, respectively, and the final amplified product of 556 bp for the distal promoter of *5-LO* gene (Fig. 1). The primers for the proximal promoter of *5-LO* gene were as follows: 5'-ATTCTCTCATAAATCRCTCTTCCTC-3' (R-556/-532) and 5'-TTGTAGAAGGGTTTGTTTAGTAGGT-3' (F+131/+107) for nested-1; 5'-CTCTCATAAATCRCTCTTCCTCTACA-3' (R-553/-528) and 5'-

AATTATTGGTTGTTAGTGGTTAYGGTGAT-3' (F+44/+16) for nested-2, with the annealing temperatures of 53 °C and 62 °C for nested-1 and nested-2 PCRs, respectively, and the final amplicon of 597 bp for the proximal promoter of *5-LO* gene (Fig. 1). Then the final PCR products (556 bp and 597 bp) were cloned into pCR II TOPO vectors using TOPO TA Cloning Kit (Invitrogen, Burlington, Ontario, Canada) following the manufacturer's instruction. One to six colonies per each condition were purified by QIAprep Spin Miniprep Kit (QIAGEN, Mississauga, Ontario, Canada) and the inserts of individual colonies were subjected to sequence analysis at McGill University and Genome Quebec Innovation Centre (Montreal, Quebec, Canada).

3.3.5. Chromatin immunoprecipitation (ChIP)

ChIP was performed using SimpleChIP™ Enzymatic Chromatin IP kit with magnetic beads (Cell Signaling Technology, Danvers, MA) according to the manufacturer's instruction. Antibodies used were rabbit monoclonal tri-methyl-histone H3 (Lys-27) antibody (1:100 dilution), rabbit polyclonal pan-methyl-histone H3 (Lys-9) antibody (1:25 dilution), rabbit monoclonal histone H3 antibody (1:50 dilution, as positive control), and normal rabbit IgG (1-2 μg, as negative control/background) (Cell Signaling Technology, Danvers, MA). The kit also contains a primer set for PCR detection of the human ribosomal protein L30 (RPL30) gene with the amplified product of 161 bp. Specific primers for *5-LO* gene detection using standard PCR were designed based on the published sequence (GenBank™ accession number NG_011437): 5'-CCAAGACAGTATGAGGAGATGCTA-3' (sense) and 5'-CGTGTATTGATCTCGGCTAAGTTT-3' (antisense) with the amplicon of 153 bp in the distal promoter region; 5'-CCAGAATCCATCCTCAGTATCAG-3' (sense) and 5'-

GACTCCCTACAAATCTAGGCACAG-3' (antisense) with the amplicon of 174 bp in the proximal promoter region; 5'-GATCTTCAGGGAACTGCATAGATT-3' (sense) and 5'-CCATGTCTCCAACAAGAATAGACA-3' (antisense) with the amplicon of 170 bp in exon 14; 5'-TAAACACCATAGGGACCCATTCTA-3' (sense) and 5'-GGGTACCTACTATGTGCAAGGAAT-3' (antisense) with the amplicon of 195 bp in exon 14; 5'-ACAGATAGATGTGGTTCATGCAGT-3' (sense) and 5'-AGATGTGTGAGAAAGCTCACTGTC-3' (antisense) with the amplicon of 154 bp in the intron between exon 3 and exon 4; 5'-AAATGTCAGAACTGAAAGGTAGGC-3' (sense) and 5'-ATCAGACTCTACCCAGAAGGAATG-3' (antisense) with the amplicon of 188 bp in the intron between exon 6 and exon 7. The PCR program was started with the initial denaturation at 95 °C for 2 min, followed by 34 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min. The PCR products were analyzed and verified by electrophoresis on 1.15% agarose gels in a Tris borate/EDTA buffer system as described previously (Faour, He et al. 2001). All gel photos were subjected to a digital imaging system (Alpha G-Imager 2000; Canberra Packard Canada, Mississauga, Ontario, Canada) for semi-quantitative measurements.

3.3.6. Statistical analysis:

Where appropriate, Student's *t* test was used to compare the statistical difference. Significance was acknowledged when the probability that the Null Hypothesis was satisfied at < 5%.

3.4. RESULTS

3.4.1. *5-LO* gene expression in mast cells and HSF

As demonstrated by (Xu, Lu et al. 2010), HSF isolated from severe hyperplastic synovia produced only small amounts of LTB_4. Since 5-LO is the key enzyme in the biosynthesis of LTs, we checked the 5-LO protein levels using Western blotting analysis in the HSF from OA and RA patients. MC/9 cells, mouse mast cells expressed high levels of 5-LO protein, were used as a positive control (Fig. 2*A*, top panel). PMA, A23187, DEX and PGE_2 were used to stimulate these different cells. PMA is the most commonly used phorbol ester. It binds to and activates protein kinase C, causing a wide range of effects in cells and tissues. PMA is also an extremely potent tumor promoter (Blumberg 1980; Blumberg 1981). A23187 is a mobile-carrier Ca^{2+} ionophore (allowing Ca^{2+} to cross the cell membrane). It was originally isolated from *Streptomyces chartreusensis* as an antibiotic. A23187 increases the intracellular Ca^{2+} level and is usually used in studies related to intracellular Ca^{2+}-mediated events. As shown in Fig. 2*A* (bottom panel), there was no 5-LO protein expression in OA and RA HSF with or without stimulation. NIH3T3 cells, the mouse embryonic fibroblasts, did not express 5-LO protein either (Fig. 2*A*, top panel). In addition, we also verified that all of these cells expressed FLAP protein through Western blotting analysis (Fig. 2*B*). FLAP is a nuclear 5-LO anchoring protein and essential for full lipoxygenase activity.

3.4.2. DNA methylation status in the promoter region of *5-LO* gene

Before further experiments in our test cell types, we first verified the technique of bisulfite sequencing as described in “Materials and Methods” in HL-60 and U937 cell lines. HL-60 cells were shown to have unmethylated *5-LO* promoter and high 5-LO

expression, while U937 cells have a highly methylated *5-LO* gene promoter and express low levels of 5-LO (Uhl, Klan et al. 2002). Consistent with this previous study, we obtained similar results in HL-60 and U937 cells (Fig. 3*B*). This demonstrated the reliability of our procedures for DNA methylation studies in test cells.

HMC-1 cells in long-term cultures lose normal high levels of *5-LO* gene expression (Colamorea, Di Paola et al. 1999) and we verified the DNA methylation profiles of *5-LO* gene in 5-LO-negative and 5-LO-positive HMC-1 cells using bisulfite sequencing. Mast cells are well known for their involvement in inflammatory and anaphylactic reactions and, since corticosteroids form a major treatment (Bradding 2003; Black, Oliver et al. 2009), we investigated the effect of DEX treatment on the expression of *5-LO* gene in the two phenotypes of HMC-1 cells (5-LO-negative and 5-LO-positive), respectively. As shown in Fig. 4*B-E*, the distal region of the *5-LO* gene promoter was highly methylated in all of the HMC-1 cells; whereas, the proximal promoter showed marked differences under various conditions. The HMC-1 cells that had been cultured continuously for many passages had much more methylated CpG islands in the proximal region of *5-LO* gene promoter (Fig. 4*D*) than the earlier cultures of HMC-1 cells (Fig. 4*B*). Interestingly, DEX treatment did not change the methylation profiles of the *5-LO* gene promoter in HMC-1 cells (Fig. 4*B* compared with Fig. 4*C*; Fig. 4*D* compared with Fig. 4*E*), although it did improve some levels of 5-LO protein expression in both phenotypes of HMC-1 cells [(Colamorea, Di Paola et al. 1999); Fig. 8]. We also performed bisulfite sequencing using the genomic DNA from OA and RA HSF to check the methylation status of *5-LO* gene promoter and verify whether 5-LO protein expression followed the same profiles as observed in HMCs and lymphocytic/myelocytic

cell lines based on DNA methylation status. Quite unexpectedly, *5-LO* gene promoter were almost completely unmethylated both in OA HSF (Fig. 5*B*) and RA HSF (Fig. 5*C*).

Since murine models are often used to simulate human diseases, we found it prudent to investigate *5-LO* promoter methylation in mouse mast cells and fibroblasts. MC/9 cells are the C57BL/6×A/J mouse F1 fetal liver mast cells, while NIH3T3 cells are Swiss mouse embryonic fibroblasts. We also included C57BL/6 mouse tail samples in this study in order to generalize our observations. Genomic DNA was isolated from the cells and tissue samples and, using the same bisulfite sequencing technique as described in "Materials and Methods", we only obtained a 597 bp PCR product from the modified mouse genomic DNA (Fig. 6*A*). The sequence of this 597 bp product amplified from the modified mouse genomic DNA was exactly the same as that amplified from the modified human genomic DNA. Sequencing analysis revealed highly methylated CpG sites in these 597 bp PCR products from MC/9 cells, NIH3T3 cells and C57BL/6 tail samples (Fig. 6*B*). Despite these results, we detected high levels of 5-LO protein in MC/9 cells, but no 5-LO protein in NIH3T3 cells (Fig. 2*A*, top panel).

3.4.3. Histone methylation status of *5-LO* gene

As mentioned before, OA and RA HSF did not express 5-LO protein (Fig. 2*A*, bottom panel); whereas, the promoter region of *5-LO* gene showed almost complete DNA hypomethylation in these cells (Fig. 5*B* and 5*C*). Therefore, other epigenetic mechanism(s), such as histone methylation, may be involved in the suppression of *5-LO* gene expression in OA and RA HSF. Because lysine methylation (Lys-9 or -27) on histone H3 has been implicated in transcriptional silencing (Lee, Teyssier et al. 2005), we investigated the histone H3 methylation status on *5-LO* gene using ChIP assay as

described in “Materials and Methods”. Based on the positions of primers we designed for ChIP analysis, we detected histone H3 (Lys-9 and -27) methylation in two introns (one is the intron between exon 3 and exon 4; the other is the intron between exon 6 and exon 7) and/or exon 14 in RA HSF (Fig. 7*A*, *lanes 3-4 versus lanes 18-19*; and *lanes 7-10 versus lanes 16-19*). However, in the *5-LO* promoter region, there was intensive tri-methylation on histone H3 (Lys-27) (Fig. 7*A*, *lanes 5-6 versus lanes 20-21*), but almost no methylation on Lys-9 residue (Fig. 7*A*, *lanes 11-12 versus lanes 20-21*). We think histone methylation, especially the tri-methylation on histone H3 (Lys-27), may play a role in the inhibition of *5-LO* gene expression in RA HSF.

As mentioned previously, DNA methylation profiles (with *versus* without DEX treatment) could not explain the rescued 5-LO expression in HMC-1 cells in the presence of physiological concentrations of DEX (Fig. 8). In order to verify if histone methylation is responsible for this recovery of 5-LO protein expression, we performed the ChIP analysis as described in “Materials and Methods” to check the histone H3 methylation status of *5-LO* gene in HMC-1 cells with or without DEX treatment. We found that HMC-1 cells with DEX treatment had less histone H3 (Lys-27) tri-methylation in exon 14 and the two intron regions (Fig. 7*B*, *lanes 22-25 versus lanes 7-10*), but almost no difference for Lys-27 tri-methylation in the *5-LO* promoter (Fig. 7*B*, *lanes 26-27 versus lanes 11-12*). On the contrary, for the Lys-9 residue, we detected much less of histone H3 methylation in the *5-LO* distal promoter region in DEX-treated HMC-1 cells than the control (HMC-1 in the absence of DEX) (Fig. 7*B*, *lane 33 versus lane 6*); while there was almost no difference on histone H3 (Lys-9) methylation in the proximal promoter, exon 14 and the two introns between HMC-1 cells with and without DEX treatment (Fig. 7*B*, *lanes 28-32 versus lanes 1-5*). Therefore, we speculate this diminishment of histone H3

methylation (in exon 14 and the two introns for Lys-27 residue; in *5-LO* distal promoter region for Lys-9 residue) may be one reason for the increased 5-LO protein expression in HMC-1 cells treated with DEX.

3.5. DISCUSSION

During a lifetime, epigenetic modifications may happen more frequently than the genetic mutations. Many diseases, such as cancers, previously thought to be caused only by genetic mutations, are now viewed as epigenetic dysregulation.

LTB_4 is a very important lipid mediator in the pathogenesis of RA, and 5-LO is the key enzyme for LTB_4 biosynthesis. In this study, we investigated the DNA methylation profiles of the *5-LO* promoter in different cells. Using the bisulfite sequencing technique, we confirmed that the promoter of *5-LO* gene was heavily methylated in U937 cell line and was almost completely unmethylated in HL-60 cells. These methylation profiles were consistent with the findings reported previously in these cell lines (Uhl, Klan et al. 2002) and testified the reliability of our technique in the DNA methylation study. The highly methylated *5-LO* gene promoter in U937 cells and the unmethylated *5-LO* promoter in HL-60 cells are well correlated with the low levels of 5-LO protein and 5-LO enzymatic activity detected in U937 and the high 5-LO activity reported in HL-60 cells, respectively (Uhl, Klan et al. 2002). Therefore, DNA (promoter) methylation status appears to play a key role in controlling the target gene expression. Indeed, methylation of the *5-LO* promoter in a luciferase chimeric construct abolished promoter activity as measured by luciferase activity (Uhl, Klan et al. 2002).

Like neutrophils, mast cells produce large amounts of LTB_4 at the sites of inflammation. Rheumatoid synovium is also infiltrated with large quantity of mast cells,

which is the major source for LTB_4 synthesis in RA patients. However, an interesting study found that the 5-LO-positive HMC-1 cells gradually lost 5-LO protein expression and 5-LO enzymatic activity after continuously culturation in medium (Colamorea, Di Paola et al. 1999). Using the HMC-1 genomic DNA kindly provided by Dr. Macchia (University of Bari, Bari, Italy), we verified the methylation profiles of *5-LO* gene promoter in HMC-1 cells by bisulfite sequencing. The 5-LO-positive HMC-1 cells (071130 and 071207DEX) had much higher unmethylated *5-LO* promoter compared with the 5-LO-negative HMC-1 cells (071203 and 071203DEX). Based on these results, we believe that DNA methylation in the promoter region of *5-LO* gene is a plausible explanation for the loss of 5-LO protein expression in HMC-1 cells in long-term cultures (071203 and 071203DEX). This continuous culturation may be viewed as an aging process for HMC-1 cells, and DNA hypermethylation has been demonstrated to be a very important epigenetic mechanism involved in aging (Issa 2000; Fraga, Ballestar et al. 2005). Although the physiological concentration of DEX treatment increased the 5-LO protein expression and the biosynthesis of 5-LO derivatives in both of the 5-LO-positive and 5-LO-negative HMC-1 cell lines (071130 and 071203) (Colamorea, Di Paola et al. 1999), we did not detect a difference in the DNA methylation status of *5-LO* gene promoter in HMC-1 cells with or without DEX treatment. It is possible that DEX may affect other epigenetic mechanisms, such as histone methylation and/or acetylation, leading to the restoration of 5-LO enzymatic activity in the 5-LO-negative HMC-1 cells. Indeed, our ChIP assay confirmed the involvement of decreased histone methylation in the DEX-treated HMC-1 cells. However, it is still possible that histone acetylation may also contribute to this process, and future experiments (ChIP assay or Western blotting analysis) are required to verify this hypothesis.

Many studies focus on DNA methylation as the major epigenetic mechanism that controls the target gene expression. But in some circumstances, histone modifications may override DNA methylation as the key regulator for gene expression. For instance, histone acetylation plays a critical role in the development of asthma, which is a chronic inflammatory disease with higher expression levels of multiple inflammatory genes. In the bronchial biopsies from patients with asthma, there were increased HAT activity and reduced HDAC enzymatic activity compared with normal subjects (Ito, Caramori et al. 2002). Since histone acetylation by HAT is associated with increased gene transcription, this observation explains the higher expression levels of inflammatory genes in asthma patients. COPD is another chronic inflammatory disease that is closely associated with histone modification. In both the macrophages and biopsy specimens from COPD patients, there was marked reduction in HDAC enzymatic activity compared with normal individuals. Since hypoacetylation induced by HDACs is associated with suppression of production of pro-inflammatory cytokines, this decreased HDAC activity in COPD patients is well correlated with the severity of disease (Ito, Ito et al. 2005).

Previous studies also reported reduced HDAC activity in the synovial tissues from RA patients compared with OA and normal subjects. The resulting histone hyperacetylation increased the transcription of genes encoding inflammatory proteins, such as tumor necrosis factor-α (TNF-α), IL-8, and matrix metalloproteinase-9 (MMP-9) in RA patients (Huber, Brock et al. 2007). Therefore, this decreased HDAC activity may play very important role in the pathogenesis of RA. The fibroblast is the major cell type inside the inflamed synovium although only a very small amount of LTB_4 was detected from the HSF in some RA patients (Xu, Lu et al. 2010). Using the same technique of bisulfite sequencing, we found that the promoter of *5-LO* gene was completely

unmethylated in HSF from both OA and RA patients. However, we did not detect any 5-LO protein in these cells. Our ChIP assay verified histone H3 (Lys-9 and Lys-27) methylation of *5-LO* gene in RA HSF, especially with the intensive Lys-27 tri-methylation in the promoter region. Since histone H3 (Lys-9 and Lys-27) methylation is associated with gene silencing (Lee, Teyssier et al. 2005), we think that histone methylation may override DNA methylation as a key regulator for *5-LO* gene expression in RA and OA HSF. In some RA patients, the alterations of histone modifications (*e.g.* histone methylation, histone acetylation) by medications or environmental factors may result in *5-LO* gene expression and the release of low level of LTB_4 from HSF. This small amount of LTB_4 may further reinforce the positive feedback loop with COX-2 and PGE_2 (Faour, He et al. 2001), contributing to the disease resolution or flare up in these RA patients.

Studies show that nucleosomes are much more abundant in exons than in introns, and preferentially positioned at intron-exon and/or exon-intron junctions (Chodavarapu, Feng et al. 2010). In our ChIP assay, we detected histone methylation in some exon and intron regions, which is consistent with several other studies revealing that different histone modifications, especially histone methylation, are enriched in exons and some introns of the target genes (Andersson, Enroth et al. 2009; Schwartz, Meshorer et al. 2009). Therefore, exons, which are characterized by their coding capacity, and introns, are also crucial in nucleosome organization. Indeed, more and more studies reported that some introns contain enhancer elements or alternative promoters, and appear to be responsible for increased gene expression in plants and many other eukaryotes (Landry, Mager et al. 2003; Rose 2008; Coulon, Chebli et al. 2010).

Previous studies reported that 5-LO mRNA was detected in synovial tissues and 5-

LO was expressed in the synovial lining layer of RA or OA patients (Hashimoto, Endo et al. 2003). LIT (LPS, ionomycin and thapsogargin) treatment strongly promoted LTB_4 synthesis in the culture medium of HSF from RA patients (Xu, Lu et al. 2010). But we did not detect any 5-LO protein using Western blotting analysis with or without stimulation. This discrepancy may be due to the different stimuli (PGE_2, A23187 and DEX) we used to treat the HSF in our study, or due to the different responses in HSF from different patients. Since all of the patients were treated differently before these studies, we should consider the influence of medications on the responses of HSF from different patients. In order to discern the connection between treatment and 5-LO expression in patients, much larger numbers of samples need to be included in these studies.

Another interesting finding in this study is that we can amplify the same fragment of *5-LO* promoter (597 bp) with the same bisulfite modified sequence from mouse genomic DNA (obtained from MC/9 cells, NIH3T3 cells and C57BL/6 mouse tails) as from human. However, the sequence of *5-LO* gene promoter in mouse genome (GenBank™ accession number AF393814) is completely different from that in human genome (GenBank™ accession number M38191). We speculate that this 597 bp fragment may be amplified from a distal part of *5-LO* gene promoter that has not been discovered yet in the mouse genome and requires further study to investigate. If this 597 bp fragment does belong to the *5-LO* gene promoter in the mouse genome, the highly methylated status of this fragment is well correlated with no expression of 5-LO protein in NIH3T3 cells, but not with MC/9 cells that expressed high level of 5-LO protein. This discrepancy in MC/9 cells may be due to the other epigenetic mechanisms that are also involved in the regulation of *5-LO* gene expression in the mouse genome. Or, maybe this

part of promoter (597 bp) is not that critical for gene expression compared with other part of *5-LO* gene promoter in the mouse genome. Therefore, the methylation status of this part of promoter (597 bp) will not determine whether the *5-LO* gene should be on or off. However, since the sequence of this 597 bp fragment is the bisulfite modified sequence, we will have to first demethylate with 5-aza-2'-deoxycytidine (AZA) to trace the original genomic sequence and the exact position of this fragment in the mouse genome before the bisulfite treatment.

The challenge of future research will be to decipher the precise epigenetic mechanisms that induce alterations in gene expression and diseases. It is also important to identify the key environmental factors that drive the susceptibility to disease. Unlike genetic mutations, epigenetic changes are readily reversible, raising the possibility of "epigenetic therapy". DNMT inhibitors can inhibit DNA methylation by DNMTs, providing many novel opportunities for cancer therapy (Brueckner and Lyko 2004). For example, AZA (decitabine), a promising chemopreventive agent, has been widely used in animal models and clinical trials in patients with cancers (Schwartsmann, Schunemann et al. 2000; Issa, Garcia-Manero et al. 2004; Gollob, Sciambi et al. 2006). Histone acetylation is also reversible by HDAC inhibitors, which can be divided into several classes — hydroxymates [trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA; vorinostat)], cyclic peptides (depsipeptide), aliphatic acids (valproic acid, butyrate) and benzamides (Acharya, Sparreboom et al. 2005). No single HDAC inhibitor is effective against all HDACs. Currently, many HDAC inhibitors have entered clinical trials for the treatment of solid or hematological malignancies. HDAC inhibitors can induce cell cycle arrest, differentiation or apoptosis in a variety of cancer cells and emerge as the very potential anticancer agents (Secrist, Zhou et al. 2003; Mei, Ho et al. 2004). The

combined pharmacological inhibition of DNA methylation and histone deacetylation usually has a synergistic effect in gene regulation and cancer therapy (Zhu and Otterson 2003; Iliopoulos, Malizos et al. 2007).

However, the issue of target specificity in epigenetic therapy is still not resolved. Some genes that require DNA methylation or histone deacetylation for silencing could also be activated by these inhibitors. For example, DNMT inhibitors that reactivate the silenced tumor suppressor genes to treat cancers may also activate the oncogenes, owing to their lack of specificity. The same limitation exists with the HDAC inhibitors, causing increased acetylation of both histone and non-histone proteins (Vogiatzi, Aimola et al. 2007; Humeniuk, Mishra et al. 2009).

3.6. CONCLUSION

Taken together, this study emphasized epigenetic regulation as a key biological process linking genetic and environmental factors in human diseases. Several international projects and organizations have also developed the interest in this field, such as the Human Epigenome Project, the Epigenome Network of Excellence and the Epigenome Society. Increasing amounts of experimental data strongly demonstrate that epigenetics play an important role in the development of not only cancer but also rheumatic diseases. And DNA methylation and histone acetylation/deacetylation have been the most thoroughly studied mechanisms. In our study, we have focused on *5-LO* gene in RA and OA patients. DNA methylation and histone modifications may influence the expression of genes involved in inflammation and/or tissue destruction, leading to disease resolution or flare up. An important characteristic of epigenetics is that, while they are stable and heritable, they are also reversible through the application of DNMTs

or HDACs inhibitors. Therefore, current and future studies will introduce new insights into the complex pathogenesis of RA and provide valuable molecular targets for the development of epigenetic drugs to treat cancers and inflammatory diseases.

Figure 1: Schematic representation of CpG island in the promoter region of *5-LO* gene. The vertical lines stand for CpG dinucleotides. The blue and red arrows show the positions of primers used to amplify PCR products. The 556 bp PCR product that corresponds to the distal promoter of *5-LO* gene contains 44 CpG dinucleotides. The 597 bp PCR product that corresponds to the proximal promoter of *5-LO* gene contains 47 CpG dinucleotides.

Fig. 1:

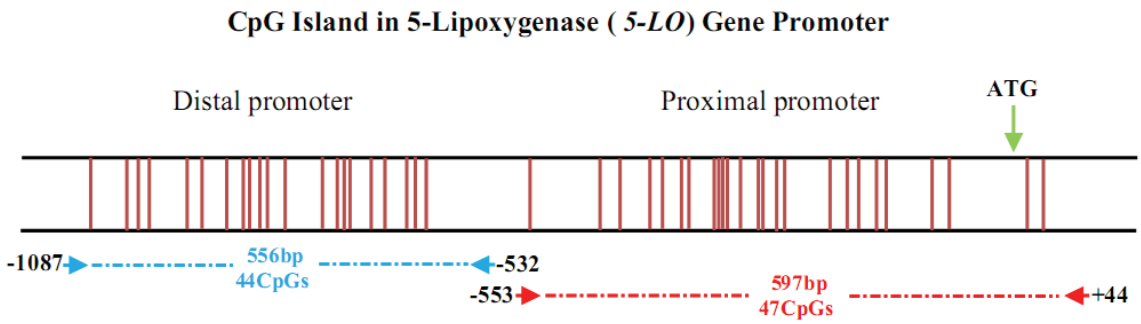


Figure 2: Western blot analysis of *5-LO* and *FLAP* gene expression. Quiescent OA and RA HSF, MC/9 cells, and NIH3T3 cells (1×10^6 cells per well in 6-well cluster plates) were stimulated with 100 nM PMA, 5 μM A23187, 1 μM DEX or 1 μM PGE_2 for 24 h in DMEM supplemented with 1% FBS at 37 °C in a humidified atmosphere of 5% CO_2, 95% air. Whole cell extracts from control and stimulated cells were collected in hot SDS-PAGE loading buffer. Fifty-70 μg of cellular protein from each conditions was subjected to Western blotting analysis for 5-LO protein (*A*) and FLAP protein (*B*).

Fig. 2A:

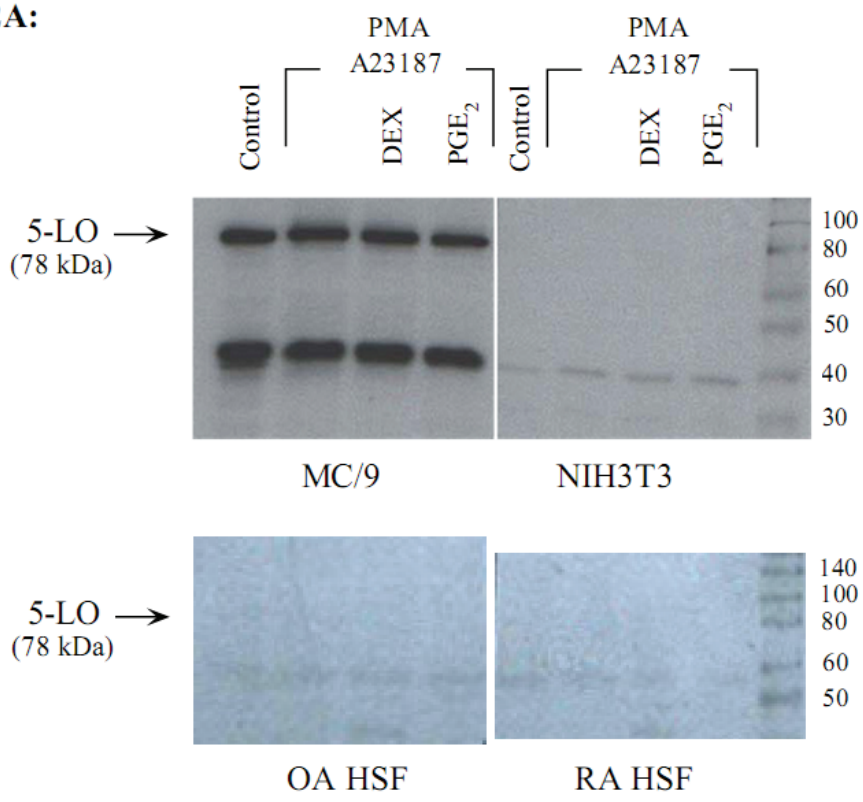

PMA
A23187
Control
DEX
PGE2
PMA
A23187
Control
DEX
PGE2
5-LO
(78 kDa)
100
80
60
50
40
30
MC/9
NIH3T3
5-LO
(78 kDa)
140
100
80
60
50
OA HSF
RA HSF

Fig. 2B:

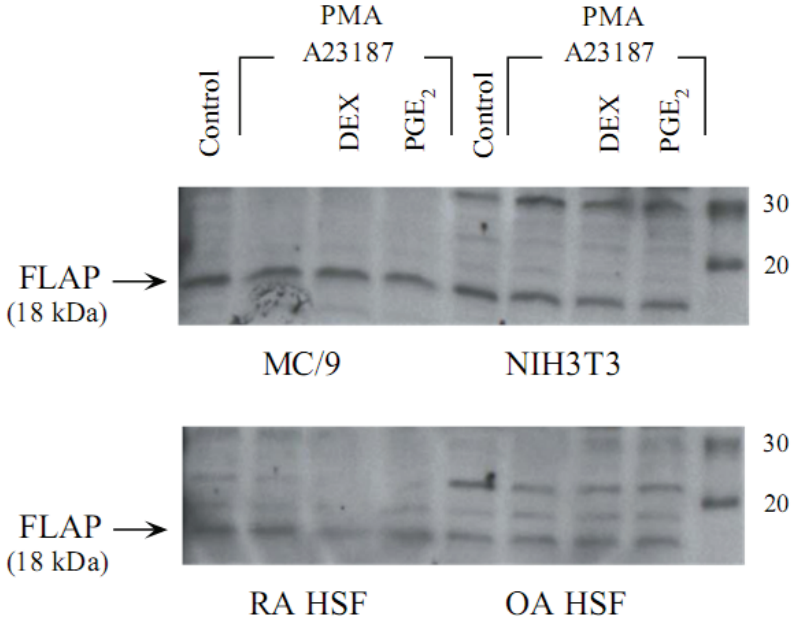

PMA
A23187
Control
DEX
PGE2
PMA
A23187
Control
DEX
PGE2
FLAP
(18 kDa)
30
20
MC/9
NIH3T3
FLAP
(18 kDa)
30
20
RA HSF
OA HSF

Figure 3: Analysis of the methylation profiles of *5-LO* gene promoter using bisulfite sequencing in HL-60 and U937 cell lines. *A*, genomic DNA isolated from HL-60 and U937 cells was subjected to bisulfite modification using the EpiTect Bisulfite Kit. The modified DNA was amplified by nested PCR using the specific primers as described in "Materials and Methods". The resulting PCR products (556 bp and 597 bp) were shown here. *B*, the PCR products were cloned into pCR II TOPO vector using TOPO TA Cloning Kit for sequencing analysis. Gene sequencing analysis revealed the methylation status of *5-LO* gene promoter in HL-60 and U937 cells. The orange circles stand for the methylated CpG dinucleotides and the white circles stand for the unmethylated CpG dinucleotides in the promoter region of *5-LO* gene. The green circles stand for the CpG dinucleotides that could not be distinguished between methylation and unmethylation, due to the specific sequence (*e.g.* too many repeated CpGs) in this area of genomic DNA that interfered severely with the gene sequencing program.

Fig. 3A:

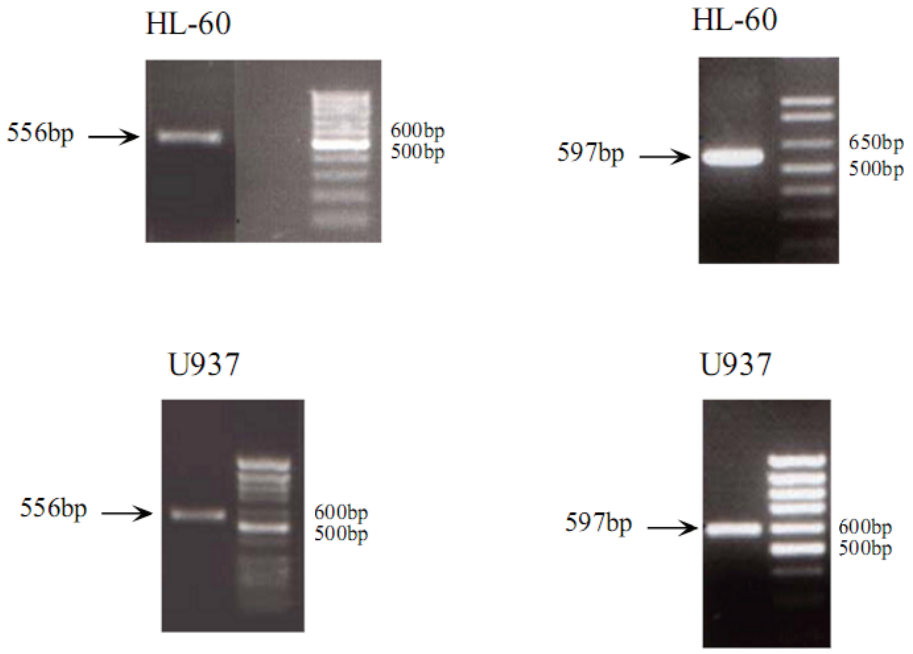

HL-60
556bp
600bp
500bp
HL-60
597bp
650bp
500bp
U937
556bp
600bp
500bp
U937
597bp
600bp
500bp

Fig. 3B:

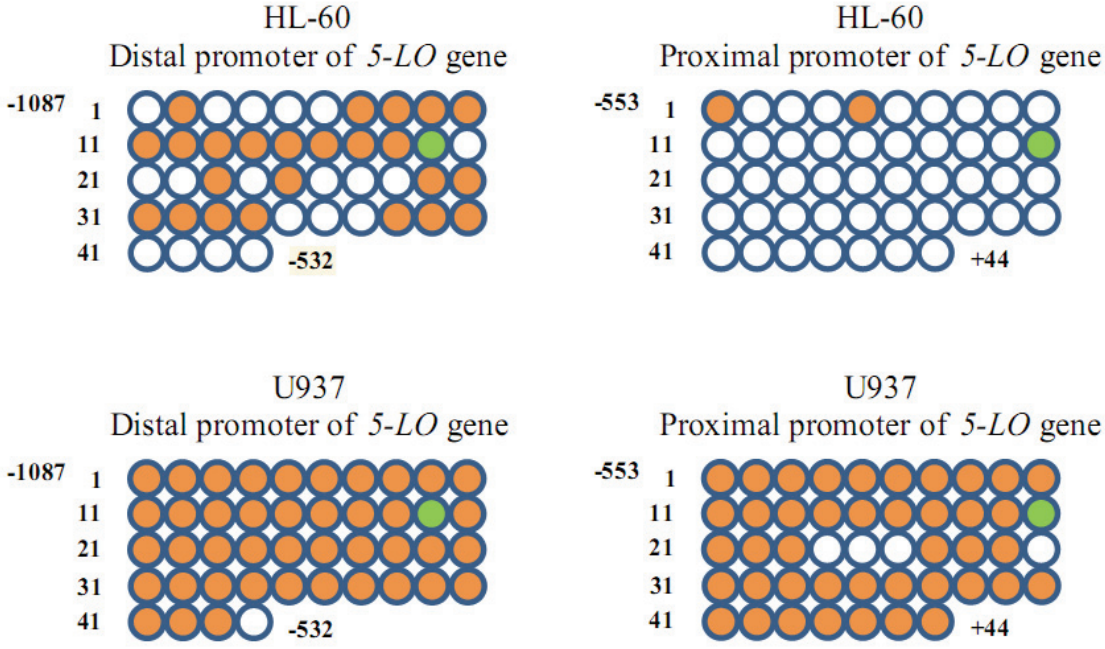

HL-60
Distal promoter of 5-LO gene
-1087
1
11
21
31
41
-532
HL-60
Proximal promoter of 5-LO gene
-553
1
11
21
31
41
+44
U937
Distal promoter of 5-LO gene
-1087
1
11
21
31
41
-532
U937
Proximal promoter of 5-LO gene
-553
1
11
21
31
41
+44

Figure 4: DNA methylation analysis of *5-LO* gene promoter in HMC-1 cells. HMC-1071130 is the subline established in 2007 from upstream frozen cells. HMC-1071130 cells expressed high levels of 5-LO protein. HMC-1071207DEX refers to the same subline (HMC-1071130) treated with DEX. HMC-1071207DEX cells expressed even higher levels of 5-LO protein than HMC-1071130 cells. HMC-1071203 refers to another subline established in 2005 and have been continuously cultured in medium since then (now is 112th passage). The expression of 5-LO protein in HMC-1071203 cells was almost abolished. HMC-1071203DEX refers to the same subline (HMC-1071203) that was treated with DEX. The expression of 5-LO protein in HMC-1071203DEX cells was partially restored after DEX treatment (Colamorea, Di Paola et al. 1999). *A*, genomic DNA isolated from these four types of HMC-1 cells was subjected to bisulfite modification. Using the modified genomic DNA, PCR products (556 bp and 597 bp) were amplified from the *5-LO* gene promoter in these four HMC-1 cells as described in "Materials and Methods". These PCR products were cloned into pCR II TOPO vectors, and gene sequencing analysis revealed that the distal promoter of *5-LO* gene was highly methylated in all of the four HMC-1 cells (*B-E*, *left* panels). For the proximal promoter of *5-LO* gene, HMC-1071130 (*B*, *right* panel) and HMC-1071207DEX (*C*, *right* panel) were almost completely unmethylated; whereas, HMC-1071203 (*D*, *right* panel) and HMC-1071203DEX (*E*, *right* panel) were highly methylated.

Fig. 4A:

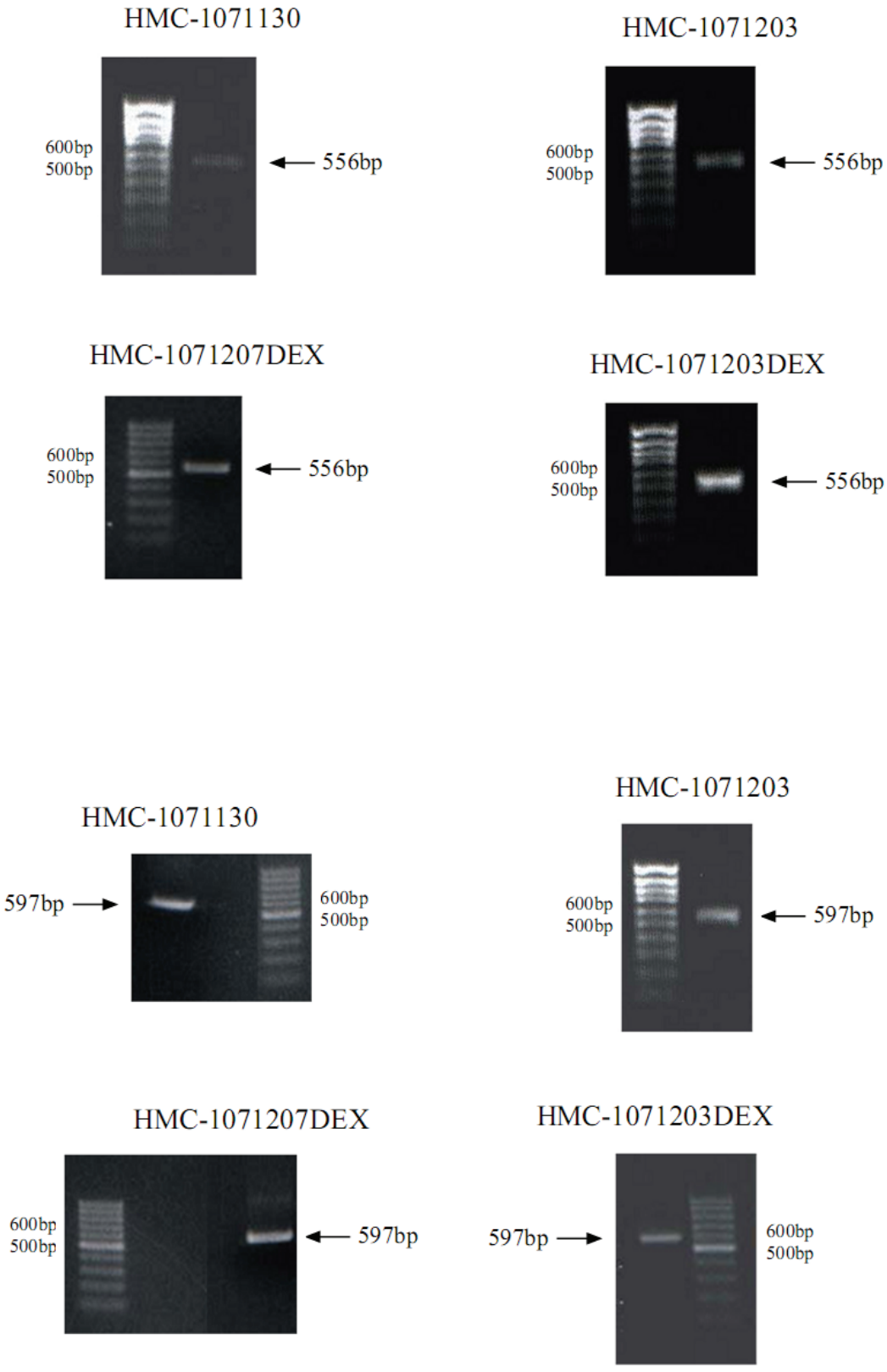

HMC-1071130
600bp
500bp
556bp
HMC-1071203
600bp
500bp
556bp
HMC-1071207DEX
600bp
500bp
556bp
HMC-1071203DEX
600bp
500bp
556bp
HMC-1071130
597bp
600bp
500bp
HMC-1071203
600bp
500bp
597bp
HMC-1071207DEX
600bp
500bp
597bp
HMC-1071203DEX
597bp
600bp
500bp

Fig. 4B:

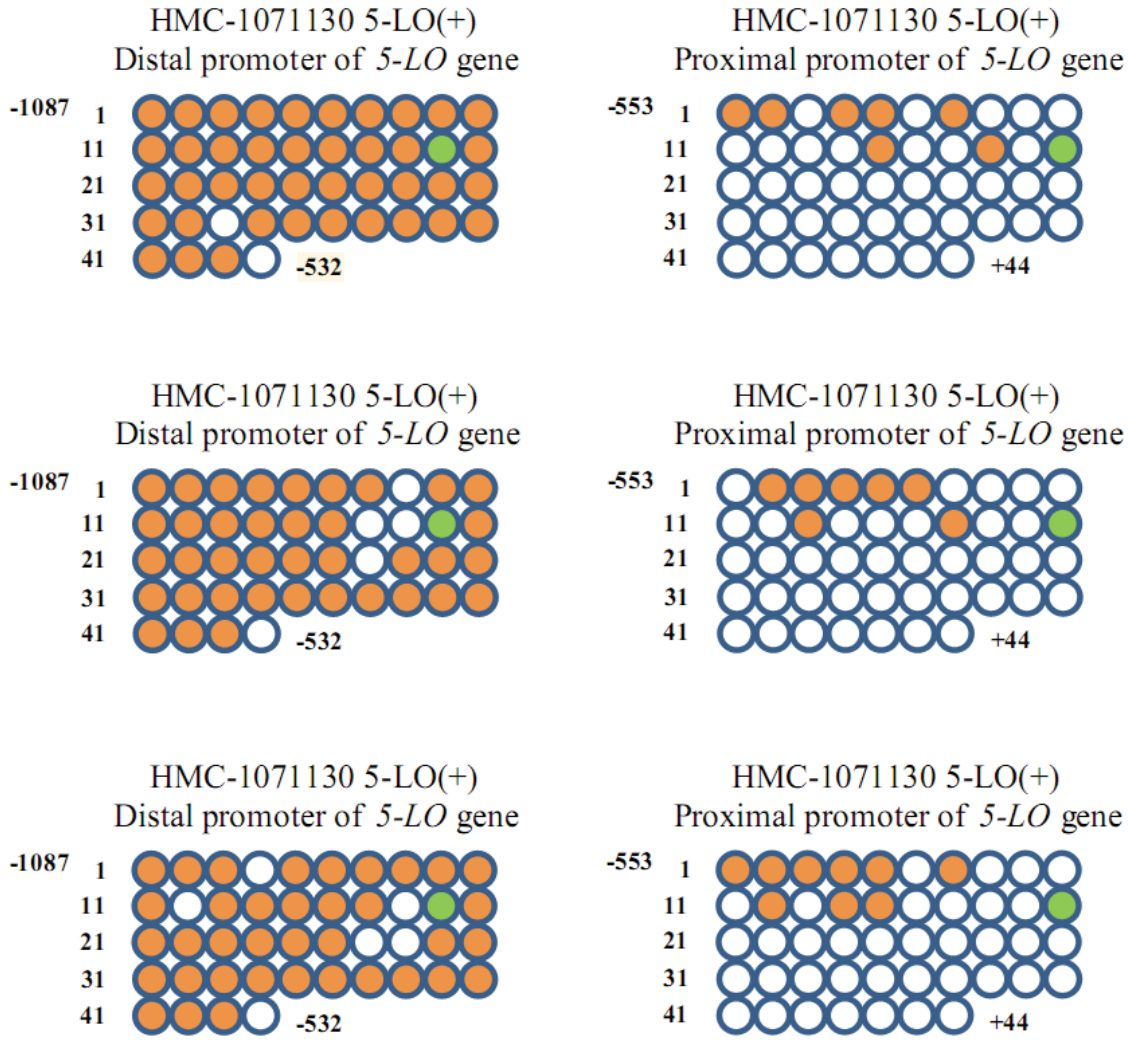

HMC-1071130 5-LO(+)
Distal promoter of 5-LO gene
-1087
1
11
21
31
41
-532
HMC-1071130 5-LO(+)
Proximal promoter of 5-LO gene
-553
1
11
21
31
41
+44
HMC-1071130 5-LO(+)
Distal promoter of 5-LO gene
-1087
1
11
21
31
41
-532
HMC-1071130 5-LO(+)
Proximal promoter of 5-LO gene
-553
1
11
21
31
41
+44
HMC-1071130 5-LO(+)
Distal promoter of 5-LO gene
-1087
1
11
21
31
41
-532
HMC-1071130 5-LO(+)
Proximal promoter of 5-LO gene
-553
1
11
21
31
41
+44

Fig. 4C:

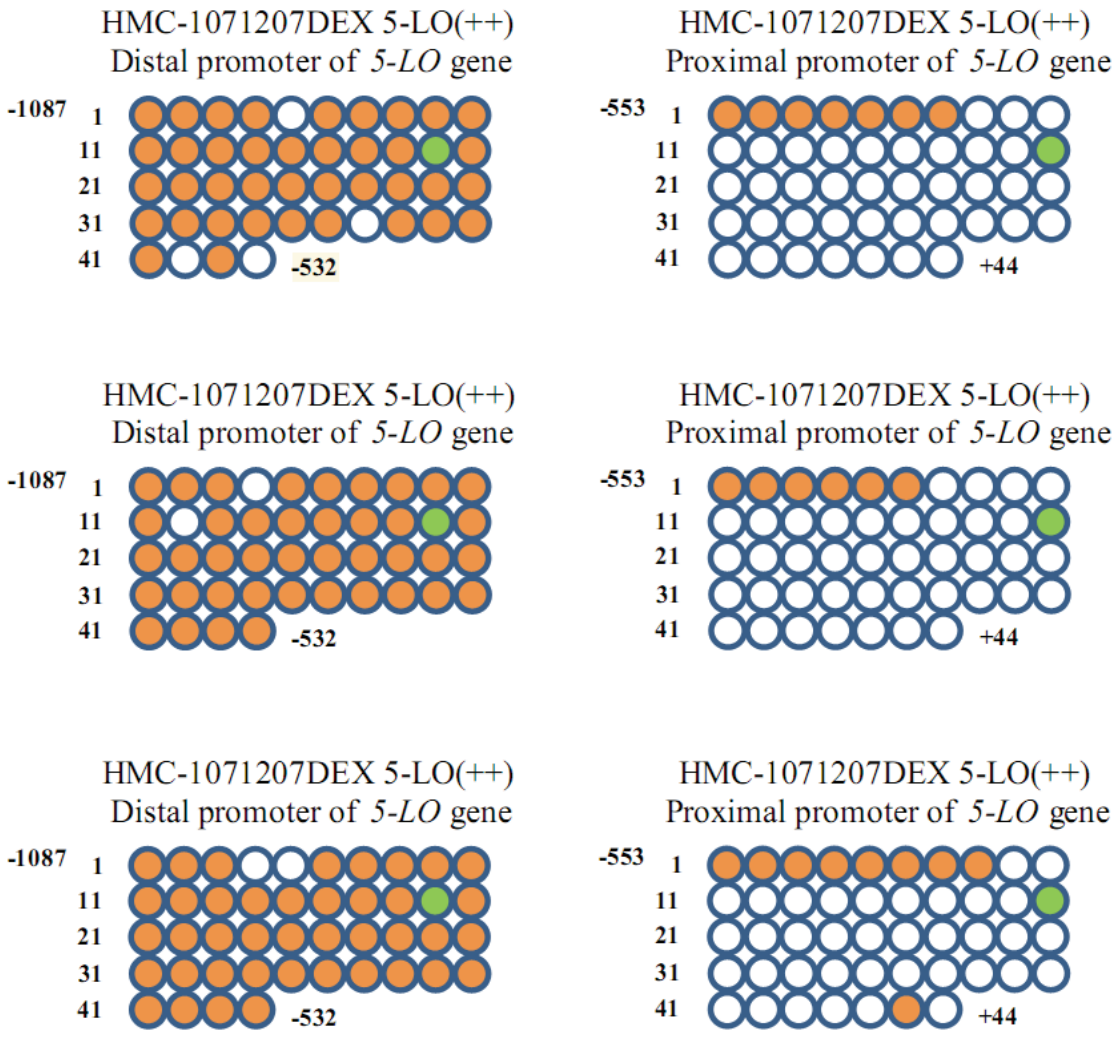

HMC-1071207DEX 5-LO(++)
Distal promoter of 5-LO gene
-1087
1
11
21
31
41
-532
HMC-1071207DEX 5-LO(++)
Proximal promoter of 5-LO gene
-553
1
11
21
31
41
+44
HMC-1071207DEX 5-LO(++)
Distal promoter of 5-LO gene
-1087
1
11
21
31
41
-532
HMC-1071207DEX 5-LO(++)
Proximal promoter of 5-LO gene
-553
1
11
21
31
41
+44
HMC-1071207DEX 5-LO(++)
Distal promoter of 5-LO gene
-1087
1
11
21
31
41
-532
HMC-1071207DEX 5-LO(++)
Proximal promoter of 5-LO gene
-553
1
11
21
31
41
+44

Fig. 4D:

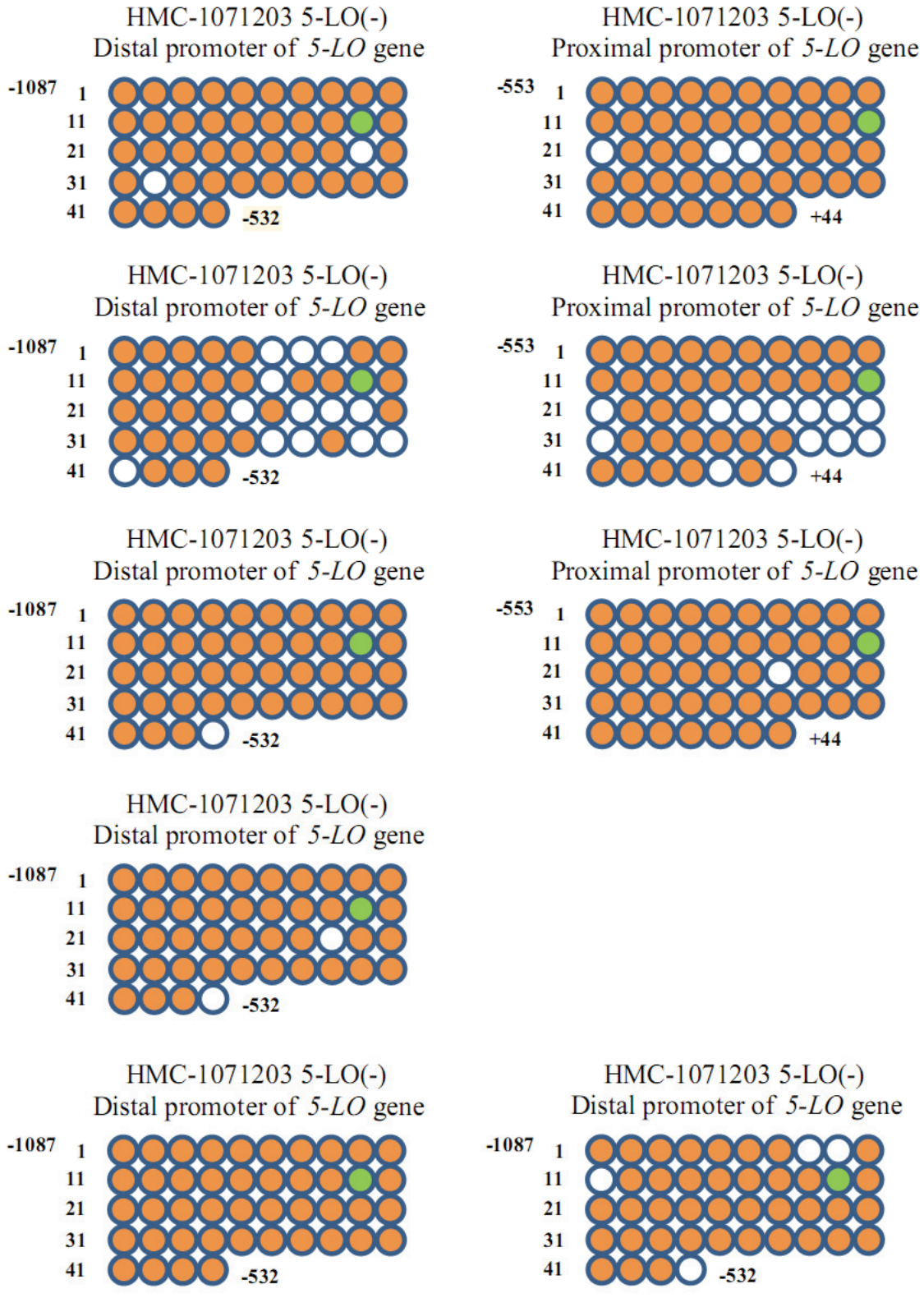

HMC-1071203 5-LO(-)
Distal promoter of 5-LO gene
-1087
1
11
21
31
41
-532
HMC-1071203 5-LO(-)
Proximal promoter of 5-LO gene
-553
1
11
21
31
41
+44
HMC-1071203 5-LO(-)
Distal promoter of 5-LO gene
-1087
1
11
21
31
41
-532
HMC-1071203 5-LO(-)
Proximal promoter of 5-LO gene
-553
1
11
21
31
41
+44
HMC-1071203 5-LO(-)
Distal promoter of 5-LO gene
-1087
1
11
21
31
41
-532
HMC-1071203 5-LO(-)
Proximal promoter of 5-LO gene
-553
1
11
21
31
41
+44
HMC-1071203 5-LO(-)
Distal promoter of 5-LO gene
-1087
1
11
21
31
41
-532
HMC-1071203 5-LO(-)
Distal promoter of 5-LO gene
-1087
1
11
21
31
41
-532
HMC-1071203 5-LO(-)
Distal promoter of 5-LO gene
-1087
1
11
21
31
41
-532

Fig. 4E:

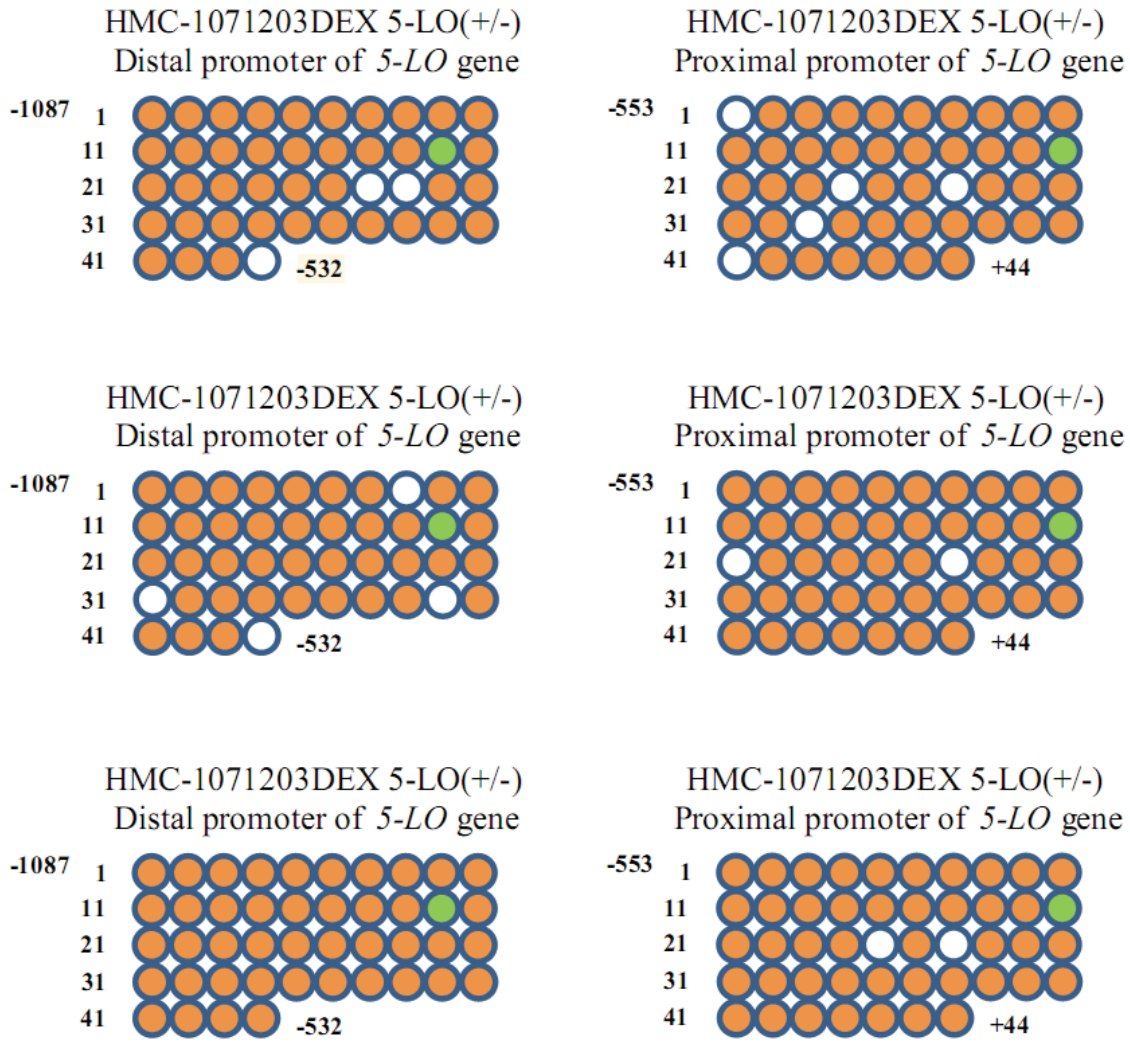

HMC-1071203DEX 5-LO(+/-)
Distal promoter of 5-LO gene
-1087
1
11
21
31
41
-532
HMC-1071203DEX 5-LO(+/-)
Proximal promoter of 5-LO gene
-553
1
11
21
31
41
+44
HMC-1071203DEX 5-LO(+/-)
Distal promoter of 5-LO gene
-1087
1
11
21
31
41
-532
HMC-1071203DEX 5-LO(+/-)
Proximal promoter of 5-LO gene
-553
1
11
21
31
41
+44
HMC-1071203DEX 5-LO(+/-)
Distal promoter of 5-LO gene
-1087
1
11
21
31
41
-532
HMC-1071203DEX 5-LO(+/-)
Proximal promoter of 5-LO gene
-553
1
11
21
31
41
+44

Figure 5: Methylation profiles of *5-LO* gene promoter in OA and RA HSF. *A*, genomic DNA isolated from OA and RA HSF was subjected to bisulfite modification and amplified by nested PCR using the specific primers as described in "Materials and Methods". The final 556 bp and 597 bp PCR products were shown here. These PCR products were cloned into pCR II TOPO vector using TOPO TA Cloning Kit following the manufacturer's instruction. Gene sequencing analysis verified that the promoter region of *5-LO* gene was completely unmethylated in both OA HSF (*B*) and RA HSF (*C*).

Fig. 5A:

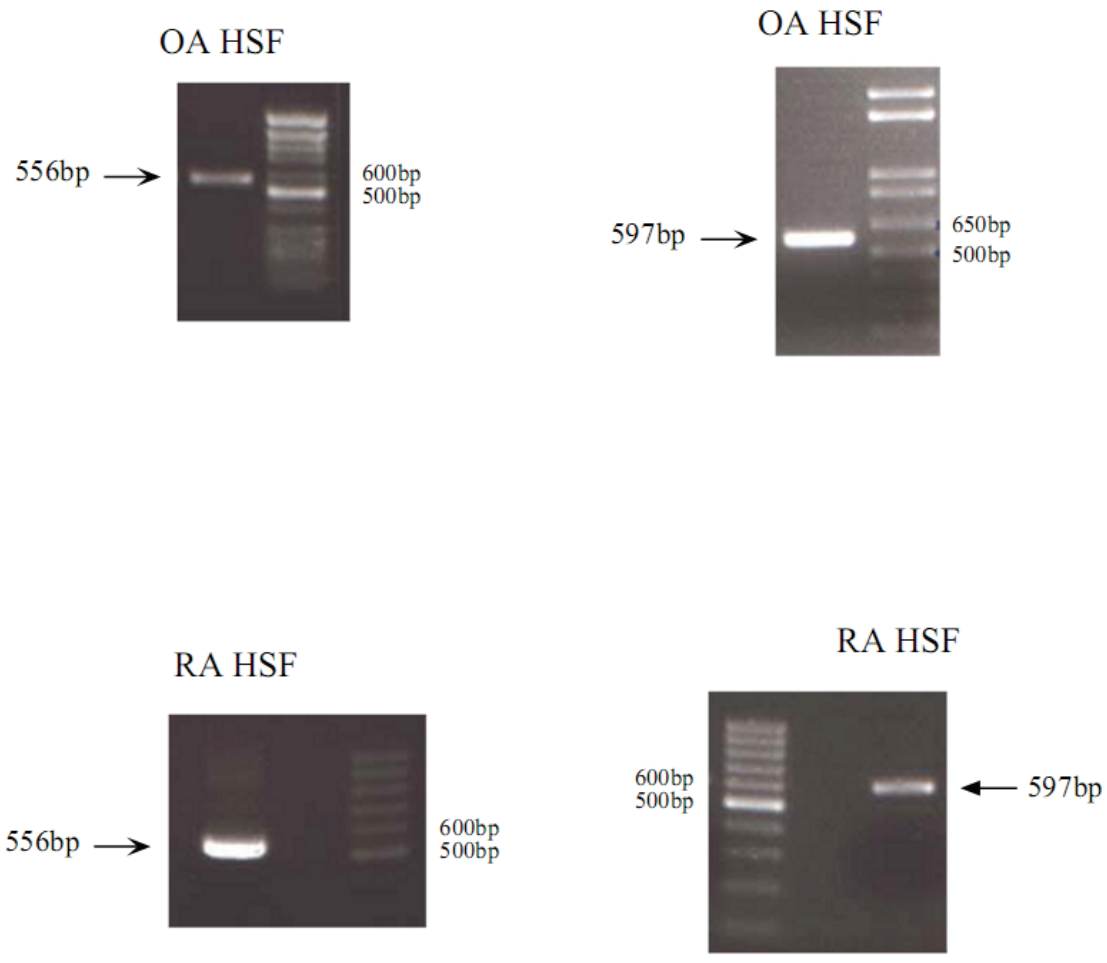


Fig. 5B:

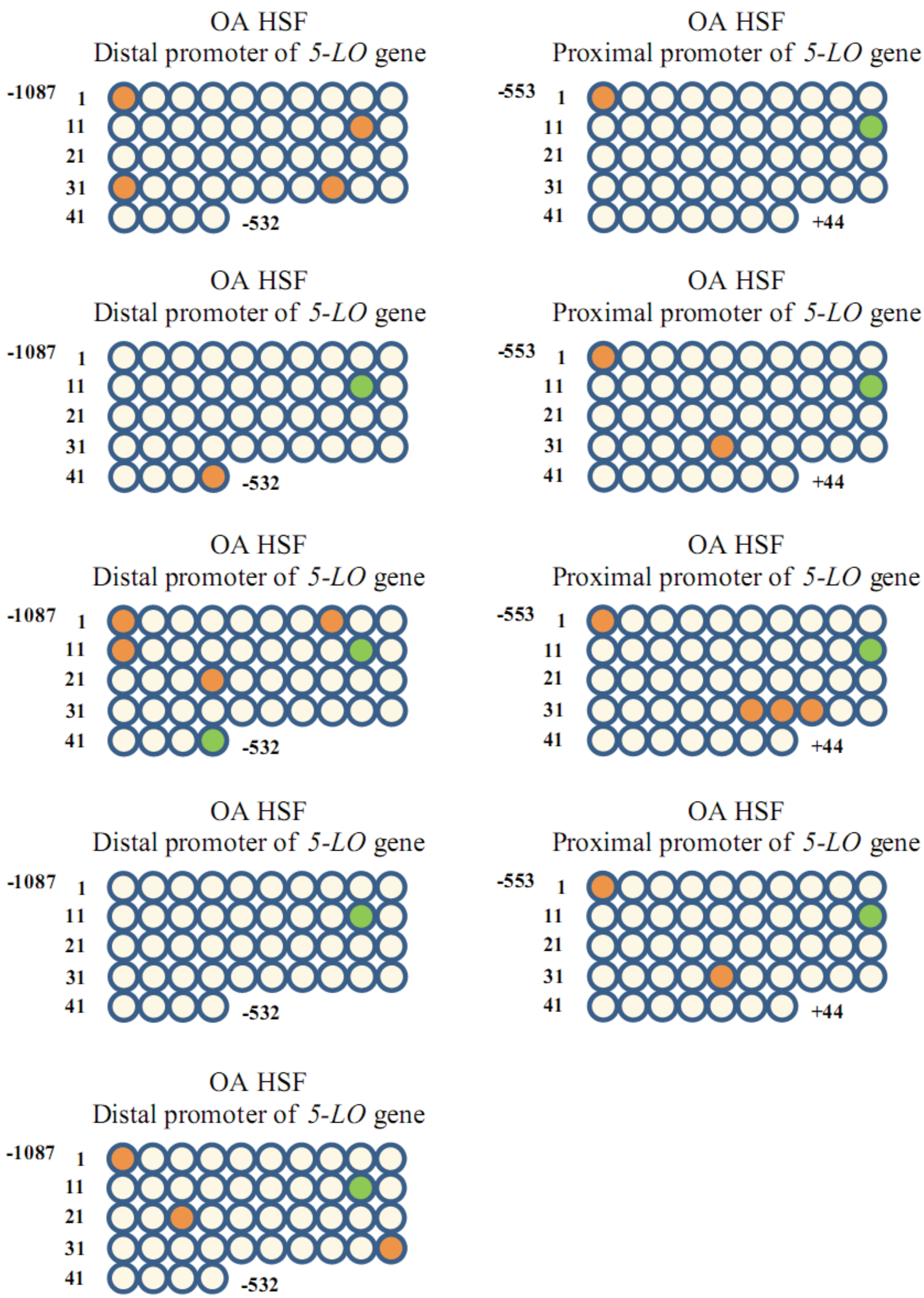

OA HSF
Distal promoter of 5-LO gene
-1087
1
11
21
31
41
-532
OA HSF
Proximal promoter of 5-LO gene
-553
1
11
21
31
41
+44

Fig. 5C:

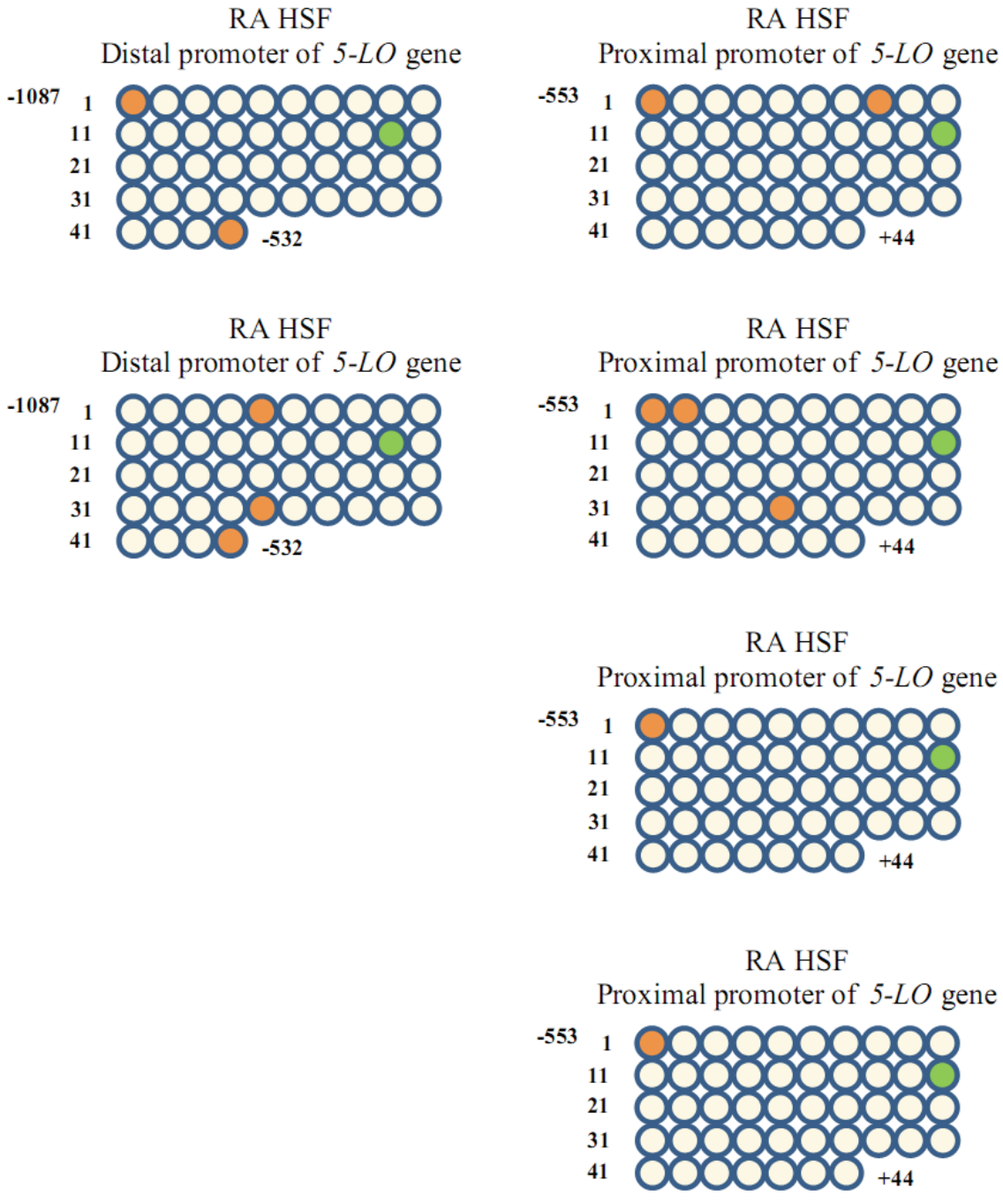

RA HSF
Distal promoter of 5-LO gene
-1087
1
11
21
31
41
-532
RA HSF
Proximal promoter of 5-LO gene
-553
1
11
21
31
41
+44
RA HSF
Distal promoter of 5-LO gene
-1087
1
11
21
31
41
-532
RA HSF
Proximal promoter of 5-LO gene
-553
1
11
21
31
41
+44
RA HSF
Proximal promoter of 5-LO gene
-553
1
11
21
31
41
+44
RA HSF
Proximal promoter of 5-LO gene
-553
1
11
21
31
41
+44

Figure 6: Methylation analysis of *5-LO* gene promoter in MC/9 cells, NIH3T3 cells and C57BL/6 mouse tail tissues. *A*, genomic DNA isolated from MC/9 cells, NIH3T3 cells and C57BL/6 mouse tail tissues was subjected to bisulfite modification. Using the modified mouse genomic DNA, only the 597 bp fragment was amplified by nested PCR with the specific primers as described in "Materials and Methods". *B*, the 597 bp PCR product was cloned into pCR II TOPO vector using TOPO TA Cloning Kit following the manufacturer's instruction. Gene sequencing analysis showed significant methylation in the 597 bp PCR products amplified from MC/9 cells, NIH3T3 cells and C57BL/6 mouse tail samples.

Fig. 6A:

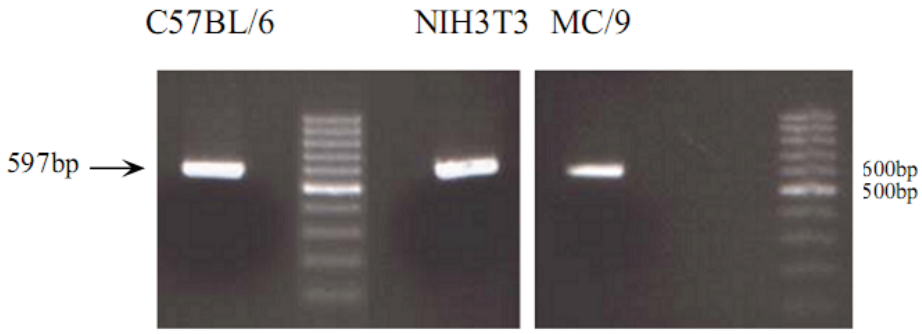

C57BL/6
NIH3T3
MC/9
597bp
500bp
500bp

Fig. 6B:

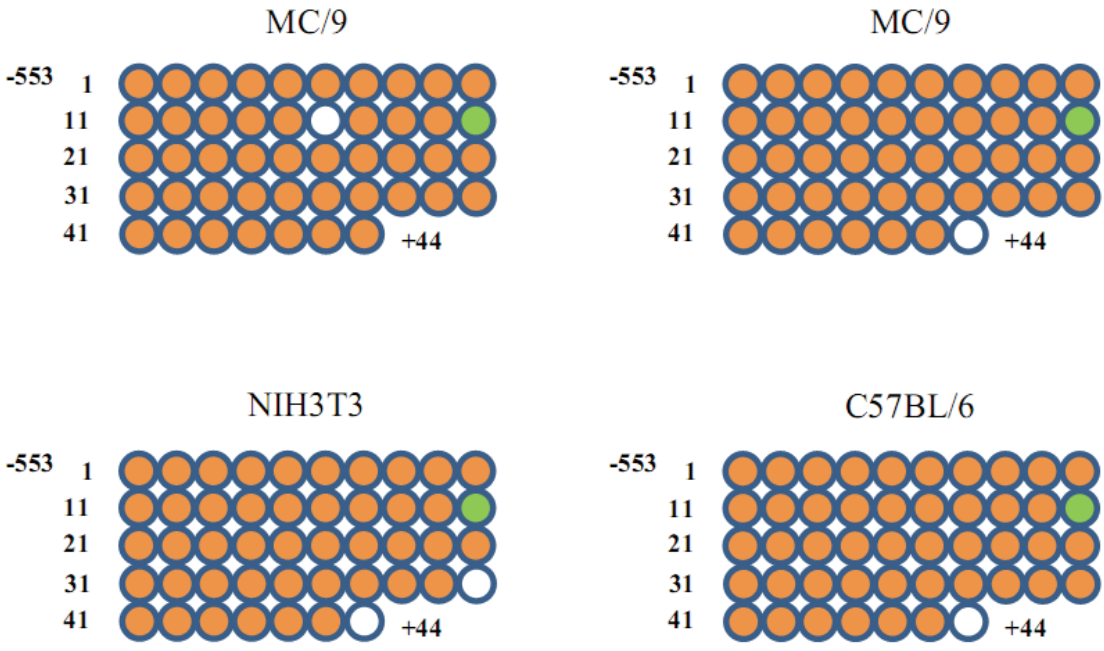

MC/9
-553
1
11
21
31
41
+44
MC/9
-553
1
11
21
31
41
+44
NIH3T3
-553
1
11
21
31
41
+44
C57BL/6
-553
1
11
21
31
41
+44

Figure 7: Histone H3 methylation analysis of *5-LO* gene in RA HSF and HMC-1 cells. ChIP assays were performed using micrococcal nuclease-digested chromatin from RA HSF or HMC-1 cells (with or without 1 μM DEX treatment for 25 days) and either tri-methyl-histone H3 (Lys-27) antibody (*A*, *lanes 1-6*; *B*, *lanes 7-12 and 22-27*), pan-methyl-histone H3 (Lys-9) antibody (*A*, *lanes 7-12*; *B*, *lanes 1-6 and 28-33*), histone H3 antibody (*A*, *lane 15*; *B*, *lanes 21 and 42*) or normal rabbit IgG (*A*, *lanes 14 and 16-21*; *B*, *lanes 20, 13-18, 34-39 and 41*) as described in "Materials and Methods". Purified DNA was analyzed by standard PCR methods using human RPL30 primers or six 5-LO primer sets as described in "Materials and Methods". PCR products were observed for each primer set in various ChIP samples with the normal rabbit IgG resulting in less or no RPL30 gene enrichment compared to histone H3 ChIP samples. The ratios of the densitometric analysis of PCR products in (*A*) RA HSF and in (*B*) HMC-1 cells (control *versus* DEX treatment) were shown as indicated.

Fig. 7A: **RA HSF**

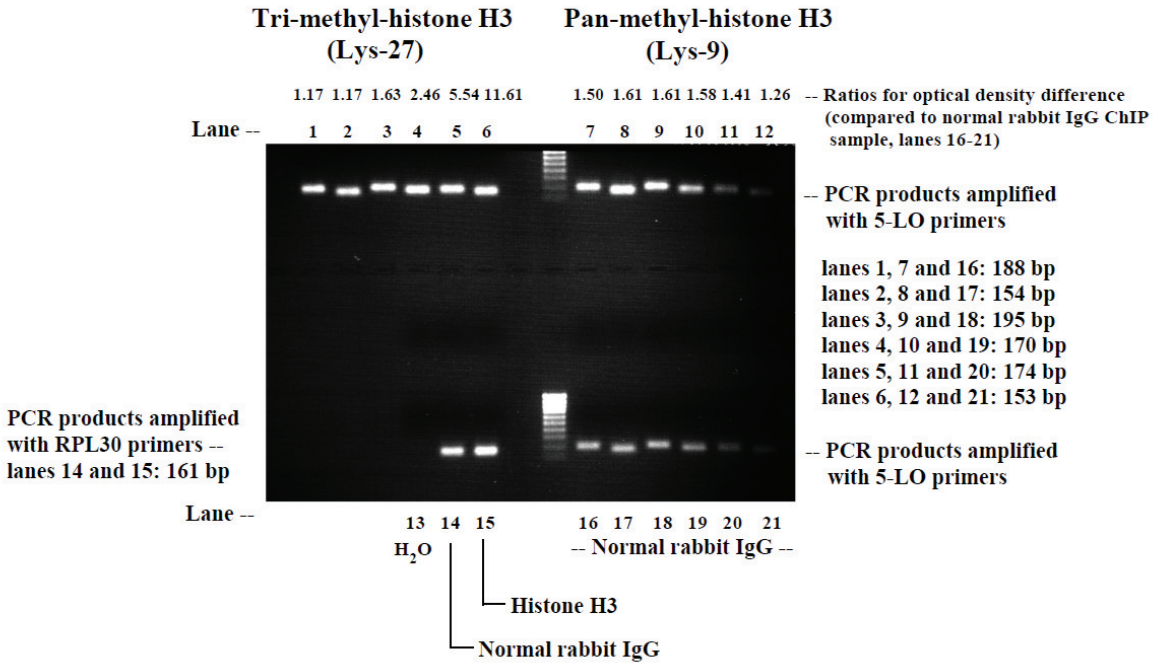
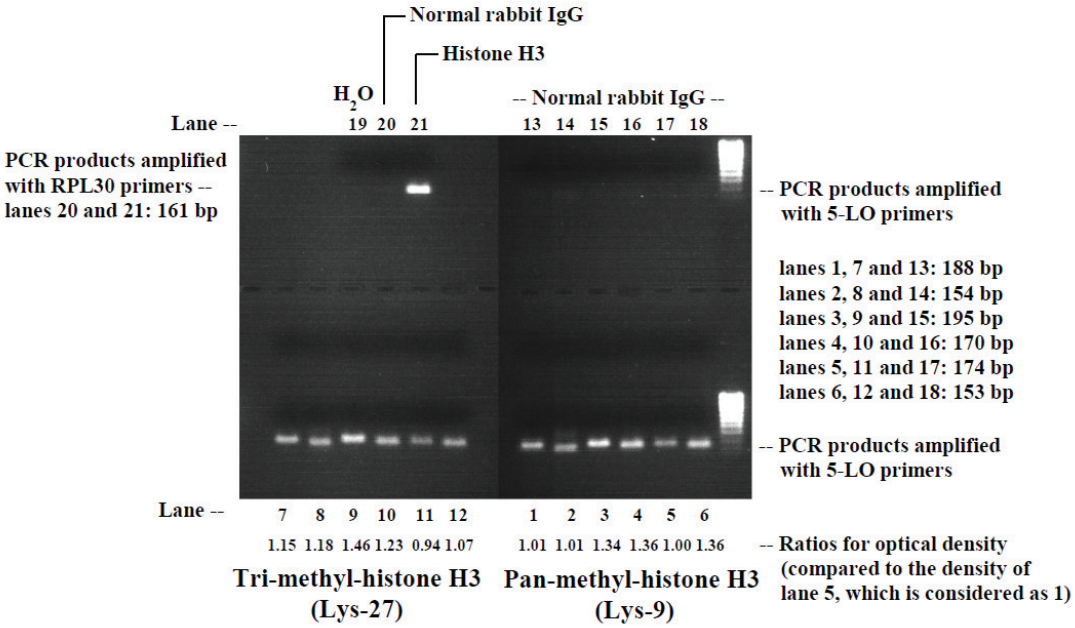

Tri-methyl-histone H3
(Lys-27)
Pan-methyl-histone H3
(Lys-9)
1.17 1.17 1.63 2.46 5.54 11.61
1.50 1.61 1.61 1.58 1.41 1.26
-- Ratios for optical density difference (compared to normal rabbit IgG ChIP sample, lanes 16-21)
Lane --
1 2 3 4 5 6
7 8 9 10 11 12
-- PCR products amplified with 5-LO primers
lanes 1, 7 and 16: 188 bp
lanes 2, 8 and 17: 154 bp
lanes 3, 9 and 18: 195 bp
lanes 4, 10 and 19: 170 bp
lanes 5, 11 and 20: 174 bp
lanes 6, 12 and 21: 153 bp
PCR products amplified with RPL30 primers -- lanes 14 and 15: 161 bp
-- PCR products amplified with 5-LO primers
Lane --
13 14 15
16 17 18 19 20 21
H2O
-- Normal rabbit IgG --
Histone H3
Normal rabbit IgG

Fig. 7B: **HMC-1 Control**



HMC-1 DEX 1μM Treatment

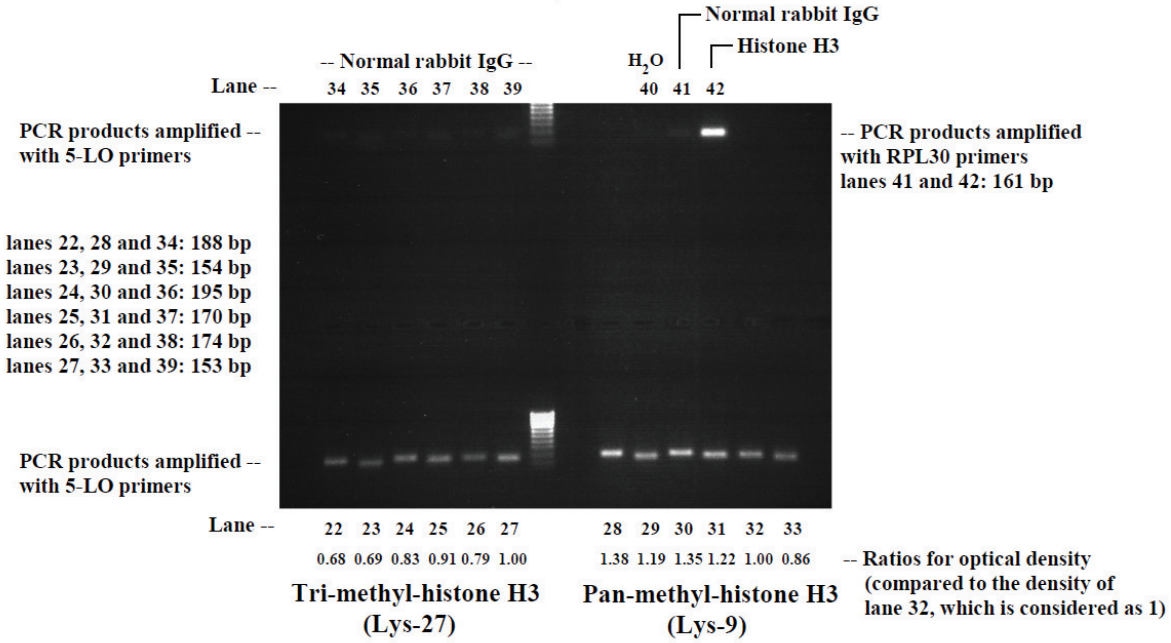
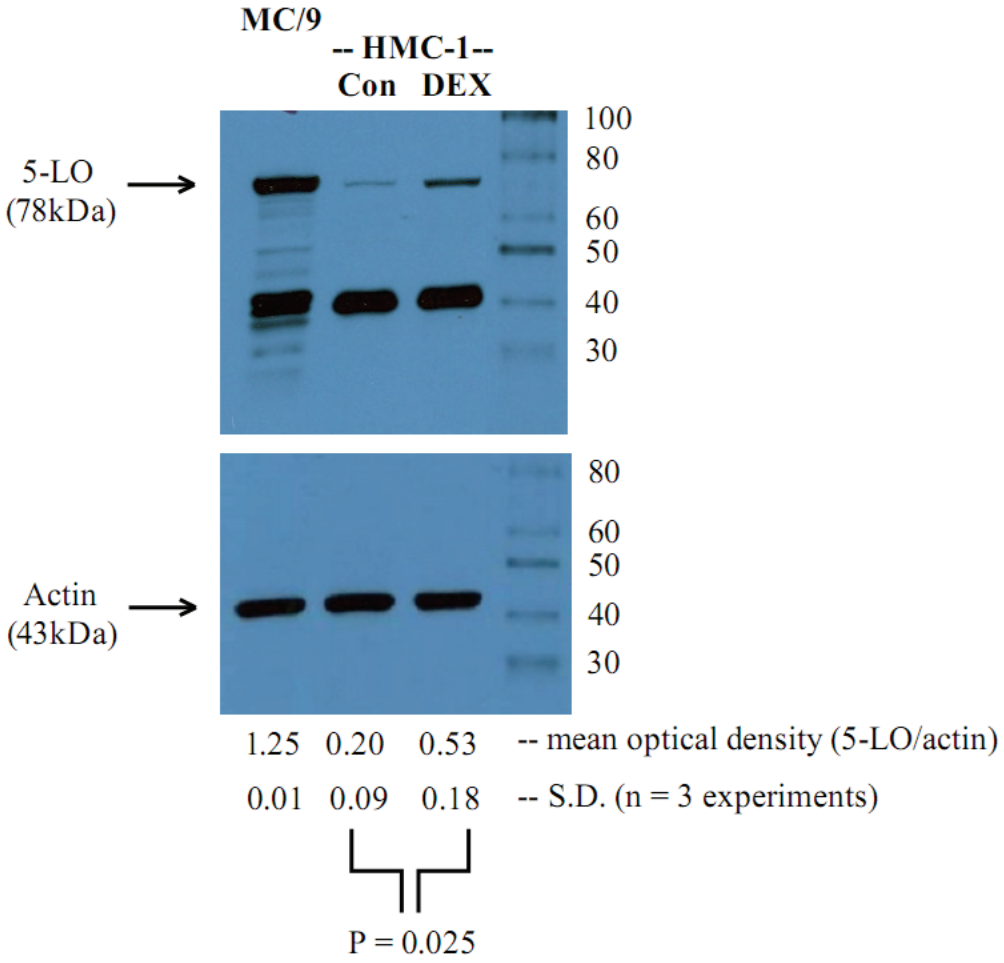


Figure 8: The rescued 5-LO protein expression in DEX treated-HMC-1 cells. HMC-1 cells were cultured with 1μM DEX in IMDM supplemented with 1.2 mM monothioglycerol, 10% heat-inactivated iron supplemented bovine calf serum and antibiotics at 37 °C in a humidified atmosphere of 5% CO_2, 95% air. Fresh medium was changed every 3-5 days. After 25 days of DEX treatment, the whole cell extracts together with untreated HMC-1 cells (*Con*) were collected in hot SDS-PAGE loading buffer. 40 μg of cellular protein was subjected to Western blotting analysis for 5-LO and actin (loading control) protein using specific polyclonal antisera as described in "Materials and Methods". MC/9 cellular protein was used as a positive control for 5-LO expression. Densitometric analysis was shown as indicated.

Fig. 8:


MC/9
-- HMC-1--
Con DEX
5-LO
(78kDa)
100
80
60
50
40
30
Actin
(43kDa)
80
60
50
40
30
1.25 0.20 0.53 -- mean optical density (5-LO/actin)
0.01 0.09 0.18 -- S.D. (n = 3 experiments)
P = 0.025

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

RA is a chronic, systemic inflammatory disorder with the principle characteristics being synovitis, cartilage and bone damage that lead to chronic pain, loss of function and disability. The pathogenesis of RA is still not fully understood. Many theories have been developed, including autoantibodies, T cells, imbalanced cytokine networks, tumor-like SF and so on. Recent studies suggest mast cells as a cellular link between autoantibodies, soluble inflammatory mediators and other effector cells in arthritis (Lee, Friend et al. 2002). Mast cells can induce vascular permeability, leukocyte recruitment and activation to promote inflammation. They can also affect stromal responses, such as proliferation and activation of SF, angiogenesis, and chondrocyte and osteoclast activation for joint remodeling. In this thesis, we focused on the role of mast cell/neutrophil-derived LTB_4 in the pathogenesis of RA.

LTB_4 may promote the development of RA through the following mechanism. Acute arthritis might be initiated by infection and/or the formation of autoantibody-mediated immune complexes inside the affected joint. The immune complexes then activate mast cells to produce LTB_4 which recruits more inflammatory cells into the synovial membrane. Interactions among these immune cells lead to the production of a variety of pro-inflammatory cytokines, chemokines, prostaglandins and degradative enzymes for perpetual joint inflammation and damage. Many of these immune cells, such as macrophages, neutrophils, dendritic cells and mast cells, can produce LTB_4 through activation by different complement and/or cytokines. Since all of these cells express LTB_4 receptors, they establish a positive feedback loop for inflammation inside the RA joint. In this thesis, we verified that mast cell/neutrophil-derived LTB_4 stabilized COX-2 mRNA through activation of the Ras/c-Raf/MEK1/2/ERK1/2 signaling pathway in IL-1β-treated HSF, providing further evidence for the role of LTB_4 in the modulation of the

inflammatory response in RA.

LTB_4 exerts various effects through BLT receptors that couple with different G protein subtypes. Studies showed the levels of BLT1 and BLT2 mRNA were significantly increased in the synovium from RA patients compared with OA patients (Hashimoto, Endo et al. 2003). In this thesis, we also investigated the expression of *BLT1* and *BLT2* genes in HSF through semi-quantitative RT-PCR and detected higher level of BLT2 mRNA than BLT1. In the case of LTB_4 stimulation, both BLT receptors mediated ERK1/2 signaling activation in HSF as verified by the BLT1 specific antagonist U75302 and the BLT2 specific antagonist LY255283. It seemed that BLT1 played more important role in the ERK1/2 activation by LTB_4 than BLT2 as demonstrated by the antagonist inhibition study. However, we could also induce strong ERK1/2 activation by a natural BLT2 specific ligand, 12-HHT, in HSF, showing that BLT2 alone could also mediate ERK1/2 signaling. In order to verify which receptor is more crucial in this stabilization effect of LTB_4 on COX-2 mRNA in our cell systems, knockdown experiments with siRNA or shRNA targeting BLT1 or BLT2, or gene knockout mice could be developed for the future studies. Moreover, PTX treatment of HSF could be used to distinguish whether the G protein involved in this stabilization process is PTX-sensitive or insensitive.

Transport of proteins and some ribonucleoproteins (RNPs) between the nucleus and cytoplasm involves the recognition of specific signal sequences by saturable transport receptors that shuttle between the nucleus and the cytoplasm, such as importins (Gorlich, Vogel et al. 1995) and exportins (Stade, Ford et al. 1997). These receptors all have an N-terminal RanGTP-binding domain, a C-terminal cargo-binding domain, and can transport cargos across nuclear pore complex (NPC) through binding the components

of NPC. Like all small GTPases, the directionality of cargo transportation through the NPC is promoted by Ran GTPase cycle. Inside the nucleus, Ran-GTP binding dissociates import receptor-cargo complexes, leading to the release of cargos from the import receptors. Then, the unloaded import receptors return to the cytoplasm to further import the specific cargos. In contrast, export receptor-cargo-RanGTP complex disassembles in the presence of Ran GTP-activating protein (Ran GAP) on the cytoplasmic side of the NPC and releases the exported cargos into the cytoplasm (Gorlich 1998; Izaurralde and Adam 1998).

Several hnRNP proteins have been demonstrated to shuttle between the nucleus and cytoplasm (Nakielny and Dreyfuss 1997). The best characterized is the hnRNP A1 protein. The M9 domain of hnRNP A1 is required for both nuclear import and export of proteins (Michael, Choi et al. 1995). The M9 domain is loosely characterized as a 38-amino acid region rich in aromatic residues and glycines. In humans, the import receptor that recognizes the M9 domain of hnRNP A1 is called transportin (Pollard, Michael et al. 1996). AUF1/hnRNP D protein, a member of hnRNP family, can also shuttle between the nucleus and cytoplasm. The CTD of AUF1 has a weak similarity to the M9 domain of hnRNP A1 and several adjacent RGG repeats might serve as RNA-binding sites. A nuclear localization signal (NLS) is also located in the CTD of p37 and p40 AUF1 isoforms. But in p42 and p45 AUF1 isoforms, nuclear export was facilitated by sequences encoded by exon 7 that disrupted the CTD region (Sarkar, Lu et al. 2003). Therefore, we observed predominately nuclear retention of p37 and p40 AUF1 isoforms with p42 AUF1 also partially distributed in the cytoplasm. However, in the case of p45 AUF1, we observed almost complete nuclear retention although it has the similar C-terminal region as p42 AUF1. The exact mechanism for this distribution of p45 AUF1 is

not clear, and the N-terminal domain encoded by exon 2 in p45 AUF1 might explain the distribution difference between p42 and p45 AUF1 isoforms. Further N-terminal deletion mutant studies are required to discern the precise mechanism.

Transfection experiments in this thesis also demonstrated that LTB_4 stabilized COX-2 mRNA through the nuclear export of RNA-binding protein p42 AUF1, a process that could be blocked by leptomycin B treatment. In the immediate future, RNA electrophoretic mobility shift assay (EMSA) could be used to verify that p42 AUF1 binds to the COX-2–3'-UTR-ARE for message stabilization (Carballo, Lai et al. 1998). Basically, we could first transfect pcDNA3-p42 AUF1 plasmid into our cell system to overexpress p42 AUF1 protein. Then cellular protein extract is incubated with the ^{32}P-labeled RNA probe that corresponds to the COX-2–3'-UTR-ARE fragment. After cross-linking and the following RNase T1 digestion, RNase-resistant RNA-protein complexes are analyzed by SDS-PAGE followed by autoradiography. Identical RNase-resistant RNA-protein complexes could also be used to perform immunoprecipitation with pan-AUF1 antibody followed by SDS-PAGE to further verify the binding of p42 AUF1 with COX-2–3'-UTR-ARE.

A number of cellular processes are regulated at the level of nucleo-cytoplasmic transport. The global regulation is suggested by the observation that NPC translocation is increased as cells pass from quiescence to proliferative state, and then to transformed condition (Feldherr and Akin 1994; Feldherr, Akin et al. 1998). Another type of regulation involves signaling pathways (Hood and Silver 1999). Among these, phosphorylation of cargos or their binding proteins by specific kinases plays a major role in the regulation of nucleo-cytoplasmic transport. Phosphorylation may change the affinity between the cargos and their specific transport receptors. For example,

phosphorylation of the cytoplasmic retention factor IκB disrupts its interaction with the transcription factor NF-κB, allowing the NLS of NF-κB to be recognized by import receptors (Mattaj and Englmeier 1998). In this thesis, we also demonstrated that activation of the Ras/c-Raf/MEK1/2/ERK1/2 signaling pathway triggered cytoplasmic translocation of p42 AUF1, which might be driven by phosphorylation of p42 AUF1. Phosphorylated p42 AUF1 might have a higher affinity for CRM1, an export receptor for leucine-rich nuclear export signals (Fornerod, Ohno et al. 1997), and lead to the cytoplasmic accumulation of p42 AUF1, further stabilizing COX-2 mRNA in IL-1β-treated HSF. However, there are few leucine amino acids in p42 AUF1 (GenBank™ accession number AF026126.1). Study showed that the CTD of AUF1 had a weak similarity to the M9 domain of hnRNP A1 (Sarkar, Lu et al. 2003) that could also function as a signal for nuclear export in mammalian cells (Michael, Choi et al. 1995) although the mechanism involved is still not fully understood. Another possibility for this nucleo-cytoplasmic shuttling of p42 AUF1 may be the existence of other nuclear export sequences in p42 AUF1 protein, and deletion mutant studies are planned in the future experiments to better understand the mechanisms involved. Moreover, identification of other nuclear export receptors may be another explanation for this p42 AUF1 shuttling process, and immunoprecipitations using cytoplasmic and nuclear protein extracts with pan-AUF1 antibody followed by mass spectrometry analysis may be very helpful in this regard.

Recent studies found that TTP can be phosphorylated by p38 MAPK and the downstream MAPK-activated kinase 2 (MK-2). *In vitro* experiments identified two major phosphorylation sites (Ser-52 and Ser-178) in mouse TTP (GenBank™ accession number M57422.1) (Chrestensen, Schroeder et al. 2004) that correspond to Ser-60 and Ser-186

sites in human TTP (GenBank^{TM} accession number M63625.1). Phosphorylation of TTP creates a functional 14-3-3 protein binding site, such that phosphorylated TTP has lower binding affinity for ARE-containing RNAs than unphosphorylated TTP, leading to decreased destabilization activity of TTP on target mRNAs (Carballo, Lai et al. 1998; Carballo, Cao et al. 2001; Mahtani, Brook et al. 2001; Sun, Stoecklin et al. 2007). On the contrary, protein phosphatase 2A (PP2A), a serine/threonine protein phosphatase associated with signal transduction, cell growth and apoptosis, could directly compete with 14-3-3 protein for TTP binding and dephosphorylation to restore the destabilization activity of TTP on target mRNAs (Sun, Stoecklin et al. 2007).

In our overexpression studies, we detected increased destabilizing effect of HuR on luciferase-COX-2–3'-UTR-ARE reporter activity under the condition of activated c-Raf/MEK1/2/ERK1/2 signaling. This result was contradictory with the traditional view of stabilization ability of HuR on target mRNAs (Brennan and Steitz 2001; Dixon, Tolley et al. 2001). We hypothesize that, in our cell culture system, HuR might be phosphorylated by the c-Raf/MEK1/2/ERK1/2 signaling cascade and this phosphorylated HuR has decreased binding affinity with ARE-containing mRNAs, causing the decreased stabilization effect on target mRNAs.

In the future studies, to determine if ERK1/2 signaling really phosphorylates HuR or p42 AUF1, we could perform cell labeling and immunoprecipitation as follows (Taylor, Thompson et al. 1995): First, cells are transfected with pcDNA3-p42 AUF1 or pcDNA-HuR-Flag constructs in the presence or absence of RafCAAX plasmid together with (^{32}P)H_3PO_4 incubation. Then, total cellular protein lysates are extracted from the labeled cells, followed by the immunoprecipitation with pan-AUF1 or anti-Flag antibody and SDS-PAGE to check if there is a shift between the c-Raf/MEK1/2/ERK1/2-activated cell

lysates and the control condition.

Some studies also reported that TTP could be phosphorylated on Ser-220 by p42 MAPK *in vitro* through a combination of protease digestion experiments and site-directed mutagenesis strategies (Taylor, Thompson et al. 1995). However, in our TTP overexpression study, the decrease of luciferase reporter activity was not subject to the c-Raf/MEK1/2/ERK1/2 signaling regulation. Further experiments using other cell systems may be performed to clarify this discrepancy. We also incorporated several TTP mutant plasmids (mutations of the Ser-60, Ser-186, and Ser-60/186 phosphorylation sites by p38 MAPK in human TTP protein) into our overexpression studies, and there was almost no difference in the decrease of luciferase reporter activities between the mutant and the wild-type constructs under the RafCAAX-induced ERK1/2 activation *versus* the control condition (data not shown), further demonstrating that p38 signaling may not be involved in our study system.

The major objectives for the treatment of RA patients are relief of clinical symptoms and preservation of joint structure and function, with the ultimate goal of disease remission. Current treatment for RA usually begins with MTX, which is very effective in reducing signs and symptoms of this disease, as well as slowing or halting radiographic progression (Braun and Rau 2009). MTX exerts anti-proliferative, anti-inflammatory and immuno-suppressive effects through the following mechanisms: (1) Reduction of cell proliferation through inhibition of the enzymes involved in folate pathway for *de novo* pyrimidine and purine synthesis; (2) Promotion of cell apoptosis by alterating the intracellular levels of reactive oxygen species; (3) Increasing endogenous release of anti-inflammatory adenosine; (4) Inhibition of the production of pro-inflammatory cytokines, such as TNF-α, IFN-γ and IL-6; (5) Decreasing the expression

of cellular adhesion molecules; (6) Suppression of the expression of RANKL (an osteoclast differentiation factor) and increased secretion of osteoprotegerin (OPG, an osteoclastogenesis inhibitory factor) to reduce the progression of bone erosion (Wessels, Huizinga et al. 2008). Other non-MTX disease-modifying antirheumatic drugs (DMARDs) also have the potential to reduce synovitis and erosive destruction in RA through various mechanisms, such as cytokine suppression, purine synthesis inhibition, and induction of apoptosis, with the adverse risks of infections and lung disease (Nandi, Kingsley et al. 2008). DMARDs can be used as monotherapy or in combination as the first-line treatment for all newly diagnosed RA (Capell, Madhok et al. 2007). Non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are often used as supplemental therapies to manage pain, inflammation and stiffness related to RA (Gaffo, Saag et al. 2006). However, 30-40% of RA patients don't respond to MTX and other DMARDs. Usually, the next step is to add BRMs that selectively inhibit specific molecules of the immune system, such as TNF-α inhibitors, IL-1 antagonists, co-stimulation blockers, and B-cell depletion therapy (anti-CD20 monoclonal antibody) (O'Dell 2004; Gaffo, Saag et al. 2006; Smolen, Aletaha et al. 2007). Although very effective, DMARDs and TNF-α blockers need to be used continuously to sustain the clinical benefits. TNF-blocking therapy also has other shortcomings, including the loss of response over time (acquired therapeutic resistance) in a significant proportion of patients, associated risk of infections and lymphoma development, as well as high cost (Geborek, Bladstrom et al. 2005; Rubbert-Roth and Finckh 2009).

Over the past two decades, studies have renewed interest in LTB_4 as a target in arthritis treatment. In the collagen-induced arthritis mouse model, the LTB_4 receptor antagonist CP-105696 markedly blocked the massive recruitment of inflammatory cells

into synovial tissue and prevented the erosion of cartilage and bone (Griffiths, Pettipher et al. 1995). Another study showed that 5-LO and LTA_4 hydrolase knockout mice were remarkably resistant to development of K/B×N serum-induced inflammatory arthritis; whereas, wild-type and LTC_4 synthase knockout mice still developed robust inflammation with synovial hyperplasia and synovial erosion into cartilage and bone. In addition, 5-LO antagonists could completely prevent arthritis from occurring in wild-type mice (Chen, Lam et al. 2006). Neutrophils from RA patients undergoing MTX treatment displayed suppressed LTB_4 production (Sperling, Benincaso et al. 1992). The FLAP inhibitor MK886 and the LTA_4 hydrolase inhibitor bestatin remarkably suppressed the production of LTB_4 from RASF (Xu, Lu et al. 2010). Although LTB_4 receptor antagonists have been dramatically effective in animal models, there is still little experience with these agents in humans as yet. Furthermore, as a major source for LTB_4, inhibition of mast cell activity and induction of their apoptosis represent other potential means to alleviate inflammation in RA patients.

Recently, epigenetic dysregulation has been accepted as an important mechanism in the pathogenesis of many diseases. Among these, DNA methylation in the promoter regions of target genes is considered to be the most important in most cases, which is also demonstrated in our DNA methylation study of *5-LO* gene promoter in HMC-1 cells (lower passage number *versus* higher passage number). However, in some conditions, histone modifications may play a driving force in the development of diseases. We didn't find DNA methylation of the *5-LO* gene promoter in OA/RA HSF that expressed no 5-LO protein. Because histone (lysine and/or arginine residues) methylation is usually associated with transcriptional silencing of target genes, we verified using ChIP assay that histone H3 (Lys-9 and -27) methylation may be responsible for the suppression of 5-

LO protein expression in these HSF cells.

In future studies, we could use the histone methylation inhibitor 3-deazaneplanocin A (DZNep) to reverse this histone modification to check if there is some changes on *5-LO* gene expression in these HSF from OA and RA patients (Miranda, Cortez et al. 2009). We could also use ChIP assays, Western blotting analysis, or the HDAC inhibitor TSA to investigate other histone modifications (*e.g.* histone acetylation) that may be involved in the regulation of *5-LO* gene expression.

As mentioned before, LTB_4 is a potent lipid mediator and functions through its specific cell surface receptors, BLT1 and -2. BLT1 is a high-affinity LTB_4 receptor and restrictly expressed in peripheral leukocytes and eosinophils, while BLT2 is widely distributed in different tissues and cell types and has lower affinity for LTB_4. Sequence analysis revealed the promoter region of *BLT1* gene has high CpG contents. It was also reported that the CpG sites of *BLT1* gene promoter were highly methylated in BLT1-nonexpressing cells, but unmethylated in BLT1-expressing cells, which provided a good explanation for the cell- and tissue-specific transcription of *BLT1* gene. Sequence analysis also found that the open reading frame (ORF) of *BLT2* gene overlaps with the promoter region of *BLT1* gene, the so-called "promoter in ORF" (Kato, Yokomizo et al. 2000). However, the biological significance of this overlap is unclear. In the case of pathological conditions, like RA and other inflammatory diseases, we speculate that the alteration of methylation status in the promoter region of *BLT1* gene may cause changes in the expression levels of BLT1 and/or BLT2 on target cells, such as SF, T cells, macrophages, and neutrophils, leading to the increased sensitivity to LTB_4 actions and reinforced inflammatory responses in the affected tissues. Indeed, transgenic mice provided strong evidence that overexpression of BLT1 in leukocytes dramatically

increased leukocytes recruitment and responsiveness in acute dermal inflammation, peritonitis, and ischemia-reperfusion-induced lung injury (Chiang, Gronert et al. 1999), suggesting the putative target of BLT receptors in suppressing excessive inflammation in disease treatment.

Since the bisulfite sequencing technique used in this thesis for *5-LO* gene promoter is very reliable for DNA methylation study in other target genes in various diseases, we could employ this technique to investigate the correlation between the methylation status of *BLT1* gene promoter with BLT1 and/or BLT2 protein expression in the development of OA and RA. Moreover, we could pursue this avenue of research in the studies of other inflammatory diseases, such as asthma (Turner, Breslow et al. 1996), psoriasis (Iversen, Kragballe et al. 1997), ulcerative colitis (Cole, Pilkington et al. 1996), and postischemic tissue injuries (Zimmerman, Guillory et al. 1990; Noiri, Yokomizo et al. 2000), in which LTB_4 and BLT receptors are also involved.

Therefore, better understanding of the epigenetic mechanisms and the development of new techniques may elucidate more links between epigenetic dysregulations with the pathogenesis of various diseases. However, unlike genetic mutations, epigenetic alterations are potentially reversible using different enzymatic inhibitors (*e.g.* DNMT inhibitor and HDAC inhibitor). Epigenetic therapy, though now controversial, may provide a good supplementation for gene therapy in many disease treatments, particularly in cancer.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

The data presented in this thesis has provided several original contributions to the existing body of scientific knowledge:

In Chapter 2:

- We recognized a bidirectional interaction between resident mast cells and SF that received scant attention in the relevant literature.
- We identified a unique autocrine/paracrine feed-back loop that serves to resolve the inflammatory response; the definition of which may be the "holy grail" of autoimmune diseases and may also contribute to further understanding of the pathophysiology of other inflammatory diseases, such as asthma and COPD.
- We elucidated a clear Ras/c-Raf/MEK1/2/ERK1/2/p42 AUF1 signaling pathway that is involved in the post-transcriptional stabilization of COX-2 mRNA by mast cell/neutrophil-derived LTB_4.
- The potential cellular and molecular interactions between infiltrating mast cells and resident HSF involve many cellular components, cytokine/chemokine networks, and immunological pathways. There is a clear potential of developing new small-molecule anti-cytokine and/or anti-inflammatory drugs for novel targets.
- Anti-TNF-α therapy is very effective in RA treatment. Considering the huge cost of anti-TNF-α therapy *versus* small-molecule anti-cytokine compounds, which could be produced at only a fraction of the cost of TNF-α blockers, our research places this in the realm of possibilities.
- Alternatively, old drugs may find new uses. As mast cells are highly pro-inflammatory cells, inhibition of their activity and/or induction of their apoptosis represent an efficient means to treat rheumatic and inflammatory diseases.

In Chapter 3:

- Epigenetic alterations may also be involved in the development of RA and/or OA.
- Under certain circumstances, DNA methylation may not be the key regulator for controlling target gene expression. Histone modifications, especially histone methylation, are getting increased attention in the pathogenesis of RA and other inflammatory diseases.
- Bisulfite sequencing technique used for DNA methylation analysis of *5-LO* gene promoter attested the reliability of methylation studies on other target genes in different inflammatory diseases.
- Epigenetic therapy, which has been widely used in many clinical trials in cancers, may also become a promising treatment for some inflammatory and/or rheumatic diseases, such as RA, lupus, and asthma.

REFERENCES:

Abbas, A. K., K. M. Murphy, et al. (1996). "Functional diversity of helper T lymphocytes." Nature **383**(6603): 787-93.

Abramovitz, M., E. Wong, et al. (1993). "5-lipoxygenase-activating protein stimulates the utilization of arachidonic acid by 5-lipoxygenase." Eur J Biochem **215**(1): 105-11.

Acharya, M. R., A. Sparreboom, et al. (2005). "Rational development of histone deacetylase inhibitors as anticancer agents: a review." Mol Pharmacol **68**(4): 917-32.

Adcock, I. M., L. Tsaprouni, et al. (2007). "Epigenetic regulation of airway inflammation." Curr Opin Immunol **19**(6): 694-700.

Ahmadzadeh, N., M. Shingu, et al. (1991). "Relationship between leukotriene B4 and immunological parameters in rheumatoid synovial fluids." Inflammation **15**(6): 497-503.

Altman, R., E. Asch, et al. (1986). "Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Diagnostic and Therapeutic Criteria Committee of the American Rheumatism Association." Arthritis Rheum **29**(8): 1039-49.

Andersson, R., S. Enroth, et al. (2009). "Nucleosomes are well positioned in exons and carry characteristic histone modifications." Genome Res **19**(10): 1732-41.

Antonelli, M., M. Bufi, et al. (1989). "Detection of leukotrienes B4, C4 and of their isomers in arterial, mixed venous blood and bronchoalveolar lavage fluid from ARDS patients." Intensive Care Med **15**(5): 296-301.

Appleby, S. B., A. Ristimaki, et al. (1994). "Structure of the human cyclo-oxygenase-2 gene." Biochem J **302 (Pt 3)**: 723-7.

Arcoleo, F., S. Milano, et al. (1995). "Effect of exogenous leukotriene B4 (LTB4) on BALB/c mice splenocyte production of Th1 and Th2 lymphokines." Int J Immunopharmacol **17**(6): 457-63.

Ashwell, J. D. (2006). "The many paths to p38 mitogen-activated protein kinase activation in the immune system." Nat Rev Immunol **6**(7): 532-40.

Bailie, M. B., T. J. Standiford, et al. (1996). "Leukotriene-deficient mice manifest enhanced lethality from Klebsiella pneumonia in association with decreased alveolar macrophage phagocytic and bactericidal activities." J Immunol **157**(12): 5221-4.

Ballestar, E., M. Esteller, et al. (2006). "The epigenetic face of systemic lupus erythematosus." J Immunol **176**(12): 7143-7.

Bannister, A. J., R. Schneider, et al. (2002). "Histone methylation: dynamic or static?" Cell **109**(7): 801-6.

Barrandon, C., B. Spiluttini, et al. (2008). "Non-coding RNAs regulating the transcriptional machinery." Biol Cell **100**(2): 83-95.

Barros, S. P. and S. Offenbacher (2009). "Epigenetics: connecting environment and genotype to phenotype and disease." J Dent Res **88**(5): 400-8.

Befus, A. D., C. Mowat, et al. (1999). "Neutrophil defensins induce histamine secretion from mast cells: mechanisms of action." J Immunol **163**(2): 947-53.

Behrens, A., W. Jochum, et al. (2000). "Oncogenic transformation by ras and fos is mediated by c-Jun N-terminal phosphorylation." Oncogene **19**(22): 2657-63.

Bendele, A., G. Sennello, et al. (1999). "Effects of interleukin 1 receptor antagonist alone and in combination with methotrexate in adjuvant arthritic rats." J Rheumatol **26**(6): 1225-9.
Bingham, C. O., 3rd (2008). "Emerging therapeutics for rheumatoid arthritis." Bull NYU Hosp Jt Dis **66**(3): 210-5.
Bird, A. (2002). "DNA methylation patterns and epigenetic memory." Genes Dev **16**(1): 6-21.
Black, J. L., B. G. Oliver, et al. (2009). "Molecular mechanisms of combination therapy with inhaled corticosteroids and long-acting beta-agonists." Chest **136**(4): 1095-100.
Blackshear, P. J. (2002). "Tristetraprolin and other CCCH tandem zinc-finger proteins in the regulation of mRNA turnover." Biochem Soc Trans **30**(Pt 6): 945-52.
Blumberg, P. M. (1980). "In vitro studies on the mode of action of the phorbol esters, potent tumor promoters: part 1." Crit Rev Toxicol **8**(2): 153-97.
Blumberg, P. M. (1981). "In vitro studies on the mode of action of the phorbol esters, potent tumor promoters, part 2." Crit Rev Toxicol **8**(3): 199-234.
Bock, C., M. Paulsen, et al. (2006). "CpG island methylation in human lymphocytes is highly correlated with DNA sequence, repeats, and predicted DNA structure." PLoS Genet **2**(3): e26.
Bogoyevitch, M. A. and B. Kobe (2006). "Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases." Microbiol Mol Biol Rev **70**(4): 1061-95.
Bortuzzo, C., R. Hanif, et al. (1996). "The effect of leukotrienes B and selected HETEs on the proliferation of colon cancer cells." Biochim Biophys Acta **1300**(3): 240-6.
Bost, F., L. Caron, et al. (2002). "Retinoic acid activation of the ERK pathway is required for embryonic stem cell commitment into the adipocyte lineage." Biochem J **361**(Pt 3): 621-7.
Boyce, J. A. (2003). "Mast cells: beyond IgE." J Allergy Clin Immunol **111**(1): 24-32; quiz 33.
Bradding, P. (2003). "The role of the mast cell in asthma: a reassessment." Curr Opin Allergy Clin Immunol **3**(1): 45-50.
Braun, J. and R. Rau (2009). "An update on methotrexate." Curr Opin Rheumatol **21**(3): 216-23.
Brennan, C. M., I. E. Gallouzi, et al. (2000). "Protein ligands to HuR modulate its interaction with target mRNAs in vivo." J Cell Biol **151**(1): 1-14.
Brennan, C. M. and J. A. Steitz (2001). "HuR and mRNA stability." Cell Mol Life Sci **58**(2): 266-77.
Brennan, F. M., K. A. Browne, et al. (1997). "Reduction of serum matrix metalloproteinase 1 and matrix metalloproteinase 3 in rheumatoid arthritis patients following anti-tumour necrosis factor-alpha (cA2) therapy." Br J Rheumatol **36**(6): 643-50.
Brewer, G. (1991). "An A + U-rich element RNA-binding factor regulates c-myc mRNA stability in vitro." Mol Cell Biol **11**(5): 2460-6.
Brueckner, B. and F. Lyko (2004). "DNA methyltransferase inhibitors: old and new drugs for an epigenetic cancer therapy." Trends Pharmacol Sci **25**(11): 551-4.
Burg, N. D. and M. H. Pillinger (2001). "The neutrophil: function and regulation in innate and humoral immunity." Clin Immunol **99**(1): 7-17.
Buzby, J. S., S. M. Lee, et al. (1996). "Increased granulocyte-macrophage colony-

stimulating factor mRNA instability in cord versus adult mononuclear cells is translation-dependent and associated with increased levels of A + U-rich element binding factor." Blood **88**(8): 2889-97.
Byrum, R. S., J. L. Goulet, et al. (1997). "Role of the 5-lipoxygenase-activating protein (FLAP) in murine acute inflammatory responses." J Exp Med **185**(6): 1065-75.
Calvanese, V., E. Lara, et al. (2009). "The role of epigenetics in aging and age-related diseases." Ageing Res Rev **8**(4): 268-76.
Camp, R. D., A. A. Coutts, et al. (1983). "Responses of human skin to intradermal injection of leukotrienes C4, D4 and B4." Br J Pharmacol **80**(3): 497-502.
Campbell, E. L., N. A. Louis, et al. (2007). "Resolvin E1 promotes mucosal surface clearance of neutrophils: a new paradigm for inflammatory resolution." FASEB J **21**(12): 3162-70.
Capell, H. A., R. Madhok, et al. (2007). "Combination therapy with sulfasalazine and methotrexate is more effective than either drug alone in patients with rheumatoid arthritis with a suboptimal response to sulfasalazine: results from the double-blind placebo-controlled MASCOT study." Ann Rheum Dis **66**(2): 235-41.
Carballo, E., H. Cao, et al. (2001). "Decreased sensitivity of tristetraprolin-deficient cells to p38 inhibitors suggests the involvement of tristetraprolin in the p38 signaling pathway." J Biol Chem **276**(45): 42580-7.
Carballo, E., W. S. Lai, et al. (1998). "Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin." Science **281**(5379): 1001-5.
Carballo, E., W. S. Lai, et al. (2000). "Evidence that tristetraprolin is a physiological regulator of granulocyte-macrophage colony-stimulating factor messenger RNA deadenylation and stability." Blood **95**(6): 1891-9.
Cassatella, M. A. (1999). "Neutrophil-derived proteins: selling cytokines by the pound." Adv Immunol **73**: 369-509.
Chaussepied, M., D. Lallemand, et al. (1998). "Upregulation of Jun and Fos family members and permanent JNK activity lead to constitutive AP-1 activation in Theileria-transformed leukocytes." Mol Biochem Parasitol **94**(2): 215-26.
Chen, C. Y., R. Gherzi, et al. (2001). "AU binding proteins recruit the exosome to degrade ARE-containing mRNAs." Cell **107**(4): 451-64.
Chen, C. Y., N. Xu, et al. (2004). "Functional dissection of hnRNP D suggests that nuclear import is required before hnRNP D can modulate mRNA turnover in the cytoplasm." RNA **10**(4): 669-80.
Chen, M., B. K. Lam, et al. (2006). "Neutrophil-derived leukotriene B4 is required for inflammatory arthritis." J Exp Med **203**(4): 837-42.
Chiang, N., K. Gronert, et al. (1999). "Leukotriene B4 receptor transgenic mice reveal novel protective roles for lipoxins and aspirin-triggered lipoxins in reperfusion." J Clin Invest **104**(3): 309-16.
Chodavarapu, R. K., S. Feng, et al. (2010). "Relationship between nucleosome positioning and DNA methylation." Nature **466**(7304): 388-92.
Chrestensen, C. A., M. J. Schroeder, et al. (2004). "MAPKAP kinase 2 phosphorylates tristetraprolin on in vivo sites including Ser178, a site required for 14-3-3 binding." J Biol Chem **279**(11): 10176-84.
Ciechanover, A. (1998). "The ubiquitin-proteasome pathway: on protein death and cell life." EMBO J **17**(24): 7151-60.
Clark, A., J. Dean, et al. (2009). "Post-transcriptional gene regulation by MAP kinases

via AU-rich elements." Front Biosci **14**: 847-71.
Cobb, B. S., A. Hertweck, et al. (2006). "A role for Dicer in immune regulation." J Exp Med **203**(11): 2519-27.
Colamorea, T., R. Di Paola, et al. (1999). "5-Lipoxygenase upregulation by dexamethasone in human mast cells." Biochem Biophys Res Commun **265**(3): 617-24.
Cole, A. T., B. J. Pilkington, et al. (1996). "Mucosal factors inducing neutrophil movement in ulcerative colitis: the role of interleukin 8 and leukotriene B4." Gut **39**(2): 248-54.
Coulon, V., K. Chebli, et al. (2010). "A novel mouse c-fos intronic promoter that responds to CREB and AP-1 is developmentally regulated in vivo." PLoS One **5**(6): e11235.
Cress, W. D. and E. Seto (2000). "Histone deacetylases, transcriptional control, and cancer." J Cell Physiol **184**(1): 1-16.
Crooks, S. W., D. L. Bayley, et al. (2000). "Bronchial inflammation in acute bacterial exacerbations of chronic bronchitis: the role of leukotriene B4." Eur Respir J **15**(2): 274-80.
Cullen, B. R. (2005). "RNAi the natural way." Nat Genet **37**(11): 1163-5.
Dean, J. L., G. Sully, et al. (2004). "The involvement of AU-rich element-binding proteins in p38 mitogen-activated protein kinase pathway-mediated mRNA stabilisation." Cell Signal **16**(10): 1113-21.
Dean, J. L., G. Sully, et al. (2002). "Identification of a novel AU-rich-element-binding protein which is related to AUF1." Biochem J **366**(Pt 3): 709-19.
Dean, J. L., R. Wait, et al. (2001). "The 3' untranslated region of tumor necrosis factor alpha mRNA is a target of the mRNA-stabilizing factor HuR." Mol Cell Biol **21**(3): 721-30.
Dember, L. M., N. D. Kim, et al. (1996). "Individual RNA recognition motifs of TIA-1 and TIAR have different RNA binding specificities." J Biol Chem **271**(5): 2783-8.
Demitsu, T., H. Katayama, et al. (1989). "Phagocytosis and bactericidal action of mouse peritoneal macrophages treated with leukotriene B4." Int J Immunopharmacol **11**(7): 801-8.
Derks, S., M. H. Lentjes, et al. (2004). "Methylation-specific PCR unraveled." Cell Oncol **26**(5-6): 291-9.
Dieker, J. and S. Muller (2009). "Epigenetic Histone Code and Autoimmunity." Clin Rev Allergy Immunol.
Ditzel, H. J. (2004). "The K/BxN mouse: a model of human inflammatory arthritis." Trends Mol Med **10**(1): 40-5.
Dixon, D. A. (2004). "Dysregulated post-transcriptional control of COX-2 gene expression in cancer." Curr Pharm Des **10**(6): 635-46.
Dixon, D. A., G. C. Balch, et al. (2003). "Regulation of cyclooxygenase-2 expression by the translational silencer TIA-1." J Exp Med **198**(3): 475-81.
Dixon, D. A., C. D. Kaplan, et al. (2000). "Post-transcriptional control of cyclooxygenase-2 gene expression. The role of the 3'-untranslated region." J Biol Chem **275**(16): 11750-7.
Dixon, D. A., N. D. Tolley, et al. (2001). "Altered expression of the mRNA stability factor HuR promotes cyclooxygenase-2 expression in colon cancer cells." J Clin Invest **108**(11): 1657-65.

Dodge, J. E., M. Okano, et al. (2005). "Inactivation of Dnmt3b in mouse embryonic fibroblasts results in DNA hypomethylation, chromosomal instability, and spontaneous immortalization." J Biol Chem **280**(18): 17986-91.
Dolhain, R. J., A. N. van der Heiden, et al. (1996). "Shift toward T lymphocytes with a T helper 1 cytokine-secretion profile in the joints of patients with rheumatoid arthritis." Arthritis Rheum **39**(12): 1961-9.
Dorsam, R. T. and J. S. Gutkind (2007). "G-protein-coupled receptors and cancer." Nat Rev Cancer **7**(2): 79-94.
Dreyfuss, G., V. N. Kim, et al. (2002). "Messenger-RNA-binding proteins and the messages they carry." Nat Rev Mol Cell Biol **3**(3): 195-205.
Duell, E. A., C. N. Ellis, et al. (1988). "Determination of 5,12, and 15-lipoxygenase products in keratomed biopsies of normal and psoriatic skin." J Invest Dermatol **91**(5): 446-50.
Dupont, C., D. R. Armant, et al. (2009). "Epigenetics: definition, mechanisms and clinical perspective." Semin Reprod Med **27**(5): 351-7.
Dykxhoorn, D. M., D. Chowdhury, et al. (2008). "RNA interference and cancer: endogenous pathways and therapeutic approaches." Adv Exp Med Biol **615**: 299-329.
Ehrlich, M. (2002). "DNA methylation in cancer: too much, but also too little." Oncogene **21**(35): 5400-13.
El-Maarri, O. (2003). "Methods: DNA methylation." Adv Exp Med Biol **544**: 197-204.
Ellis, L., P. W. Atadja, et al. (2009). "Epigenetics in cancer: targeting chromatin modifications." Mol Cancer Ther **8**(6): 1409-20.
Espel, E. (2005). "The role of the AU-rich elements of mRNAs in controlling translation." Semin Cell Dev Biol **16**(1): 59-67.
Esteller, M. (2002). "CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future." Oncogene **21**(35): 5427-40.
Esteller, M. (2007). "Epigenetic gene silencing in cancer: the DNA hypermethylome." Hum Mol Genet **16 Spec No 1**: R50-9.
Fallon, M. D., M. P. Whyte, et al. (1981). "Systemic mastocytosis associated with generalized osteopenia. Histopathological characterization of the skeletal lesion using undecalcified bone from two patients." Hum Pathol **12**(9): 813-20.
Fan, X. C. and J. A. Steitz (1998). "HNS, a nuclear-cytoplasmic shuttling sequence in HuR." Proc Natl Acad Sci U S A **95**(26): 15293-8.
Fan, X. C. and J. A. Steitz (1998). "Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs." EMBO J **17**(12): 3448-60.
Faour, W. H., N. Alaaeddine, et al. (2005). "Early growth response factor-1 mediates prostaglandin E2-dependent transcriptional suppression of cytokine-induced tumor necrosis factor-alpha gene expression in human macrophages and rheumatoid arthritis-affected synovial fibroblasts." J Biol Chem **280**(10): 9536-46.
Faour, W. H., Q. He, et al. (2006). "Prostaglandin E2 stimulates p53 transactivational activity through specific serine 15 phosphorylation in human synovial fibroblasts. Role in suppression of c/EBP/NF-kappaB-mediated MEKK1-induced MMP-1 expression." J Biol Chem **281**(29): 19849-60.
Faour, W. H., Y. He, et al. (2001). "Prostaglandin E(2) regulates the level and stability of cyclooxygenase-2 mRNA through activation of p38 mitogen-activated protein

kinase in interleukin-1 beta-treated human synovial fibroblasts." J Biol Chem **276**(34): 31720-31.
Faour, W. H., A. Mancini, et al. (2003). "T-cell-derived interleukin-17 regulates the level and stability of cyclooxygenase-2 (COX-2) mRNA through restricted activation of the p38 mitogen-activated protein kinase cascade: role of distal sequences in the 3'-untranslated region of COX-2 mRNA." J Biol Chem **278**(29): 26897-907.
Farfel, Z., H. R. Bourne, et al. (1999). "The expanding spectrum of G protein diseases." N Engl J Med **340**(13): 1012-20.
Feldherr, C., D. Akin, et al. (1998). "The nuclear import factor p10 regulates the functional size of the nuclear pore complex during oogenesis." J Cell Sci **111 (Pt 13)**: 1889-96.
Feldherr, C. M. and D. Akin (1994). "Role of nuclear trafficking in regulating cellular activity." Int Rev Cytol **151**: 183-228.
Feldmann, M. and R. N. Maini (2001). "Anti-TNF alpha therapy of rheumatoid arthritis: what have we learned?" Annu Rev Immunol **19**: 163-96.
Fink, M. P., B. P. O'Sullivan, et al. (1993). "A novel leukotriene B4-receptor antagonist in endotoxin shock: a prospective, controlled trial in a porcine model." Crit Care Med **21**(12): 1825-37.
Flynn, M. A., M. Qiao, et al. (1999). "Avian osteoclast cells are stimulated to resorb calcified matrices by and possess receptors for leukotriene B4." Calcif Tissue Int **64**(2): 154-9.
Foley, D. L., J. M. Craig, et al. (2009). "Prospects for epigenetic epidemiology." Am J Epidemiol **169**(4): 389-400.
Fornerod, M., M. Ohno, et al. (1997). "CRM1 is an export receptor for leucine-rich nuclear export signals." Cell **90**(6): 1051-60.
Forse, R. A. (2000). "Biology of heterotrimeric G-protein signaling." Crit Care Med **28**(4 Suppl): N53-9.
Fraga, M. F., E. Ballestar, et al. (2005). "Epigenetic differences arise during the lifetime of monozygotic twins." Proc Natl Acad Sci U S A **102**(30): 10604-9.
Fraga, M. F. and M. Esteller (2002). "DNA methylation: a profile of methods and applications." Biotechniques **33**(3): 632, 634, 636-49.
Fretland, D. J., C. P. Anglin, et al. (1995). "Pharmacological activity of the second generation leukotriene B4 receptor antagonist, SC-53228: effects on acute colonic inflammation and hepatic function in rodents." Inflammation **19**(5): 503-15.
Frommer, M., L. E. McDonald, et al. (1992). "A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands." Proc Natl Acad Sci U S A **89**(5): 1827-31.
Fukuda, M., S. Asano, et al. (1997). "CRM1 is responsible for intracellular transport mediated by the nuclear export signal." Nature **390**(6657): 308-11.
Funk, C. D., S. Hoshiko, et al. (1989). "Characterization of the human 5-lipoxygenase gene." Proc Natl Acad Sci U S A **86**(8): 2587-91.
Gaffo, A., K. G. Saag, et al. (2006). "Treatment of rheumatoid arthritis." Am J Health Syst Pharm **63**(24): 2451-65.
Galli, S. J., S. Nakae, et al. (2005). "Mast cells in the development of adaptive immune responses." Nat Immunol **6**(2): 135-42.
Gauthier, M. L., C. R. Pickering, et al. (2005). "p38 regulates cyclooxygenase-2 in human mammary epithelial cells and is activated in premalignant tissue." Cancer

___ **65**(5): 1792-9.
Geborek, P., A. Bladstrom, et al. (2005). "Tumour necrosis factor blockers do not increase overall tumour risk in patients with rheumatoid arthritis, but may be associated with an increased risk of lymphomas." Ann Rheum Dis **64**(5): 699-703.
Giehl, K. (2005). "Oncogenic Ras in tumour progression and metastasis." Biol Chem **386**(3): 193-205.
Goldsmith, Z. G. and D. N. Dhanasekaran (2007). "G protein regulation of MAPK networks." Oncogene **26**(22): 3122-42.
Goll, M. G. and T. H. Bestor (2005). "Eukaryotic cytosine methyltransferases." Annu Rev Biochem **74**: 481-514.
Gollob, J. A., C. J. Sciambi, et al. (2006). "Phase I trial of sequential low-dose 5-aza-2'-deoxycytidine plus high-dose intravenous bolus interleukin-2 in patients with melanoma or renal cell carcinoma." Clin Cancer Res **12**(15): 4619-27.
Gopalakrishnan, S., B. O. Van Emburgh, et al. (2008). "DNA methylation in development and human disease." Mutat Res **647**(1-2): 30-8.
Gorlich, D. (1998). "Transport into and out of the cell nucleus." EMBO J **17**(10): 2721-7.
Gorlich, D., F. Vogel, et al. (1995). "Distinct functions for the two importin subunits in nuclear protein import." Nature **377**(6546): 246-8.
Goto, H., Y. Tomono, et al. (1999). "Identification of a novel phosphorylation site on histone H3 coupled with mitotic chromosome condensation." J Biol Chem **274**(36): 25543-9.
Gouble, A., S. Grazide, et al. (2002). "A new player in oncogenesis: AUF1/hnRNPD overexpression leads to tumorigenesis in transgenic mice." Cancer Res **62**(5): 1489-95.
Gray, S. G. and T. J. Ekstrom (2001). "The human histone deacetylase family." Exp Cell Res **262**(2): 75-83.
Greenberg, A. K., S. Basu, et al. (2002). "Selective p38 activation in human non-small cell lung cancer." Am J Respir Cell Mol Biol **26**(5): 558-64.
Grethe, S. and M. I. Porn-Ares (2006). "p38 MAPK regulates phosphorylation of Bad via PP2A-dependent suppression of the MEK1/2-ERK1/2 survival pathway in TNF-alpha induced endothelial apoptosis." Cell Signal **18**(4): 531-40.
Griffiths, R. J., E. R. Pettipher, et al. (1995). "Leukotriene B4 plays a critical role in the progression of collagen-induced arthritis." Proc Natl Acad Sci U S A **92**(2): 517-21.
Gupta, S., T. Barrett, et al. (1996). "Selective interaction of JNK protein kinase isoforms with transcription factors." EMBO J **15**(11): 2760-70.
Han, J., J. D. Lee, et al. (1994). "A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells." Science **265**(5173): 808-11.
Hashimoto, A., H. Endo, et al. (2003). "Differential expression of leukotriene B4 receptor subtypes (BLT1 and BLT2) in human synovial tissues and synovial fluid leukocytes of patients with rheumatoid arthritis." J Rheumatol **30**(8): 1712-8.
Hay, R. T. (2005). "SUMO: a history of modification." Mol Cell **18**(1): 1-12.
Hayashi, M. and J. D. Lee (2004). "Role of the BMK1/ERK5 signaling pathway: lessons from knockout mice." J Mol Med **82**(12): 800-8.
Hayatsu, H. (2008). "The bisulfite genomic sequencing used in the analysis of epigenetic states, a technique in the emerging environmental genotoxicology research." Mutat Res **659**(1-2): 77-82.

He, W., J. P. Pelletier, et al. (2002). "Synthesis of interleukin 1beta, tumor necrosis factor-alpha, and interstitial collagenase (MMP-1) is eicosanoid dependent in human osteoarthritis synovial membrane explants: interactions with antiinflammatory cytokines." J Rheumatol **29**(3): 546-53.
Hebert, M. J., T. Takano, et al. (1996). "Sequential morphologic events during apoptosis of human neutrophils. Modulation by lipoxygenase-derived eicosanoids." J Immunol **157**(7): 3105-15.
Hendzel, M. J., Y. Wei, et al. (1997). "Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation." Chromosoma **106**(6): 348-60.
Hennig, R., X. Z. Ding, et al. (2002). "5-Lipoxygenase and leukotriene B(4) receptor are expressed in human pancreatic cancers but not in pancreatic ducts in normal tissue." Am J Pathol **161**(2): 421-8.
Hewagama, A. and B. Richardson (2009). "The genetics and epigenetics of autoimmune diseases." J Autoimmun **33**(1): 3-11.
Hochberg, M. C., R. W. Chang, et al. (1992). "The American College of Rheumatology 1991 revised criteria for the classification of global functional status in rheumatoid arthritis." Arthritis Rheum **35**(5): 498-502.
Hochstrasser, M. (2000). "Biochemistry. All in the ubiquitin family." Science **289**(5479): 563-4.
Hochstrasser, M. (2000). "Evolution and function of ubiquitin-like protein-conjugation systems." Nat Cell Biol **2**(8): E153-7.
Holliday, R. (2006). "Epigenetics: a historical overview." Epigenetics **1**(2): 76-80.
Hood, J. K. and P. A. Silver (1999). "In or out? Regulating nuclear transport." Curr Opin Cell Biol **11**(2): 241-7.
Houseley, J., J. LaCava, et al. (2006). "RNA-quality control by the exosome." Nat Rev Mol Cell Biol **7**(7): 529-39.
Huang, C., K. Jacobson, et al. (2004). "MAP kinases and cell migration." J Cell Sci **117**(Pt 20): 4619-28.
Huber, L. C., M. Brock, et al. (2007). "Histone deacetylase/acetylase activity in total synovial tissue derived from rheumatoid arthritis and osteoarthritis patients." Arthritis Rheum **56**(4): 1087-93.
Humeniuk, R., P. J. Mishra, et al. (2009). "Molecular targets for epigenetic therapy of cancer." Curr Pharm Biotechnol **10**(2): 161-5.
Iliopoulos, D., K. N. Malizos, et al. (2007). "Epigenetic regulation of leptin affects MMP-13 expression in osteoarthritic chondrocytes: possible molecular target for osteoarthritis therapeutic intervention." Ann Rheum Dis **66**(12): 1616-21.
Illingworth, R. S. and A. P. Bird (2009). "CpG islands--'a rough guide'." FEBS Lett **583**(11): 1713-20.
Invernizzi, P. (2007). "The X chromosome in female-predominant autoimmune diseases." Ann N Y Acad Sci **1110**: 57-64.
Issa, J. P. (2000). "CpG-island methylation in aging and cancer." Curr Top Microbiol Immunol **249**: 101-18.
Issa, J. P., G. Garcia-Manero, et al. (2004). "Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2'-deoxycytidine (decitabine) in hematopoietic malignancies." Blood **103**(5): 1635-40.

Ito, K., G. Caramori, et al. (2002). "Expression and activity of histone deacetylases in human asthmatic airways." Am J Respir Crit Care Med **166**(3): 392-6.
Ito, K., M. Ito, et al. (2005). "Decreased histone deacetylase activity in chronic obstructive pulmonary disease." N Engl J Med **352**(19): 1967-76.
Iversen, L., K. Kragballe, et al. (1997). "Significance of leukotriene-A4 hydrolase in the pathogenesis of psoriasis." Skin Pharmacol **10**(4): 169-77.
Iwamoto, K. and T. Kato (2009). "Epigenetic profiling in schizophrenia and major mental disorders." Neuropsychobiology **60**(1): 5-11.
Izaurralde, E. and S. Adam (1998). "Transport of macromolecules between the nucleus and the cytoplasm." RNA **4**(4): 351-64.
Jaenisch, R. and A. Bird (2003). "Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals." Nat Genet **33 Suppl**: 245-54.
Jang, B. C., U. Munoz-Najar, et al. (2003). "Leptomycin B, an inhibitor of the nuclear export receptor CRM1, inhibits COX-2 expression." J Biol Chem **278**(5): 2773-6.
Jaskelioff, M. and C. L. Peterson (2003). "Chromatin and transcription: histones continue to make their marks." Nat Cell Biol **5**(5): 395-9.
Johansson, N., R. Ala-aho, et al. (2000). "Expression of collagenase-3 (MMP-13) and collagenase-1 (MMP-1) by transformed keratinocytes is dependent on the activity of p38 mitogen-activated protein kinase." J Cell Sci **113 Pt 2**: 227-35.
Johnson, E. N. and K. M. Druey (2002). "Heterotrimeric G protein signaling: role in asthma and allergic inflammation." J Allergy Clin Immunol **109**(4): 592-602.
Jones, O. T. (1993). "The mechanism of the production of superoxide by phagocytes." Mol Chem Neuropathol **19**(1-2): 177-84.
Jordana, M., A. D. Befus, et al. (1988). "Effect of histamine on proliferation of normal human adult lung fibroblasts." Thorax **43**(7): 552-8.
Junttila, M. R., R. Ala-Aho, et al. (2007). "p38alpha and p38delta mitogen-activated protein kinase isoforms regulate invasion and growth of head and neck squamous carcinoma cells." Oncogene **26**(36): 5267-79.
Jutel, M., K. Blaser, et al. (2005). "Histamine in allergic inflammation and immune modulation." Int Arch Allergy Immunol **137**(1): 82-92.
Kamohara, M., J. Takasaki, et al. (2000). "Molecular cloning and characterization of another leukotriene B4 receptor." J Biol Chem **275**(35): 27000-4.
Kaneda, M., M. Okano, et al. (2004). "Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting." Nature **429**(6994): 900-3.
Karouzakis, E., R. E. Gay, et al. (2009). "Epigenetic control in rheumatoid arthritis synovial fibroblasts." Nat Rev Rheumatol **5**(5): 266-72.
Kato, K., T. Yokomizo, et al. (2000). "Cell-specific transcriptional regulation of human leukotriene B(4) receptor gene." J Exp Med **192**(3): 413-20.
Kato, T. (2009). "Epigenomics in psychiatry." Neuropsychobiology **60**(1): 2-4.
Kawakami, A., Q. Tian, et al. (1992). "Identification and functional characterization of a TIA-1-related nucleolysin." Proc Natl Acad Sci U S A **89**(18): 8681-5.
Kedersha, N. L., M. Gupta, et al. (1999). "RNA-binding proteins TIA-1 and TIAR link the phosphorylation of eIF-2 alpha to the assembly of mammalian stress granules." J Cell Biol **147**(7): 1431-42.
Kelly, M., J. M. Hwang, et al. (2007). "Modulating leukocyte recruitment in inflammation." J Allergy Clin Immunol **120**(1): 3-10.

Kiener, H. P., R. Hofbauer, et al. (2000). "Tumor necrosis factor alpha promotes the expression of stem cell factor in synovial fibroblasts and their capacity to induce mast cell chemotaxis." Arthritis Rheum **43**(1): 164-74.
Kim, K. I., S. H. Baek, et al. (2002). "Versatile protein tag, SUMO: its enzymology and biological function." J Cell Physiol **191**(3): 257-68.
Kim, V. N. (2005). "Small RNAs: classification, biogenesis, and function." Mol Cells **19**(1): 1-15.
Kimura, H. and K. Shiota (2003). "Methyl-CpG-binding protein, MeCP2, is a target molecule for maintenance DNA methyltransferase, Dnmt1." J Biol Chem **278**(7): 4806-12.
Kinet, J. P. (1999). "The high-affinity IgE receptor (Fc epsilon RI): from physiology to pathology." Annu Rev Immunol **17**: 931-72.
Klose, R. J. and A. P. Bird (2006). "Genomic DNA methylation: the mark and its mediators." Trends Biochem Sci **31**(2): 89-97.
Koch, A. E. (2003). "Angiogenesis as a target in rheumatoid arthritis." Ann Rheum Dis **62 Suppl 2**: ii60-7.
Konstan, M. W., R. W. Walenga, et al. (1993). "Leukotriene B4 markedly elevated in the epithelial lining fluid of patients with cystic fibrosis." Am Rev Respir Dis **148**(4 Pt 1): 896-901.
Kornberg, R. D. (1974). "Chromatin structure: a repeating unit of histones and DNA." Science **184**(139): 868-71.
Kouzarides, T. (2007). "Chromatin modifications and their function." Cell **128**(4): 693-705.
Kristensen, L. S. and L. L. Hansen (2009). "PCR-based methods for detecting single-locus DNA methylation biomarkers in cancer diagnostics, prognostics, and response to treatment." Clin Chem **55**(8): 1471-83.
Krutzfeldt, J. and M. Stoffel (2006). "MicroRNAs: a new class of regulatory genes affecting metabolism." Cell Metab **4**(1): 9-12.
Kulmacz, R. J., W. A. van der Donk, et al. (2003). "Comparison of the properties of prostaglandin H synthase-1 and -2." Prog Lipid Res **42**(5): 377-404.
Kunkel, H. G., E. C. Franklin, et al. (1959). "Studies on the isolation and characterization of the "rheumatoid factor"." J Clin Invest **38**(2): 424-34.
Kyriakis, J. M. and J. Avruch (2001). "Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation." Physiol Rev **81**(2): 807-69.
Lai, W. S., E. A. Kennington, et al. (2003). "Tristetraprolin and its family members can promote the cell-free deadenylation of AU-rich element-containing mRNAs by poly(A) ribonuclease." Mol Cell Biol **23**(11): 3798-812.
Lai, W. S., J. S. Parker, et al. (2006). "Novel mRNA targets for tristetraprolin (TTP) identified by global analysis of stabilized transcripts in TTP-deficient fibroblasts." Mol Cell Biol **26**(24): 9196-208.
Laird, P. W. (2003). "The power and the promise of DNA methylation markers." Nat Rev Cancer **3**(4): 253-66.
Lan, K. P., C. J. Wang, et al. (2004). "Induced eosinophilia and proliferation in Angiostrongylus cantonensis-infected mouse brain are associated with the induction of JAK/STAT1, IAP/NF-kappaB and MEKK1/JNK signals." J Helminthol **78**(4): 311-7.

Landry, J. R., D. L. Mager, et al. (2003). "Complex controls: the role of alternative promoters in mammalian genomes." Trends Genet **19**(11): 640-8.
Lawrence, M. C., K. McGlynn, et al. (2007). "Differential regulation of CHOP-10/GADD153 gene expression by MAPK signaling in pancreatic beta-cells." Proc Natl Acad Sci U S A **104**(28): 11518-25.
Lee, D. M., D. S. Friend, et al. (2002). "Mast cells: a cellular link between autoantibodies and inflammatory arthritis." Science **297**(5587): 1689-92.
Lee, D. Y., C. Teyssier, et al. (2005). "Role of protein methylation in regulation of transcription." Endocr Rev **26**(2): 147-70.
Leppa, S. and D. Bohmann (1999). "Diverse functions of JNK signaling and c-Jun in stress response and apoptosis." Oncogene **18**(45): 6158-62.
Levy, B. D., C. B. Clish, et al. (2001). "Lipid mediator class switching during acute inflammation: signals in resolution." Nat Immunol **2**(7): 612-9.
Levy, N. S., S. Chung, et al. (1998). "Hypoxic stabilization of vascular endothelial growth factor mRNA by the RNA-binding protein HuR." J Biol Chem **273**(11): 6417-23.
Li, C. Y. and J. Y. Baek (2002). "Mastocytosis and fibrosis: role of cytokines." Int Arch Allergy Immunol **127**(2): 123-6.
Li, E., T. H. Bestor, et al. (1992). "Targeted mutation of the DNA methyltransferase gene results in embryonic lethality." Cell **69**(6): 915-26.
Li, N. L., D. Q. Zhang, et al. (2000). "Isolation and characteristics of autoreactive T cells specific to aggrecan G1 domain from rheumatoid arthritis patients." Cell Res **10**(1): 39-49.
Li, Y., A. Ferrante, et al. (1996). "Neutrophil oxygen radical generation. Synergistic responses to tumor necrosis factor and mono/polyunsaturated fatty acids." J Clin Invest **97**(7): 1605-9.
Liao, K. P., L. Alfredsson, et al. (2009). "Environmental influences on risk for rheumatoid arthritis." Curr Opin Rheumatol **21**(3): 279-83.
Lipsky, P. E. (1999). "Role of cyclooxygenase-1 and -2 in health and disease." Am J Orthop **28**(3 Suppl): 8-12.
Liu, J. and A. Lin (2005). "Role of JNK activation in apoptosis: a double-edged sword." Cell Res **15**(1): 36-42.
Loflin, P., C. Y. Chen, et al. (1999). "Unraveling a cytoplasmic role for hnRNP D in the in vivo mRNA destabilization directed by the AU-rich element." Genes Dev **13**(14): 1884-97.
Lu, J. Y. and R. J. Schneider (2004). "Tissue distribution of AU-rich mRNA-binding proteins involved in regulation of mRNA decay." J Biol Chem **279**(13): 12974-9.
Lunde, B. M., C. Moore, et al. (2007). "RNA-binding proteins: modular design for efficient function." Nat Rev Mol Cell Biol **8**(6): 479-90.
Luster, A. D. (1998). "Chemokines--chemotactic cytokines that mediate inflammation." N Engl J Med **338**(7): 436-45.
Ma, W. J., S. Cheng, et al. (1996). "Cloning and characterization of HuR, a ubiquitously expressed Elav-like protein." J Biol Chem **271**(14): 8144-51.
Mahtani, K. R., M. Brook, et al. (2001). "Mitogen-activated protein kinase p38 controls the expression and posttranslational modification of tristetraprolin, a regulator of tumor necrosis factor alpha mRNA stability." Mol Cell Biol **21**(19): 6461-9.
Mancini, A. and J. A. Di Battista (2007). "Tristetraprolin: a molecular link in

prostaglandin E2-mediated COX-2 post-transcriptional gene regulation." Inflamm. Res. **56**(**3 Suppl**): S374.
Mancini, A. and J. A. Di Battista (2008). "Tristetraprolin: a prostaglandin E2-responsive bifunctional regulator of cyclooxygenase-2 expression." Osteoarthritis and Cartilage **16**(**4 Suppl**): S16.
Mancuso, P., P. Nana-Sinkam, et al. (2001). "Leukotriene B4 augments neutrophil phagocytosis of Klebsiella pneumoniae." Infect Immun **69**(4): 2011-6.
Marmorstein, R. and R. C. Trievel (2009). "Histone modifying enzymes: structures, mechanisms, and specificities." Biochim Biophys Acta **1789**(1): 58-68.
Marshall, J. S. (2004). "Mast-cell responses to pathogens." Nat Rev Immunol **4**(10): 787-99.
Martin, T. R., B. P. Pistorese, et al. (1989). "Effects of leukotriene B4 in the human lung. Recruitment of neutrophils into the alveolar spaces without a change in protein permeability." J Clin Invest **84**(5): 1609-19.
Matsukawa, A., C. M. Hogaboam, et al. (1999). "Endogenous monocyte chemoattractant protein-1 (MCP-1) protects mice in a model of acute septic peritonitis: cross-talk between MCP-1 and leukotriene B4." J Immunol **163**(11): 6148-54.
Matsumoto, I., D. M. Lee, et al. (2003). "Low prevalence of antibodies to glucose-6-phosphate isomerase in patients with rheumatoid arthritis and a spectrum of other chronic autoimmune disorders." Arthritis Rheum **48**(4): 944-54.
Matsumoto, I., M. Maccioni, et al. (2002). "How antibodies to a ubiquitous cytoplasmic enzyme may provoke joint-specific autoimmune disease." Nat Immunol **3**(4): 360-5.
Mattaj, I. W. and L. Englmeier (1998). "Nucleocytoplasmic transport: the soluble phase." Annu Rev Biochem **67**: 265-306.
Mazan-Mamczarz, K., Y. Kuwano, et al. (2009). "Identification of a signature motif in target mRNAs of RNA-binding protein AUF1." Nucleic Acids Res **37**(1): 204-14.
McInnes, I. B. and G. Schett (2007). "Cytokines in the pathogenesis of rheumatoid arthritis." Nat Rev Immunol **7**(6): 429-42.
McKay, M. M. and D. K. Morrison (2007). "Integrating signals from RTKs to ERK/MAPK." Oncogene **26**(22): 3113-21.
Mei, S., A. D. Ho, et al. (2004). "Role of histone deacetylase inhibitors in the treatment of cancer (Review)." Int J Oncol **25**(6): 1509-19.
Meissner, T., E. Krause, et al. (2004). "Ratjadone and leptomycin B block CRM1-dependent nuclear export by identical mechanisms." FEBS Lett **576**(1-2): 27-30.
Mekori, Y. A. (2004). "The mastocyte: the "other" inflammatory cell in immunopathogenesis." J Allergy Clin Immunol **114**(1): 52-7.
Mekori, Y. A. and D. D. Metcalfe (1999). "Mast cell-T cell interactions." J Allergy Clin Immunol **104**(3 Pt 1): 517-23.
Melien, O. (2007). "Heterotrimeric G proteins and disease." Methods Mol Biol **361**: 119-44.
Metcalfe, D. D., D. Baram, et al. (1997). "Mast cells." Physiol Rev **77**(4): 1033-79.
Metz, M., M. A. Grimbaldeston, et al. (2007). "Mast cells in the promotion and limitation of chronic inflammation." Immunol Rev **217**: 304-28.
Metzger, E., M. Wissmann, et al. (2005). "LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription." Nature **437**(7057): 436-9.
Michael, W. M., M. Choi, et al. (1995). "A nuclear export signal in hnRNP A1: a signal-

mediated, temperature-dependent nuclear protein export pathway." Cell **83**(3): 415-22.
Ming, X. F., G. Stoecklin, et al. (2001). "Parallel and independent regulation of interleukin-3 mRNA turnover by phosphatidylinositol 3-kinase and p38 mitogen-activated protein kinase." Mol Cell Biol **21**(17): 5778-89.
Miranda, T. B., C. C. Cortez, et al. (2009). "DZNep is a global histone methylation inhibitor that reactivates developmental genes not silenced by DNA methylation." Mol Cancer Ther **8**(6): 1579-88.
Montuschi, P. and P. J. Barnes (2002). "Exhaled leukotrienes and prostaglandins in asthma." J Allergy Clin Immunol **109**(4): 615-20.
Mor, A. and M. R. Philips (2006). "Compartmentalized Ras/MAPK signaling." Annu Rev Immunol **24**: 771-800.
Morita, H., K. Takeda, et al. (1999). "Immunosuppressive effect of leukotriene B(4) receptor antagonist in vitro." Biochem Biophys Res Commun **264**(2): 321-6.
Mukherjee, D., M. Gao, et al. (2002). "The mammalian exosome mediates the efficient degradation of mRNAs that contain AU-rich elements." EMBO J **21**(1-2): 165-74.
Muller-Ladner, U., J. Kriegsmann, et al. (1996). "Synovial fibroblasts of patients with rheumatoid arthritis attach to and invade normal human cartilage when engrafted into SCID mice." Am J Pathol **149**(5): 1607-15.
Murayama, T. and M. Ui (1983). "Loss of the inhibitory function of the guanine nucleotide regulatory component of adenylate cyclase due to its ADP ribosylation by islet-activating protein, pertussis toxin, in adipocyte membranes." J Biol Chem **258**(5): 3319-26.
Nakielny, S. and G. Dreyfuss (1997). "Nuclear export of proteins and RNAs." Curr Opin Cell Biol **9**(3): 420-9.
Nandi, P., G. H. Kingsley, et al. (2008). "Disease-modifying antirheumatic drugs other than methotrexate in rheumatoid arthritis and seronegative arthritis." Curr Opin Rheumatol **20**(3): 251-6.
Nathan, D., K. Ingvarsdottir, et al. (2006). "Histone sumoylation is a negative regulator in Saccharomyces cerevisiae and shows dynamic interplay with positive-acting histone modifications." Genes Dev **20**(8): 966-76.
Nepom, G. T., P. Byers, et al. (1989). "HLA genes associated with rheumatoid arthritis. Identification of susceptibility alleles using specific oligonucleotide probes." Arthritis Rheum **32**(1): 15-21.
Neu, I., J. Mallinger, et al. (1992). "Leukotrienes in the cerebrospinal fluid of multiple sclerosis patients." Acta Neurol Scand **86**(6): 586-7.
Neumann, E., S. R. Barnum, et al. (2002). "Local production of complement proteins in rheumatoid arthritis synovium." Arthritis Rheum **46**(4): 934-45.
Neves, S. R., P. T. Ram, et al. (2002). "G protein pathways." Science **296**(5573): 1636-9.
Noiri, E., T. Yokomizo, et al. (2000). "An in vivo approach showing the chemotactic activity of leukotriene B(4) in acute renal ischemic-reperfusion injury." Proc Natl Acad Sci U S A **97**(2): 823-8.
O'Dell, J. R. (2004). "Therapeutic strategies for rheumatoid arthritis." N Engl J Med **350**(25): 2591-602.
O'Garra, A. and P. Vieira (2004). "Regulatory T cells and mechanisms of immune system control." Nat Med **10**(8): 801-5.
Oda, A., M. Miyata, et al. (1994). "Antibodies to 65Kd heat-shock protein were elevated

in rheumatoid arthritis." Clin Rheumatol **13**(2): 261-4.
Offermanns, S. (2001). "In vivo functions of heterotrimeric G-proteins: studies in Galpha-deficient mice." Oncogene **20**(13): 1635-42.
Ogilvie, R. L., M. Abelson, et al. (2005). "Tristetraprolin down-regulates IL-2 gene expression through AU-rich element-mediated mRNA decay." J Immunol **174**(2): 953-61.
Okano, M., D. W. Bell, et al. (1999). "DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development." Cell **99**(3): 247-57.
Okuno, T., Y. Iizuka, et al. (2008). "12(S)-Hydroxyheptadeca-5Z, 8E, 10E-trienoic acid is a natural ligand for leukotriene B4 receptor 2." J Exp Med **205**(4): 759-66.
Okuno, T., T. Yokomizo, et al. (2005). "Leukotriene B4 receptor and the function of its helix 8." J Biol Chem **280**(37): 32049-52.
Orton, R. J., O. E. Sturm, et al. (2005). "Computational modelling of the receptor-tyrosine-kinase-activated MAPK pathway." Biochem J **392**(Pt 2): 249-61.
Otto, J. C. and W. L. Smith (1994). "The orientation of prostaglandin endoperoxide synthases-1 and -2 in the endoplasmic reticulum." J Biol Chem **269**(31): 19868-75.
Pages, G., P. Lenormand, et al. (1993). "Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation." Proc Natl Acad Sci U S A **90**(18): 8319-23.
Pap, T., U. Muller-Ladner, et al. (2000). "Fibroblast biology. Role of synovial fibroblasts in the pathogenesis of rheumatoid arthritis." Arthritis Res **2**(5): 361-7.
Peng, S. S., C. Y. Chen, et al. (1998). "RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein." EMBO J **17**(12): 3461-70.
Phillips, K., N. Kedersha, et al. (2004). "Arthritis suppressor genes TIA-1 and TTP dampen the expression of tumor necrosis factor alpha, cyclooxygenase 2, and inflammatory arthritis." Proc Natl Acad Sci U S A **101**(7): 2011-6.
Phillips, R. S., S. B. Ramos, et al. (2002). "Members of the tristetraprolin family of tandem CCCH zinc finger proteins exhibit CRM1-dependent nucleocytoplasmic shuttling." J Biol Chem **277**(13): 11606-13.
Picot, D., P. J. Loll, et al. (1994). "The X-ray crystal structure of the membrane protein prostaglandin H2 synthase-1." Nature **367**(6460): 243-9.
Piecyk, M., S. Wax, et al. (2000). "TIA-1 is a translational silencer that selectively regulates the expression of TNF-alpha." EMBO J **19**(15): 4154-63.
Pierce, K. L., R. T. Premont, et al. (2002). "Seven-transmembrane receptors." Nat Rev Mol Cell Biol **3**(9): 639-50.
Pillai, R. S., S. N. Bhattacharyya, et al. (2007). "Repression of protein synthesis by miRNAs: how many mechanisms?" Trends Cell Biol **17**(3): 118-26.
Pollard, V. W., W. M. Michael, et al. (1996). "A novel receptor-mediated nuclear protein import pathway." Cell **86**(6): 985-94.
Pope, R. M. (2002). "Apoptosis as a therapeutic tool in rheumatoid arthritis." Nat Rev Immunol **2**(7): 527-35.
Porras, A., S. Zuluaga, et al. (2004). "P38 alpha mitogen-activated protein kinase sensitizes cells to apoptosis induced by different stimuli." Mol Biol Cell **15**(2): 922-33.
Poubelle, P. E., J. Stankova, et al. (1991). "Leukotriene B4 up-regulates IL-6 rather than

IL-1 synthesis in human monocytes." Agents Actions **34**(1-2): 42-5.
Preuss, U., G. Landsberg, et al. (2003). "Novel mitosis-specific phosphorylation of histone H3 at Thr11 mediated by Dlk/ZIP kinase." Nucleic Acids Res **31**(3): 878-85.
Rae, S. A. and M. J. Smith (1981). "The stimulation of lysosomal enzyme secretion from human polymorphonuclear leucocytes by leukotriene B4." J Pharm Pharmacol **33**(9): 616-7.
Raman, M., W. Chen, et al. (2007). "Differential regulation and properties of MAPKs." Oncogene **26**(22): 3100-12.
Rantapaa-Dahlqvist, S., B. A. de Jong, et al. (2003). "Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis." Arthritis Rheum **48**(10): 2741-9.
Rao, D. D., J. S. Vorhies, et al. (2009). "siRNA vs. shRNA: similarities and differences." Adv Drug Deliv Rev **61**(9): 746-59.
Reddy, K. B., S. M. Nabha, et al. (2003). "Role of MAP kinase in tumor progression and invasion." Cancer Metastasis Rev **22**(4): 395-403.
Ristimaki, A., S. Garfinkel, et al. (1994). "Induction of cyclooxygenase-2 by interleukin-1 alpha. Evidence for post-transcriptional regulation." J Biol Chem **269**(16): 11769-75.
Ritchlin, C. (2000). "Fibroblast biology. Effector signals released by the synovial fibroblast in arthritis." Arthritis Res **2**(5): 356-60.
Roberts, P. J. and C. J. Der (2007). "Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer." Oncogene **26**(22): 3291-310.
Robertson, K. D. (2005). "DNA methylation and human disease." Nat Rev Genet **6**(8): 597-610.
Robertson, K. D. and A. P. Wolffe (2000). "DNA methylation in health and disease." Nat Rev Genet **1**(1): 11-9.
Rocca, B. and G. A. FitzGerald (2002). "Cyclooxygenases and prostaglandins: shaping up the immune response." Int Immunopharmacol **2**(5): 603-30.
Rodriguez, A., E. Vigorito, et al. (2007). "Requirement of bic/microRNA-155 for normal immune function." Science **316**(5824): 608-11.
Rola-Pleszczynski, M., L. Gagnon, et al. (1983). "Leukotriene B4 augments human natural cytotoxic cell activity." Biochem Biophys Res Commun **113**(2): 531-7.
Rose, A. B. (2008). "Intron-mediated regulation of gene expression." Curr Top Microbiol Immunol **326**: 277-90.
Rouse, J., P. Cohen, et al. (1994). "A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins." Cell **78**(6): 1027-37.
Roux, P. P. and J. Blenis (2004). "ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions." Microbiol Mol Biol Rev **68**(2): 320-44.
Rubbert-Roth, A. and A. Finckh (2009). "Treatment options in patients with rheumatoid arthritis failing initial TNF inhibitor therapy: a critical review." Arthritis Res Ther **11 Suppl 1**: S1.
Rumsaeng, V., W. W. Cruikshank, et al. (1997). "Human mast cells produce the CD4+ T lymphocyte chemoattractant factor, IL-16." J Immunol **159**(6): 2904-10.
Saklatvala, J. (2004). "The p38 MAP kinase pathway as a therapeutic target in

inflammatory disease." Curr Opin Pharmacol **4**(4): 372-7.
Salmon, M., D. Scheel-Toellner, et al. (1997). "Inhibition of T cell apoptosis in the rheumatoid synovium." J Clin Invest **99**(3): 439-46.
Sanders, R. D., D. Brian, et al. (2008). "G-protein-coupled receptors." Handb Exp Pharmacol(182): 93-117.
Sandler, H. and G. Stoecklin (2008). "Control of mRNA decay by phosphorylation of tristetraprolin." Biochem Soc Trans **36**(Pt 3): 491-6.
Sangha, O. (2000). "Epidemiology of rheumatic diseases." Rheumatology (Oxford) **39 Suppl 2**: 3-12.
Sarkar, B., J. Y. Lu, et al. (2003). "Nuclear import and export functions in the different isoforms of the AUF1/heterogeneous nuclear ribonucleoprotein protein family." J Biol Chem **278**(23): 20700-7.
Sarkar, B., Q. Xi, et al. (2003). "Selective degradation of AU-rich mRNAs promoted by the p37 AUF1 protein isoform." Mol Cell Biol **23**(18): 6685-93.
Sawaoka, H., D. A. Dixon, et al. (2003). "Tristetraprolin binds to the 3'-untranslated region of cyclooxygenase-2 mRNA. A polyadenylation variant in a cancer cell line lacks the binding site." J Biol Chem **278**(16): 13928-35.
Scheven, B. A., J. S. Milne, et al. (1999). "Macrophage-inflammatory protein-1alpha regulates preosteoclast differentiation in vitro." Biochem Biophys Res Commun **254**(3): 773-8.
Schulze-Koops, H. and J. R. Kalden (2001). "The balance of Th1/Th2 cytokines in rheumatoid arthritis." Best Pract Res Clin Rheumatol **15**(5): 677-91.
Schwartsmann, G., H. Schunemann, et al. (2000). "A phase I trial of cisplatin plus decitabine, a new DNA-hypomethylating agent, in patients with advanced solid tumors and a follow-up early phase II evaluation in patients with inoperable non-small cell lung cancer." Invest New Drugs **18**(1): 83-91.
Schwartz, S., E. Meshorer, et al. (2009). "Chromatin organization marks exon-intron structure." Nat Struct Mol Biol **16**(9): 990-5.
Sebolt-Leopold, J. S., D. T. Dudley, et al. (1999). "Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo." Nat Med **5**(7): 810-6.
Secrist, J. P., X. Zhou, et al. (2003). "HDAC inhibitors for the treatment of cancer." Curr Opin Investig Drugs **4**(12): 1422-7.
Sengupta, S., B. C. Jang, et al. (2003). "The RNA-binding protein HuR regulates the expression of cyclooxygenase-2." J Biol Chem **278**(27): 25227-33.
Serhan, C. N., N. Chiang, et al. (2008). "Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators." Nat Rev Immunol **8**(5): 349-61.
Serhan, C. N., J. Z. Haeggstrom, et al. (1996). "Lipid mediator networks in cell signaling: update and impact of cytokines." FASEB J **10**(10): 1147-58.
Sharon, P. and W. F. Stenson (1984). "Enhanced synthesis of leukotriene B4 by colonic mucosa in inflammatory bowel disease." Gastroenterology **86**(3): 453-60.
Shi, Y., F. Lan, et al. (2004). "Histone demethylation mediated by the nuclear amine oxidase homolog LSD1." Cell **119**(7): 941-53.
Shigeyama, Y., T. Pap, et al. (2000). "Expression of osteoclast differentiation factor in rheumatoid arthritis." Arthritis Rheum **43**(11): 2523-30.
Shouda, T., T. Yoshida, et al. (2001). "Induction of the cytokine signal regulator SOCS3/CIS3 as a therapeutic strategy for treating inflammatory arthritis." J Clin Invest **108**(12): 1781-8.

Shyu, A. B. and M. F. Wilkinson (2000). "The double lives of shuttling mRNA binding proteins." Cell **102**(2): 135-8.
Slotkin, R. K. and R. Martienssen (2007). "Transposable elements and the epigenetic regulation of the genome." Nat Rev Genet **8**(4): 272-85.
Smith, W. L. (1992). "Prostanoid biosynthesis and mechanisms of action." Am J Physiol **263**(2 Pt 2): F181-91.
Smolen, J. S., D. Aletaha, et al. (2007). "New therapies for treatment of rheumatoid arthritis." Lancet **370**(9602): 1861-74.
Smolen, J. S. and G. Steiner (2003). "Therapeutic strategies for rheumatoid arthritis." Nat Rev Drug Discov **2**(6): 473-88.
Smyth, E. M., T. Grosser, et al. (2009). "Prostanoids in health and disease." J Lipid Res **50 Suppl**: S423-8.
Sokka, T., B. Abelson, et al. (2008). "Mortality in rheumatoid arthritis: 2008 update." Clin Exp Rheumatol **26**(5 Suppl 51): S35-61.
Song, L. and R. S. Tuan (2006). "MicroRNAs and cell differentiation in mammalian development." Birth Defects Res C Embryo Today **78**(2): 140-9.
Spencer, A. G., E. Thuresson, et al. (1999). "The membrane binding domains of prostaglandin endoperoxide H synthases 1 and 2. Peptide mapping and mutational analysis." J Biol Chem **274**(46): 32936-42.
Sperling, R. I., A. I. Benincaso, et al. (1992). "Acute and chronic suppression of leukotriene B4 synthesis ex vivo in neutrophils from patients with rheumatoid arthritis beginning treatment with methotrexate." Arthritis Rheum **35**(4): 376-84.
Stade, K., C. S. Ford, et al. (1997). "Exportin 1 (Crm1p) is an essential nuclear export factor." Cell **90**(6): 1041-50.
Stamp, L. K., M. J. James, et al. (2004). "Interleukin-17: the missing link between T-cell accumulation and effector cell actions in rheumatoid arthritis?" Immunol Cell Biol **82**(1): 1-9.
Stastny, P. (1976). "Mixed lymphocyte cultures in rheumatoid arthritis." J Clin Invest **57**(5): 1148-57.
Steiner, G. and J. Smolen (2002). "Autoantibodies in rheumatoid arthritis and their clinical significance." Arthritis Res **4 Suppl 2**: S1-5.
Steiner, G., M. Tohidast-Akrad, et al. (1999). "Cytokine production by synovial T cells in rheumatoid arthritis." Rheumatology (Oxford) **38**(3): 202-13.
Sterner, D. E. and S. L. Berger (2000). "Acetylation of histones and transcription-related factors." Microbiol Mol Biol Rev **64**(2): 435-59.
Stuart, J. M., A. S. Townes, et al. (1984). "Collagen autoimmune arthritis." Annu Rev Immunol **2**: 199-218.
Stuart, R. W. and M. K. Racke (2002). "Targeting T cell costimulation in autoimmune disease." Expert Opin Ther Targets **6**(3): 275-89.
Sully, G., J. L. Dean, et al. (2004). "Structural and functional dissection of a conserved destabilizing element of cyclo-oxygenase-2 mRNA: evidence against the involvement of AUF-1 [AU-rich element/poly(U)-binding/degradation factor-1], AUF-2, tristetraprolin, HuR (Hu antigen R) or FBP1 (far-upstream-sequence-element-binding protein 1)." Biochem J **377**(Pt 3): 629-39.
Sun, L., G. Stoecklin, et al. (2007). "Tristetraprolin (TTP)-14-3-3 complex formation protects TTP from dephosphorylation by protein phosphatase 2a and stabilizes tumor necrosis factor-alpha mRNA." J Biol Chem **282**(6): 3766-77.

Suter, M. A. and K. M. Aagaard-Tillery (2009). "Environmental influences on epigenetic profiles." Semin Reprod Med **27**(5): 380-90.

Suzuki, T., K. T. J, et al. (2002). "Phosphorylation of three regulatory serines of Tob by Erk1 and Erk2 is required for Ras-mediated cell proliferation and transformation." Genes Dev **16**(11): 1356-70.

Tager, A. M., S. K. Bromley, et al. (2003). "Leukotriene B4 receptor BLT1 mediates early effector T cell recruitment." Nat Immunol **4**(10): 982-90.

Tanabe, T. and N. Tohnai (2002). "Cyclooxygenase isozymes and their gene structures and expression." Prostaglandins Other Lipid Mediat **68-69**: 95-114.

Taupin, J. L., Q. Tian, et al. (1995). "The RNA-binding protein TIAR is translocated from the nucleus to the cytoplasm during Fas-mediated apoptotic cell death." Proc Natl Acad Sci U S A **92**(5): 1629-33.

Taylor, G. A., M. J. Thompson, et al. (1995). "Phosphorylation of tristetraprolin, a potential zinc finger transcription factor, by mitogen stimulation in intact cells and by mitogen-activated protein kinase in vitro." J Biol Chem **270**(22): 13341-7.

Taylor, G. A., M. J. Thompson, et al. (1996). "Mitogens stimulate the rapid nuclear to cytosolic translocation of tristetraprolin, a potential zinc-finger transcription factor." Mol Endocrinol **10**(2): 140-6.

Tedla, N., H. W. Wang, et al. (1998). "Regulation of T lymphocyte trafficking into lymph nodes during an immune response by the chemokines macrophage inflammatory protein (MIP)-1 alpha and MIP-1 beta." J Immunol **161**(10): 5663-72.

Tetlow, L. C., D. J. Adlam, et al. (2001). "Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes." Arthritis Rheum **44**(3): 585-94.

Tian, Q., M. Streuli, et al. (1991). "A polyadenylate binding protein localized to the granules of cytolytic lymphocytes induces DNA fragmentation in target cells." Cell **67**(3): 629-39.

Toda, A., T. Yokomizo, et al. (2002). "Leukotriene B4 receptors." Prostaglandins Other Lipid Mediat **68-69**: 575-85.

Tsai, S. C., R. Adamik, et al. (1984). "Effects of guanyl nucleotides and rhodopsin on ADP-ribosylation of the inhibitory GTP-binding component of adenylate cyclase by pertussis toxin." J Biol Chem **259**(24): 15320-3.

Tsankova, N., W. Renthal, et al. (2007). "Epigenetic regulation in psychiatric disorders." Nat Rev Neurosci **8**(5): 355-67.

Turner, C. R., R. Breslow, et al. (1996). "In vitro and in vivo effects of leukotriene B4 antagonism in a primate model of asthma." J Clin Invest **97**(2): 381-7.

Uhl, J., N. Klan, et al. (2002). "The 5-lipoxygenase promoter is regulated by DNA methylation." J Biol Chem **277**(6): 4374-9.

Ulrey, C. L., L. Liu, et al. (2005). "The impact of metabolism on DNA methylation." Hum Mol Genet **14 Spec No 1**: R139-47.

van Hoof, A. and R. Parker (2002). "Messenger RNA degradation: beginning at the end." Curr Biol **12**(8): R285-7.

van Pelt, J. P., E. M. de Jong, et al. (1997). "The regulation of CD11b integrin levels on human blood leukocytes and leukotriene B4-stimulated skin by a specific leukotriene B4 receptor antagonist (LY293111)." Biochem Pharmacol **53**(7): 1005-12.

Verheijden, G. F., A. W. Rijnders, et al. (1997). "Human cartilage glycoprotein-39 as a candidate autoantigen in rheumatoid arthritis." Arthritis Rheum **40**(6): 1115-25.
Viola, A. and A. D. Luster (2008). "Chemokines and their receptors: drug targets in immunity and inflammation." Annu Rev Pharmacol Toxicol **48**: 171-97.
Vogiatzi, P., P. Aimola, et al. (2007). "Epigenome-derived drugs: recent advances and future perspectives." Drug News Perspect **20**(10): 627-33.
Vossenaar, E. R., A. J. Zendman, et al. (2004). "Citrullination, a possible functional link between susceptibility genes and rheumatoid arthritis." Arthritis Res Ther **6**(1): 1-5.
Wagner, B. J., C. T. DeMaria, et al. (1998). "Structure and genomic organization of the human AUF1 gene: alternative pre-mRNA splicing generates four protein isoforms." Genomics **48**(2): 195-202.
Walton, K. J., J. M. Duncan, et al. (2002). "Heparin acts synergistically with interleukin-11 to induce STAT3 activation and in vitro osteoclast formation." Blood **100**(7): 2530-6.
Wang, H., L. Zhai, et al. (2006). "Histone H3 and H4 ubiquitylation by the CUL4-DDB-ROC1 ubiquitin ligase facilitates cellular response to DNA damage." Mol Cell **22**(3): 383-94.
Wang, W., M. C. Caldwell, et al. (2000). "HuR regulates cyclin A and cyclin B1 mRNA stability during cell proliferation." EMBO J **19**(10): 2340-50.
Wang, W., H. Furneaux, et al. (2000). "HuR regulates p21 mRNA stabilization by UV light." Mol Cell Biol **20**(3): 760-9.
Wellbrock, C., M. Karasarides, et al. (2004). "The RAF proteins take centre stage." Nat Rev Mol Cell Biol **5**(11): 875-85.
Wessels, J. A., T. W. Huizinga, et al. (2008). "Recent insights in the pharmacological actions of methotrexate in the treatment of rheumatoid arthritis." Rheumatology (Oxford) **47**(3): 249-55.
Weston, C. R. and R. J. Davis (2007). "The JNK signal transduction pathway." Curr Opin Cell Biol **19**(2): 142-9.
Weyand, C. M., K. C. Hicok, et al. (1992). "The influence of HLA-DRB1 genes on disease severity in rheumatoid arthritis." Ann Intern Med **117**(10): 801-6.
Wilson, G. M., J. Lu, et al. (2003). "Regulation of A + U-rich element-directed mRNA turnover involving reversible phosphorylation of AUF1." J Biol Chem **278**(35): 33029-38.
Wilusz, C. J., M. Wormington, et al. (2001). "The cap-to-tail guide to mRNA turnover." Nat Rev Mol Cell Biol **2**(4): 237-46.
Wissmann, M., N. Yin, et al. (2007). "Cooperative demethylation by JMJD2C and LSD1 promotes androgen receptor-dependent gene expression." Nat Cell Biol **9**(3): 347-53.
Wojdacz, T. K. and L. L. Hansen (2006). "Techniques used in studies of age-related DNA methylation changes." Ann N Y Acad Sci **1067**: 479-87.
Wong, N., R. Morley, et al. (2008). "Archived Guthrie blood spots as a novel source for quantitative DNA methylation analysis." Biotechniques **45**(4): 423-30.
Wu, C. and J. R. Morris (2001). "Genes, genetics, and epigenetics: a correspondence." Science **293**(5532): 1103-5.
Xu, N., C. Y. Chen, et al. (2001). "Versatile role for hnRNP D isoforms in the differential regulation of cytoplasmic mRNA turnover." Mol Cell Biol **21**(20):

6960-71.
Xu, S., H. Lu, et al. (2010). "Regulation of TNFalpha and IL1beta in rheumatoid arthritis synovial fibroblasts by leukotriene B4." Rheumatol Int **30**(9): 1183-9.
Yamashita, A., T. C. Chang, et al. (2005). "Concerted action of poly(A) nucleases and decapping enzyme in mammalian mRNA turnover." Nat Struct Mol Biol **12**(12): 1054-63.
Yang, X. J. (2004). "Lysine acetylation and the bromodomain: a new partnership for signaling." Bioessays **26**(10): 1076-87.
Yoder, J. A., C. P. Walsh, et al. (1997). "Cytosine methylation and the ecology of intragenomic parasites." Trends Genet **13**(8): 335-40.
Yokomizo, T., T. Izumi, et al. (1997). "A G-protein-coupled receptor for leukotriene B4 that mediates chemotaxis." Nature **387**(6633): 620-4.
Yokomizo, T., K. Kato, et al. (2000). "A second leukotriene B(4) receptor, BLT2. A new therapeutic target in inflammation and immunological disorders." J Exp Med **192**(3): 421-32.
Yoon, S. and R. Seger (2006). "The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions." Growth Factors **24**(1): 21-44.
Yoshimura, A., T. Naka, et al. (2007). "SOCS proteins, cytokine signalling and immune regulation." Nat Rev Immunol **7**(6): 454-65.
Young, L. E., S. Sanduja, et al. (2009). "The mRNA binding proteins HuR and tristetraprolin regulate cyclooxygenase 2 expression during colon carcinogenesis." Gastroenterology **136**(5): 1669-79.
Yuryev, A. and L. P. Wennogle (2003). "Novel raf kinase protein-protein interactions found by an exhaustive yeast two-hybrid analysis." Genomics **81**(2): 112-25.
Zarubin, T. and J. Han (2005). "Activation and signaling of the p38 MAP kinase pathway." Cell Res **15**(1): 11-8.
Zawia, N. H., D. K. Lahiri, et al. (2009). "Epigenetics, oxidative stress, and Alzheimer disease." Free Radic Biol Med **46**(9): 1241-9.
Zebisch, A., A. P. Czernilofsky, et al. (2007). "Signaling through RAS-RAF-MEK-ERK: from basics to bedside." Curr Med Chem **14**(5): 601-23.
Zhai, B., Q. He, et al. (2008). "Molecular and cellular mechanisms regulating cross-talk between the cyclooxygenase and lipoxygenase biosynthetic axes." Osteoarthritis and Cartilage **16**(**4 Suppl**): S189.
Zhang, L., W. Zhou, et al. (1997). "Gene expression profiles in normal and cancer cells." Science **276**(5316): 1268-72.
Zhang, Y. and C. Dong (2007). "Regulatory mechanisms of mitogen-activated kinase signaling." Cell Mol Life Sci **64**(21): 2771-89.
Zhang, Y. and D. Reinberg (2001). "Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails." Genes Dev **15**(18): 2343-60.
Zhao, M., L. New, et al. (1999). "Regulation of the MEF2 family of transcription factors by p38." Mol Cell Biol **19**(1): 21-30.
Zhu, B., Y. Zheng, et al. (2005). "Monoubiquitination of human histone H2B: the factors involved and their roles in HOX gene regulation." Mol Cell **20**(4): 601-11.
Zhu, W. G. and G. A. Otterson (2003). "The interaction of histone deacetylase inhibitors and DNA methyltransferase inhibitors in the treatment of human cancer cells." Curr Med Chem Anticancer Agents **3**(3): 187-99.

Zimmerman, B. J., D. J. Guillory, et al. (1990). "Role of leukotriene B4 in granulocyte infiltration into the postischemic feline intestine." Gastroenterology **99**(5): 1358-63.

Zimmermann, S. and K. Moelling (1999). "Phosphorylation and regulation of Raf by Akt (protein kinase B)." Science **286**(5445): 1741-4.

Zvaifler, N. J. (1973). "The immunopathology of joint inflammation in rheumatoid arthritis." Adv Immunol **16**(0): 265-336.

APPENDIX