The Role of Retinoic Acid Receptor Gamma in Retinoid-Induced Limb Dysmorphogenesis

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ABSTRACT

Retinol (vitamin A) and its active metabolite, all-*trans* retinoic acid, signal through nuclear retinoic acid and retinoid X receptor (RAR/RXR) heterodimers. These complexes regulate the expression of genes involved in developmental processes such as limb development. In excess, retinoids are potent teratogens and cause marked reductive effects on the developing limb. The goal of this thesis was to elucidate the molecular mechanisms underlying retinoid-induced limb dysmorphogenesis. Specifically, using an *in vitro* limb culture system, I examined the involvement of one RAR isoform, RARγ, in mediating retinoid insult.

My first objective was to examine how limbs deficient in RARγ responded to exogenous retinoid exposure. I showed that RARγ-null limbs (on an RARα1-null background) exhibited less severe limb defects following retinoid insult when compared to their wild-type counterparts. Additionally, the absence of RARγ abolished the retinoid-induced misregulation of genes important for chondrogenesis (*Sox9* and *Col2a1*) and limb outgrowth (*Meis-1* and *-2*).

The next objective set out to determine how pharmacological activation of RARγ affected limb development. The RARγ-selective agonist (BMS-189961) caused limb dysmorphology (namely, effects on cartilage) that was comparable to pan-RAR activation with all-*trans* retinoic acid. A chondrogenesis-focused gene array analysis identified *Mgp* and *Gdf10* as two RARγ-responsive genes that may mediate retinoid-induced limb insult.

Subsequently, I assessed the functional involvement of *Mgp* in mediating retinoid teratogenicity. Limbs were treated with all-*trans* retinoic acid and warfarin (an inhibitor of MGP); warfarin co-treatment rescued limbs from retinoid-induced insult.

My final objective was to determine the importance of *Gdf10* in mediating limb development. Recombinant human Gdf10-soaked beads were implanted into distal limb structures; ectopic overexpression of Gdf10 in the web (but not the digital ray) resulted in marked proximal limb malformations.

Collectively, these studies have illustrated the importance of RARγ in retinoid teratology and have identified several potential mechanisms by which retinoids cause limb defects.

RÉSUMÉ

Le rétinol (vitamine A) et son métabolite actif, l'acide rétinoïque all-trans, se fixe sur des hétérodimères de récepteur nucléaire à l'acide rétinoïque (RAR/RXR) pour transmettre son signal. Ces complexes ligand/récepteurs régulent l'expression de gènes impliqués dans le développement, dont, celui des membres. Un excès de rétinoïdes peut avoir un effet tératogène puissant et causer une réduction significative du développement des membres. Le but de ce travail de thèse a été d'élucider les mécanismes moléculaires responsables de la dysmorphogenèse induite par les rétinoïdes sur les membres.

Dans un premier temps, nous avons étudié les effets d'une exposition à des rétinoïdes exogènes sur le développement des membres issus d'animaux transgéniques dont le gène RARγ a été inactivé (sur un fond génétique inactivé pour le gène RARα1). Nous avons montré que les effets négatifs des rétinoïdes sont significativement diminués lorsque les membres exposés sont déficients en RARγ, comparés aux animaux sauvages. De plus, l'absence de RARγ entraîne une disparition de l'effet de l'exposition aux rétinoïdes sur l'expression de gènes fondamentaux pour la chondrogenèse (*Sox9* et *Col2a1*) et la croissance des membres (*Meis-1* et *-2*).

Dans un second objectif, nous avons déterminé comment l'activation pharmacologique de RARγ induit des anomalies de développement des membres. L'agoniste sélectif de RARγ (BMS-189961) induit des anomalies morphologiques des membres (en particulier sur le cartilage) comparables à celles induites par l'activation des tous les RAR par l'acide rétinoïque all-trans. Une analyse de puces à ADN ciblées sur les gènes de la chondrogenèse a montré que les gènes *Mgp* et *Gdf-10* sont impliqués dans la réponse à l'acide rétinoïque et pourraient être impliqués dans l'induction des effets délétères sur les membres.

Enfin, nous avons évalué la fonction biologique d'un des gènes activé par la voie de signalisation de RARγ, *Mgp*. Afin de démontrer l'importance des *Mgp* dans les effet tératogènes des rétinoïdes, les membres en développement sont cultivés en présence d'acide rétinoïque all-trans et d'un inhibiteur du MGP : la warfarine. Le

co-traitement avec la warfarine permet de protéger les membres contres les effets négatifs des rétinoïdes. D'autre part, afin de comprendre le rôle du *Gdf-10* dans le développement des membres, nous avons implanté des billes contenant du *Gdf-10* recombinant dans la partie distale des membres en développement. La surexpression du *Gdf-10* dans la région interdigitale (mais non pas dans la région des doigts) induit des malformations marquées dans la partie proximale du membre en développement.

L'ensemble de ces résultats montre l'importance de RARγ dans la transduction du signal tératogène des rétinoïdes et a permis d'identifier plusieurs mécanismes d'action possibles expliquant l'effet négatif des rétinoïdes sur le développement des membres.

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ABBREVIATIONS USED IN THIS THESIS

ActR	Activin receptor
AER	Apical ectodermal ridge
ADH	Alcohol dehydrogenase
AF	Activator function
BMP	Bone morphogenetic protein
BMS961	Bristol-Myers Squibb compound-189961
BSA	Bovine serum albumin
Col2a1	Type II(alpha1) collagen
CREB	cAMP response element binding
CRABP	Cellular retinoic acid binding protein
CRBP	Cellular retinol binding protein
Ctsk	Cathepsin K
CYP	Cytochrome P450 enzyme
DBD	DNA-binding domain
Dcn	Decorin
DV	Dorsoventral
EC_{50}	Median effective concentration
ECM	Extracellular matrix
E	Embryonic day
En-1	Engrailed-1
FGF	Fibroblast growth factor
GD	Gestational day
GDF	Growth differentiation factor
GLA	Gamma-carboxyglutamic acid
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HMG	High mobility group
Hox	Homeotic gene
Ihh	Indian hedgehog
ISH	*In situ* hybridization

LBD	Ligand-binding domain
LPM	Lateral plate mesoderm
LRAT	Lecithin:retinol acyltransferase
Meis	Myeloid ecotropic viral integration site
MGP	Matrix GLA protein
MKP-1	MAPK (mitogen-activated protein kinase) phosphatase 1
MMP	Matrix metalloproteinase
NLS	Nuclear localization sequence
NRE	Negative response element
OC	Osteocalcin
PBS	Phosphate-buffered saline
PBX	Pre B-cell leukemia transcription factor 1
PD	Proximodistal
PPAR	Peroxisome proliferator-activated receptor
pSmad	Phosphorylated Smad protein
PTH	Parathyroid hormone
PZ	Progress zone
qRT-PCR	Quantitative real-time polymerase chain reaction
RA	Retinoic acid
RALDH	Retinaldehyde dehydrogenase
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RARKO	Retinoic acid receptor knockout
RBP	Retinol-binding protein
RNAi	RNA interference
ROR	Retinoid-related orphan receptor
RXR	Rexinoid receptor
Shh	Sonic hedgehog
Sox	SRY (sex determining region Y)-related gene
TBX	T-box protein 5
TGF-β	Transforming growth factor beta

VAD	Vitamin A deficiency
VEGF	Vascular endothelial growth factor
VKD	Vitamin K-dependent
ZPA	Zone of polarizing activity

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PREFACE

Format of the Thesis

This is a manuscript-based thesis, which conforms to the 'Thesis Preparation and Submission Guideline' of the Faculty of Graduate Studies and Research at McGill University.

This thesis consists of 6 chapters. Chapter One is the Introduction and reviews many facets of retinoid biology, teratology as well as limb development. It also provides the reader with the rationale, hypothesis and accompanying objectives that drove the studies presented within this dissertation.

Chapters Two to Five (inclusively) comprise the data chapters that address my hypothesis. Chapter Two is published in Birth Defects Research Part A (2006. 76: 39-45). Chapter Three is published in Toxicological Sciences (2008. 106: 223-232). Chapters Four and Five are manuscripts in preparation.

Chapter Six contains the Discussion that provides an integration of the results, presents short-term and long term future studies, final conclusions as well as a list of Original Contributions. References are provided at the end of each chapter.

Chapter 1

Introduction

1.1 Vitamin A and its Derivatives

Vitamin A (retinol) is required for numerous embryonic and adult processes. Derivatives sharing similar structural and functional characteristics to vitamin A are commonly referred to as retinoids. Physiological concentrations of retinoids must be tightly regulated since dietary vitamin A deficiency and excessive intake both lead to clinical pathologies during embryo development and adult life. Several naturally occurring retinol isomers and retinoid metabolites exhibit bioactivity (Fig. 1.1).

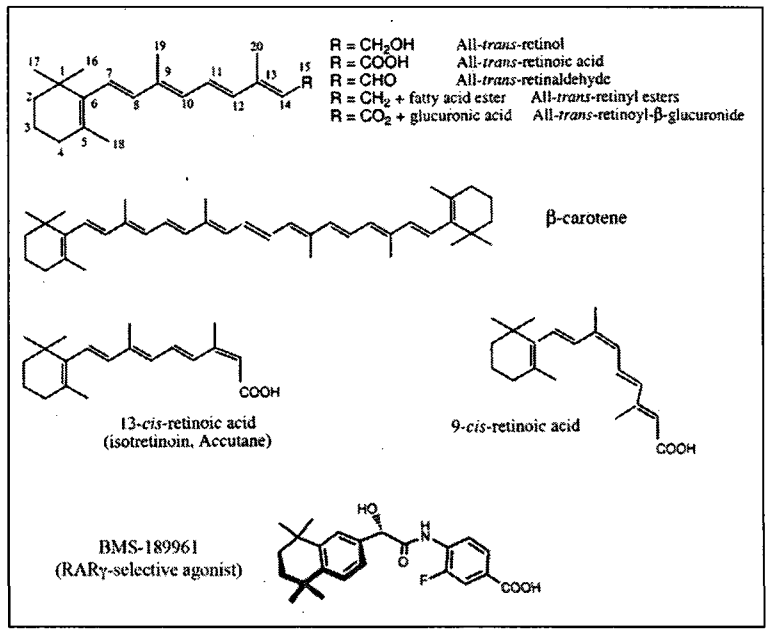

Figure 1.1. Several natural and synthetic retinoid-related compounds (adapted from Collins and Mao, 1999).

All-*trans* retinoic acid (RA) has been shown to be the most potent retinoid under physiological conditions, playing important roles in countless physiological processes (reviewed in Maden, 2000). Moreover, thousands of synthetic retinoid

analogs have been developed as therapeutic interventions targeting various diseases (a topic that will be discussed later).

1.1.1 Formation, absorption, distribution, metabolism and excretion

Retinoids are not produced *de novo* and therefore must be obtained through the diet from animal (preformed retinol and fatty acid retinyl esters) and plant (carotenoids) sources. In most industrialized nations, 75% of the dietary vitamin A comes from preformed sources, largely derived from multivitamins, fish liver oils, fortified dairy products and snack foods, whereas in developing nations, 70-90% of vitamin A is obtained from plant sources (Harrison, 2005).

Upon ingestion, the majority of animal-derived retinyl esters are converted into retinol via a hydrolysis reaction in the intestinal lumen. Conversely, in the case of plant-derived carotenoids (technically defined as terpenoids and not retinoids), 10% of all known carotenoids, such as β-carotene, are precursors of vitamin A that, upon cleavage by dioxygenase in the duodenum, yield one or more physiologically pertinent retinaldehyde molecules. Carotenoid cleavage is a tightly regulated process, making vitamin A toxicity from provitamin A sources highly unlikely (Fraser and Bramley, 2004). Conversely, absorption and storage of preformed vitamin A quickly becomes inefficient when vitamin A pathology develops.

Once converted, retinol and retinaldehyde products bind to cellular retinol-binding protein (CRBP) type II to prevent oxidation and degradation. In the presence of lecithin:retinol acyltransferase (LRAT), the retinol bound to CRBP-II becomes converted into retinyl esters such as palmitate (Herr and Ong, 1992). These retinyl esters are packaged into chylomicrons (organized aggregates of phospholipids and

triacylglycerol) and shuttled to the liver, where 80% of total retinol and retinyl esters are stored in vertebrates (Blomhoff et al., 1982).

In the liver, the esters are bound by CRBP-I and hydrolyzed to retinol and, due to their hydrophobic nature, must complex with retinol-binding protein (RBP, Kanai et al., 1968) for delivery to the hepatic perisinusoidal stellate cells where retinol is then re-esterified and stored in lipid droplets. Additionally, RBP-binding protects free retinol from chemical and enzymatic degradation. When needed by target tissues, retinyl esters are re-hydrolyzed to retinol and secreted into the circulation bound to RBP and transthyretin, a high molecular weight transport protein that prevents the glomerular filtration of retinol. Upon arrival at its target, retinol can then be released and bioactivated via a series of enzymatic reactions (Blomhoff et al., 1984).

With the exception of several target tissues such as the eye (where retinol is isomerized to retinal chromophores such as 11-*cis* retinal), retinol is most often oxidized in a two-step process to form all-*trans* RA (Fig. 1.2; Kim et al., 1992; Napoli, 1996). The first step entails the rate-limiting conversion of retinol into all-*trans* retinaldehyde by several sets of enzymes.

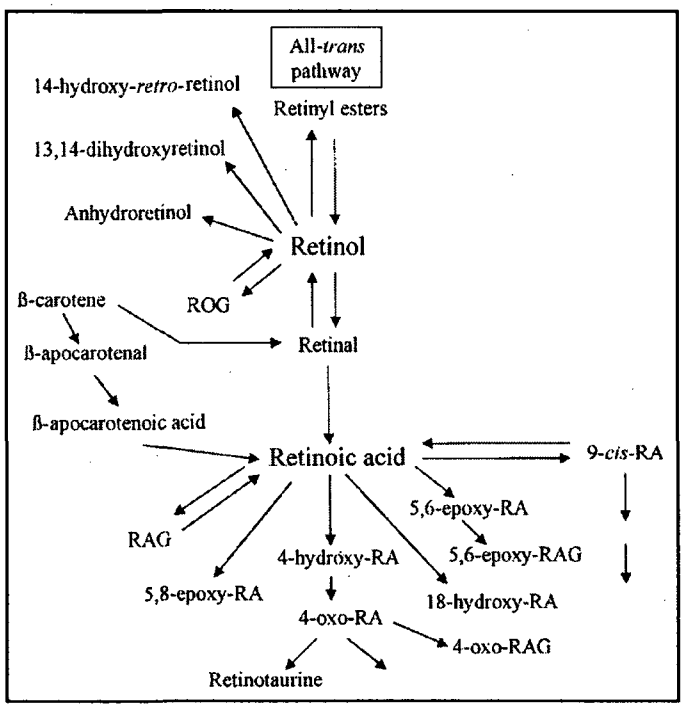

Figure 1.2. The key players in the metabolism and catabolism of all-*trans* retinoic acid (adapted from Collins and Mao, 1999).

Ample evidence has illustrated the involvement of cytosolic medium-chain alcohol dehydrogenases (ADH1, ADH3 and ADH4) in this first oxidation step of free retinol. ADH3 is ubiquitously expressed and ablation leads to reduced growth and survival that can be rescued by dietary vitamin A supplementation. ADH1 and ADH4 are expressed in a tissue specific manner and ablation of these enzymes does not affect normal growth, but does perturb the response of the organism to vitamin deficiency or excess (Duester et al., 2003).

Membrane-bound short-chain dehydrogenase/reductases (SDR) can also partake in the retinol-to-retinaldehyde conversion, but require the retinol to be bound to CRBP-I, whereas ADHs react only with free retinol. SDRs are expressed in many

vitamin A-sensitive cell types and are often co-expressed with CRBP-I (Everts et al., 2005).

The second oxidation converting all-*trans* retinaldehyde into all-*trans* RA is an irreversible step. It has been demonstrated that the retinaldehyde dehydrogenases (RALDH) 1, RALDH2 and RALDH3 are responsible for the production of all-*trans* RA. RALDH1, 2 and 3 are expressed in specific tissues and exhibit different roles during retinoid metabolism. RALDH1 is highly expressed in retina and in adult epithelial tissues, but no major effects are seen in RALDH1 knockout embryos (Fan et al., 2003). It has been postulated that RALDH1 is involved in the catabolism of excess retinol (Molotkov and Duester, 2003). RALDH2 is expressed in various cell types, both in embryonic and adult tissues (Niederreither et al., 1997) and RALDH2-null mice die *in utero*, but can be rescued by all-*trans* RA supplementation during gestation (Niederreither et al., 1999). Thus, RALDH2 appears to be responsible for all-*trans* RA production in the majority of the embryo. RALDH3 is expressed in the retina, lens and brain and RALDH3-null mice die immediately postpartum due to defects in nasal development but can also be rescued by exogenous all-*trans* RA treatment (Dupe et al., 2003). RALDH4 has been recently characterized, but has been shown to be more active with 9-*cis* retinal, illustrating its role in the production of 9-*cis* RA. However, the bioactive properties of 9-*cis* RA have yet to be observed in mammalian species. Interestingly, studies have implicated 9-*cis* RA as an important morphogen during frog (Kraft et al., 1994) and chick (Thaller et al., 1993) development.

Cellular retinoic-acid binding proteins I (CRABP-I) and CRABP-II bind all-*trans* RA and are expressed in various sites of the developing embryo and in the adult. CRABPs play a role in limiting the access of all-*trans* RA to the nucleus, the subcellular locale where retinoids exert the majority of their effects (Boylan and Gudas, 1992).

Apart from the CRABP-mediated sequestration of all-*trans* RA, the catabolism of all-*trans* RA is paramount for the regulation of its action. Three cytochrome P450s; CYP26A1, CYP26B1 and CYP26C1 (Swindell and Eichele, 1999; White et al., 2000; Taimi et al., 2004), have been cloned and identified to degrade all-*trans* RA into more polar hydroxylated metabolites, which are then eliminated by urine, bile, and feces (Barua and Olson, 1986; Niederreither et al., 2002a). Notably, some of the degradation products (i.e., 4-hydroxy-RA, 4-oxo-RA) have been shown to exhibit observable levels of bioactivity when administered exogenously (Reijntjes et al., 2005), yet their endogenous physiological function is controversial. The CYP26 enzymes are generally expressed in a non-overlapping fashion, suggesting unique functions for each in the degradation of all-*trans* RA (Reijntjes et al., 2004). Importantly, the expression domains are localized in a complementary fashion to the all-*trans* RA-producing RALDH enzymes, demonstrating a tightly regulated tug-of-war between production and degradation that leads to the generation of distinct gradients of all-*trans* RA during development and adulthood (Swindell et al., 1999).

1.1.2 Retinoid receptors

The majority of retinoid action is mediated by the binding of all-*trans* RA to two members of the ligand-dependent nuclear transcription factor superfamily: retinoic acid (RAR) and rexinoid (RXR) nuclear receptors. Through the induction of nuclear receptor activity, retinoids can regulate the activation and/or repression of target genes. Three subtypes of RARs and RXRs (α, β and γ) exist and each is encoded by distinct genes. In addition, alternative splicing and differential promoter usage can produce multiple isomers of each subtype (Leid et al., 1992). In the case of RARs, α1-2, β1-4 and γ1-2 exist, while only two RXR isoforms of each subtype exist (α1-2, β1-2 and γ1-2).

Structurally, RARs and RXRs consist of 6 conserved domains (A-F), each bearing particular functions (Fig. 1.3). The sequence differences within the A and B regions (amino-terminal) confer the variation between the different RAR and RXR isoforms as well as contain a ligand-independent (constitutively active) transcriptional activation function domain (AF-1). The C region houses a secondary dimerization sequence as well as two zinc fingers that directly interact with chromatin, commonly known as the DNA binding domain (DBD). The D region contains the nuclear localization sequence and acts as a hinge between the DBD and the ligand binding-domain (LBD) situated in Region E. The E region contains the primary dimerization sequence and the ligand-dependent transcriptional activation function domain (AF-2). The carboxy-terminal F region is only found in RARs (not RXRs) and its function is unknown (Bastien and Rochette-Egly, 2004).

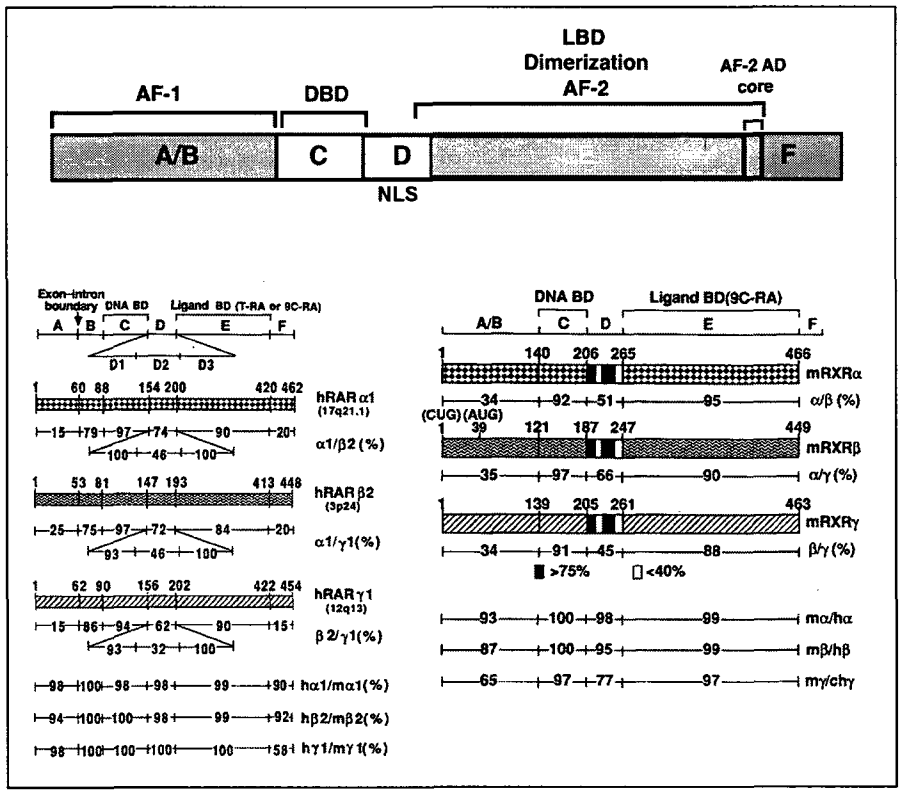

Figure 1.3. The structurally-conserved domains of RARs and RXRs and their respective sequence homologies; AF, activator function; DBD, DNA binding domain; NLS, nuclear localization sequence; LBD, ligand binding domain. (adapted from Bastien and Rochette-Egly, 2004 and Leid et al., 1992).

1.1.3 Mechanism of retinoid action

Retinoid receptors function as heterodimers of RARs and RXRs that associate with specific sequences of DNA known as retinoic acid response elements (RARE) in the promoters of target genes. RAREs consist of two direct PuG(G/T)TCA repeats separated by 1 to 5 base pairs (DR1-5); DR5 and DR2 RAREs are the most common, but RAR/RXR heterodimers bind DR1 as well. RXRs can function as homodimers and preferentially bind DR1 RAREs, but their existence and physiological roles *in vivo* are questionable. In fact, *in vitro* binding studies have shown that all-*trans* RA and 9-*cis* RA are high affinity ligands for RAR whereas only

9-*cis* RA binds RXRs and RARs (Allenby et al., 1993). Given the abundance of all-*trans* RA *in vivo* and the lack of evidence suggesting a biological role for 9-*cis* RA, the most important activator of RAR/RXR function appears to be all-*trans* RA binding to RAR (Durand et al., 1992). In other words, RXRs play a requisite role in retinoid signaling insomuch as the RXR must be present to form RAR/RXR heterodimers and thus initiate retinoid signaling, but RXR activation alone does not lead to similar signaling outcomes (Elmazar et al., 1997).

In the absence of ligand, RAR/RXR heterodimers are found predominantly in the nucleus. They are associated to specific RAREs bound to transcriptional corepressors NCoR and SMRT (Glass and Rosenfeld, 2000), which in turn recruit multi-unit complexes containing histone deacetylase (HDAC) activity, tightening nucleosome-DNA interactions and silencing chromatin (Fig. 1.4; Dilworth and Chambon, 2001).

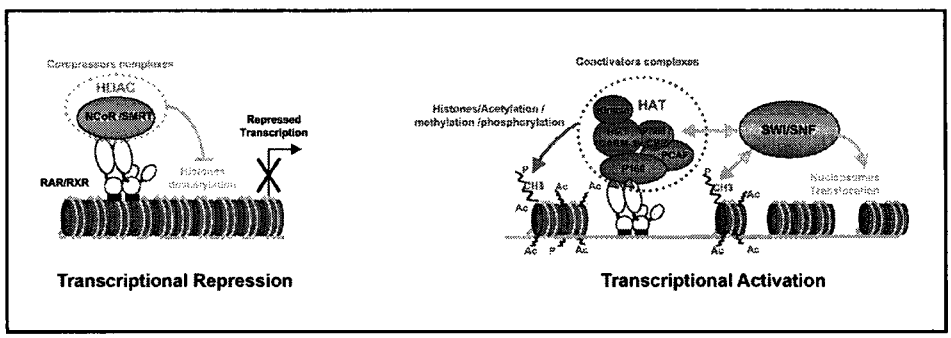

Figure 1.4. The switch that controls the transcriptional activation of retinoid receptors (adapted from Bastien and Rochette-Egly, 2004).

In contrast, when all-*trans* RA binds the RAR, several conformational changes in the receptor lead to the dissociation of the corepressor complexes and the subsequent binding of coactivators (i.e., p300, CBP and CARM-1) to the AF-2 domain; these proteins are able to acetylate and methylate chromatin, decompacting and remodeling repressive chromatin, facilitating the association of transcriptional machinery, ultimately leading to the upregulation of gene transcription (Dilworth and Chambon, 2001). The exception to this ligand-dependent response is when RAR/RXR heterodimers are associated to DR1 RAREs (Kurokawa et al., 1995). Such DR1 RAREs have been alternatively classified as negative response elements since ligand binding can induce repression rather than activation of target genes (Jepsen et al., 2000).

Ultimately, these observations highlight the importance of liganded and unliganded retinoid receptors alike, illustrating the biological need for properly regulated concentrations of retinoids during development and adulthood.

1.1.4 Efficiency of retinoid action

Given the importance of proper retinoid signaling, multiple mechanisms have evolved to control the extent and efficiency of retinoid action. Several mechanisms, including the tight balance of ligand production versus degradation and the control of ligand availability by CRBP/CRABP binding, have been addressed above, yet several other mechanisms affecting more downstream events (namely following receptor activation) have been recently characterized.

The first implicates the ubiquitin-proteosome pathway in a paradoxical system of proteolytic and non-proteolytic processes, both of which have yet to be fully

described. Primarily, studies have identified the ubiquitin-dependent proteolysis of transcriptional activators following retinoid-dependent transcription (DeMartino and Slaughter, 1999). Additionally, RARs and RXRs appear to be degraded by the proteosome in response to retinoids (Zhu et al., 1999). Hence, it is suggested that this level of regulation controls the magnitude and duration of retinoid-induced gene transcription (Bastien and Rochette-Egly, 2004). From a non-proteolytic standpoint, there is evidence suggesting that several ubiquitin ligases can associate with RARs (vom Baur et al., 1996), becoming integral components of the basic transcription machinery. The 19S component of the proteosome has been shown to associate with transcription activators and aid in the elongation process (Ferdous et al., 2001). Thus, the ubiquitin-proteosome pathway plays a two-faced role in the regulation of retinoid signaling by ubiquitinating and degrading receptor heterodimers and transcription factors or associating with and aiding the transcriptional machinery.

Another way by which retinoid-mediated transcription can be regulated is through phosphorylation of RAR/RXR heterodimers. RARs and RXRs can act as substrates for numerous kinases with phosphorylation playing an integral role in the functionality of several retinoid receptors (Gianni et al., 2001). Specifically, components of the transcriptional machinery can phosphorylate retinoid receptors, alter their conformation and either encourage transcriptional activity (Gianni et al., 2002) or induce receptor degradation (Kopf et al., 2000). Additionally, transcriptional corepressors such as SMRT, can be phosphorylated, decreasing binding efficiency to RAR/RXR heterodimers (Hong and Privalsky, 2000). Conversely, phosphorylation of transcriptional activators leads to the enhancement of receptor binding and

enzyme activity (Font de Mora and Brown, 2000). All in all, the phosphorylation of the many players partaking in retinoid signaling provides numerous checkpoints by which transcriptional efficiency can be regulated.

1.1.5 Non-classical actions of retinoids

Recent studies have demonstrated that retinoids can elicit their biological activity not only through RAR/RXR heterodimers, but also via novel non-canonical signaling pathways. Apart from classically heterodimerizing with RARs, RXRs are able to associate with numerous other nuclear receptors including the vitamin D and peroxisome-proliferator activated receptors (PPARs). This promiscuity can lead to the indirect transcriptional regulation of a broader set of target genes (Leid et al., 1992). RARs have been shown also to bind with other partners such as SF-1 (Barnea and Bergman, 2000) and Sp1 (Loudig et al., 2000), in turn associating with response elements other than RAREs, thus affecting additional targets.

In addition, retinoid receptors can crosstalk with other signaling pathways in a negative fashion; for example, recent work has addressed the repressive roles of retinoid activity on AP-1-dependent gene transcription (Shaulian and Karin, 2002). Unfortunately, the mechanisms responsible for the anti-AP-1 activity are elusive, yet several explanations have been proposed: (1) retinoids compete for common coactivators, (2) retinoid activity can inhibit JNK activity, (3) RARs may prevent proper Jun-Fos dimerization from occurring, or (4) retinoid activity competes for transcriptional machinery.

Recently, several other nuclear receptors, including RAR-related orphan receptor (ROR) β (Stehlin-Gaon et al., 2003), PPARβ and PPARδ (Shaw et al.,

2003), have been shown to respond to an all-*trans* RA stimulus. In the case of RORβ, all-*trans* RA binding led to the inhibition of receptor activity, while ligand binding to PPARβ/δ induced transcriptional activation.

Additionally, observations describing the immediate early effects (in the time span of seconds to minutes, rather than hours as in the case of transcriptional activation) of retinoid exposure on biological outcome have highlighted the non-genomic nuclear receptor-independent modes of retinoid action. Specifically, cAMP response element binding-protein (CREB) can be activated in a time frame that is too short (15-30 min after all-*trans* RA treatment) to encompass transcriptional and translational activation (Aggarwal et al., 2006).

In sum, these newly characterized non-classical mechanisms (genomic and non-genomic alike) are still poorly understood, yet clearly illustrate the broader complexity of retinoid action.

1.1.6 Retinoid balance

Because retinoids play important roles during development, their concentrations must be controlled in order for proper embryogenesis to occur. In fact, seminal work reporting the embryonic outcomes following vitamin A deficiency (VAD) and/or excess retinoid exposure has exemplified the necessary regulation of retinoid balance (reviewed in Clagett-Dame and DeLuca, 2002).

1.1.6.1 Vitamin A deficiency

VAD was first shown to be deleterious to embryonic development in 1933, when Hale (Hale, 1933) fed pregnant pigs a stock vitamin A-deficient diet for the first 30 days of gestation. *Postpartum*, all pigs were deemed blind with many exhibiting

major eye defects. In addition, other craniofacial defects (i.e., cleft lip) were also observed. Importantly, supplementation with retinoid-rich cod liver oil or green fodder was sufficient to prevent the observed defects. Follow-up studies in other species like the rat were done and identified a wide array of defects affecting numerous facets of reproductive, skeletal and cardiovascular development; the term 'vitamin A deficiency syndrome' was coined to describe these observations (Warkany and Schraffenberger, 1944). More recent studies have developed robust diet-induced VAD in rat (Warkany, 1954) and quail (Dong and Zile, 1995) models. Genetic ablation of retinoid-synthesizing RALDHs in mice (Niederreither et al., 2002b) and of specific RARs to disrupt retinoid signaling (Mark et al., 1997) have been examined also, further illustrating the importance of retinoid action during embryogenesis.

1.1.6.2 Retinoid excess

Like retinoid deficiency, retinoid excess can be detrimental to the developing conceptus. Retinoids are well known teratogens that can disrupt embryogenesis. The first account of vitamin A-induced teratogenesis was described by Cohlan (Cohlan, 1953). Briefly, when pregnant rats were administered 'natural vitamin A' (most likely retinyl esters) from gestational day (GD) 2 to 16, malformations, including exencephaly, spina bifida, hydrocephaly and truncations of facial structures, were observed. Several years later, Kochhar treated pregnant mice with all-*trans* RA on varying gestational days and described the teratogenic outcomes (Kochhar, 1967). Since then, the teratogenicity of excess retinol or all-*trans* RA has been well characterized in numerous species (Geelen, 1979). Furthermore, the

types and severity of the retinoid-induced phenotypes manifested were shown to be highly dependent on the time and dose of retinoid insult (Shenefelt, 1972).

From a clinical standpoint, humans are most commonly exposed to excess retinoid during pharmacological intervention, but specific concerns associated with supplemental vitamin A use and over-consumption of foods containing high retinoid levels (i.e., liver) may be warranted as well (Miller et al., 1998). Upwards of 10,000 retinoids have been synthesized and developed for potential pharmaceutical uses in the treatment of several malignancies (i.e., acute promyelocytic leukemia) and management of various skin diseases (i.e., acne and psoriasis). With respect to the latter, 13-*cis* RA (isotretinoin, Accutane®) is a retinoid analog that has been extremely successful in the treatment of severe cystic acne *vulgaris*, and is often prescribed to women of childbearing age making its teratogenic potential quite worrisome (Honein et al., 2004). While several risk management programs are in place to control the exposure of women of reproductive age to excess retinoid exposure, there are still major concerns regarding the continued occurrence of devastating fetal effects. Specifically, there is a broad spectrum of anomalies (referred to as 'isotretinoin embryopathy') in >35% of exposed infants. Of these cases, a large percentage (>70%) will suffer not only from physical malformations, but also from cognitive deficits. In cases where no physical anomalies are observed, 40% will exhibit impaired learning ability (Adams and Lammer, 1993).

In sum, identifying and understanding the mechanisms by which retinoids collectively regulate morphogenesis will be paramount in developing methods for birth defect prevention.

1.2 The Limb: Our Model System

Limb development is an extensively characterized process and is a well-accepted model for morphogenesis (Cohn and Tickle, 1996). Moreover, the signaling mechanisms controlling limb sculpting and patterning are also involved in morphogenetic processing governing numerous other organs, whether they be retinoid-sensitive or not (Lee et al., 2004; Yamada, 2005). Thus, examining how the limb responds to insult is an excellent model to study the teratogenicity of retinoids.

The use of organ cultures, namely the *in vitro* limb bud culture, has provided an exceptional system, facilitating the examination of direct toxicant effects on limb development. In contrast to *in vivo* treatment where maternal factors (metabolism and transport) come into play, *in vitro* assessment provides researchers with precise control over the dose and time of a teratogenic exposure to limbs. Given the developmental variation *in utero*, explantation before treatment allows for the *a priori* selection of limbs, ensuring the use of a uniform developmental stage (Nakamura, 1975; Neubert and Barrach, 1977). In 1929, Fells and Robison (Fell and Robison, 1929) were the first to explant and culture limb buds *in vitro*, demonstrating an inconsistent, yet successful maturation of undifferentiated limb tissue into cartilage. More satisfactory techniques were devised several years later where mouse limb bud cultures were used to study the effects of 1-azetidine-2-carboxylic acid on limb development *in vitro* (Kochhar and Aydelotte, 1974). Limb buds (usually forelimbs) from different species, including chicken (Karkinen-Jaaskelainen, 1976), rabbit (Neubert and Barrach, 1977) and even human (Rajan, 1974), have been used;

however, consistently reproducible results using murine limb buds have been obtained and therefore mice are used most commonly.

The *in vitro* exposure of limbs obtained from murine early-to-mid-organogenesis stage embryos to retinoids has been shown to cause a multitude of reductive malformations on growth and cartilage formation (Kochhar and Aydelotte, 1974). These observations coincided with effects seen following *in utero* maternal retinoid exposure at similar gestational stages (Kochhar, 1973). Notably, there are differences between limb development *in vitro* and *in utero:* (1) given that the limbs are explanted, blood supply *in vitro* is completely eliminated, making long term sustainability of these cultures quite difficult; (2) limb development *in vitro* occurs much more slowly than *in utero;* and (3) several alterations in the development of the cartilage anlagen may appear *in vitro*. Nevertheless, the overall limb structure remains clearly recognizable (Friedman, 1987). The plethora of work standardizing the *in vitro* limb culture illustrates the technical and biological strengths of this particular system in assessing retinoid-induced teratogenicity.

1.3 The Developing Limb

Limb development is a very intricate process that involves the transformation of an unspecialized group of undifferentiated cells (limb primordia) into a complex heterogeneous three-dimensional structure consisting of three major segments: the proximal stylopod (humerus or femur), the middle zeugopod (radius and ulna or tibia and fibula) and the distal autopod (hand or foot plate; Fig. 1.5).

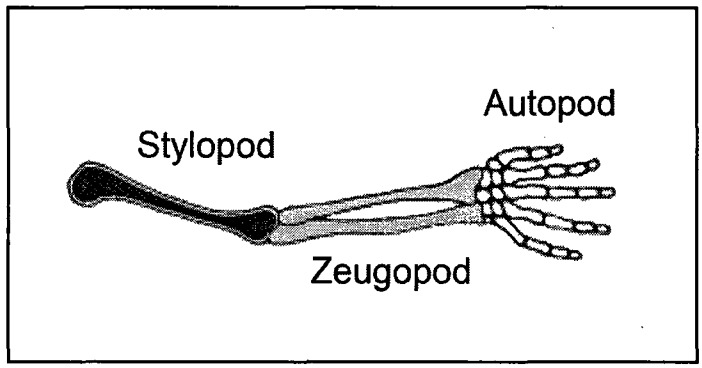

Figure 1.5. The basic skeletal structure of a mammalian forelimb (adapted from Capdevila and Izpisua Belmonte, 2001).

From the primordial stage to its completion, a multitude of signaling mechanisms participate in supporting the various facets of limb development, from outgrowth and patterning to differentiation and programmed cell death (Tickle, 2000; Gurrieri et al., 2002). These cellular events are evolutionarily conserved and are used in many instances during embryogenesis to help sculpt not only limbs, but also many other organs as well (e.g. kidneys and teeth). Downstream genes, which are differentially expressed in one organ (i.e., the limb) but not another (i.e., the kidney), dictate the final morphogenetic outcomes. Evidently, while limb and kidney morphology may differ, the underlying molecular mechanisms controlling their organogenesis are surprisingly similar (Capdevila and Izpisua Belmonte, 2001).

1.3.1 Timing of limb development

Limb development is a highly conserved process and occurs at specific species-dependent stages of organogenesis. In the mouse (20 day gestation; the model system used in this thesis), forelimb initiation starts at embryonic day (ED) 9.5 (after neural tube closure and embryo turning) and differentiation of the majority of

limb components is completed by approximately ED 14.5 (Fig. 1.6; Weston et al., 2003b).

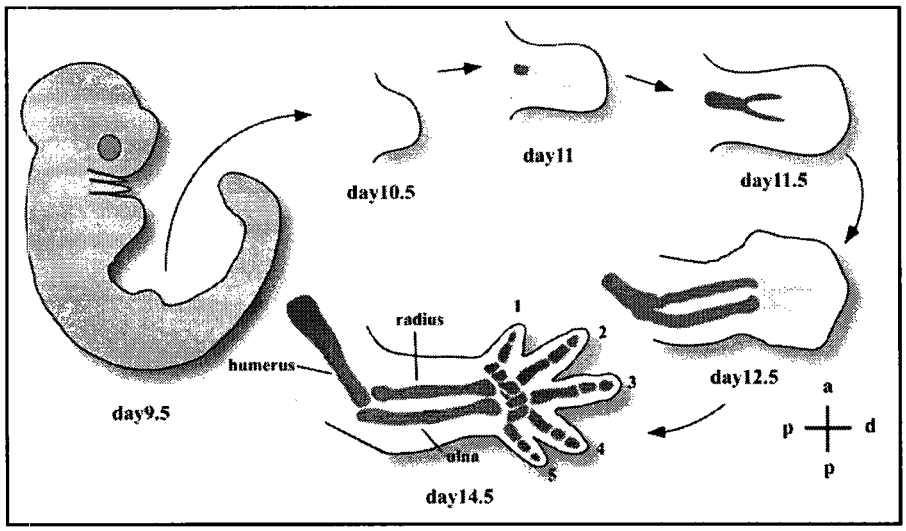

Figure 1.6. The timing and progression of forelimb development in the embryonic mouse (from Weston and Underhill, 2003).

Hindlimb development occurs at the same rate, but proceeds roughly 24 hours after initial forelimb budding. Comparatively, forelimb development occurs around ED 27 (first trimester) in humans (Otis and Brent, 1954) and ED 2 in chick (Hamburger and Hamilton, 1951).

1.3.2 Limb initiation and induction

Pairs of limb buds consisting of undifferentiated mesenchymal cells encased by a thin layer of ectoderm originate in the lateral plate mesoderm (LPM), along the embryonic trunk (Searls and Janners, 1971). The positional specification of limb bud initiation is not understood fully, but several studies support the involvement of homeodomain transcription factors (known as *Hox* genes) that confer positional

identity along the anterior-posterior (AP) axis and allocate the limb fields. Various *Hox* genes (Deschamps et al., 1999), as well as T-box (*Tbx*) transcription factors (Isaac et al., 1998), are differentially expressed along the embryonic AP axis, and, in turn, dictate where the forelimb and hindlimb fields will form. Interestingly, all-*trans* RA has been shown to be involved in controlling the expression of *Hox* genes during limb bud initiation (Marshall et al., 1996).

Once the limb fields are specified, the cells within the LPM divide actively while non-limb LPM divide more slowly (Searls and Janners, 1971), leading to the noticeable protrusion of the limb primordium; this stage is known as limb induction. A key signaling molecule, fibroblast growth factor (*Fgf*) 8 is expressed in the intermediate mesoderm and it turn controls *Wnt* signaling. *Wnts* (*Wnt-2c* in forelimbs; *Wnt-8c* in hindlimbs) stimulate and maintain expression of *Fgf10* in the LPM. Through a positive feedback loop, *Fgf10* induces *Fgf8* expression not in the intermediate, but rather the overlying surface ectoderm, leading to the formation of the apical ectodermal ridge (AER; Fig. 1.7).

The *Fgf10*-dependent induction of the AER is absolutely required for limb development because limbs do not form (amelia) in *Fgf10*-null mice (Min et al., 1998).

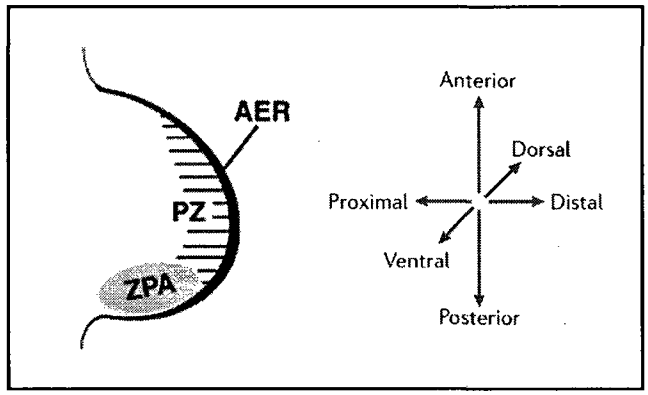

Figure 1.7. Schematic cross-section of the developing limb at E10, illustrating the locations of the apical ectodermal ridge (AER), progress zone (PZ) and zone of polarizing activity (ZPA; adapted from Capdevila and Izpisua Belmonte, 2001).

1.3.3 Proximodistal (PD) axis

The AER is a very important strip of epithelium located at the distal tip of the limb bud; it is located in such a way that it separates the dorsal and ventral sides of the limb. Signals emanating from the AER interact with the underlying mesenchyme and are absolutely required for limb outgrowth and patterning (Guo et al., 2003). Several *Fgfs* (and other genes) are involved in AER function, with *Fgf8* being the best characterized. While AER removal can lead to limb truncation, FGF supplementation following AER removal can rescue such malformations (Niswander et al., 1993). In addition to regulating outgrowth, *Fgf*s are thought also to sustain the directly underlying population of undifferentiated mesenchymal progenitors that is commonly referred to as the progress zone (PZ; Mahmood et al., 1995).

The majority of the components of the developed limb arise from the highly proliferative PZ (Fig. 1.7). Many genes are involved in PZ function, but particular importance not only lays in the temporal-spatial regulation of these genes, but also in how long cells remain within the PZ. The 'PZ model' of limb outgrowth suggests that cells exiting the PZ early acquire proximal values and in turn differentiate into proximal structures and vice versa (Summerbell et al., 1973; Wolpert, 2002).

As the limb outgrows, the proximal mesenchyme also provides a signaling center that can help determine PD cell fate. Of particular interest to my work, the proximal expression of two *Hox*-related genes, *Meis-1* and *-2* has been demonstrated (Mercader et al., 1999). In particular, ectopic expression of *Meis* genes in the distal limb domains inhibited distalization, leading to limb truncations and differentiation of more proximal structures (Capdevila et al., 1999). Mechanistically, MEIS can associate with and induce the nuclear localization of PBX, another set of *Hox*-related proteins. Once in the nucleus, MEIS and PBX can trimerize with particular *Hox* genes (i.e., *Hox10* and *11*) although the specific mechanisms of these trimeric complexes have not been fully characterized (Jacobs et al., 1999; Shanmugam et al., 1999).

1.3.4 Anteroposterior (AP) axis

The signal center that controls anteroposterior patterning is the zone of polarizing activity (ZPA), a population of mesenchymal cells located in the posterior distal margin (adjacent to the presumptive fifth digit) of the developing limb bud (Fig. 7). Sonic hedgehog (SHH) is a morphogen secreted by the ZPA; the expression gradient of *Shh* is considered to play a key role in AP patterning (Ogura et al., 1996).

To control expression of *Shh*, the zinc-finger transcriptional repressor GLI3R is reciprocally expressed in the anterior region of the limb and inhibits SHH activity (Theil et al., 1999).

SHH-soaked beads implanted into the anterior portion of the limb bud result in mirror-image duplications, a phenotype also seen when tissue from the ZPA is grafted into the anterior limb. Ectopic application of all-*trans* RA in the anterior margin leads to similar effects, indicating the role of all-*trans* RA in the regulation of ZPA function (Tickle et al., 1982). Whereas all-*trans* RA was initially thought to directly regulate *Shh* expression, *Hoxb-8* and *dHand* (a basic helix-loop-helix transcription factor) are expressed in the ZPA and shown to function upstream of *Shh* (Stratford et al., 1997; Fernandez-Teran et al., 2000).

1.3.5 Dorsoventral (DV) axis

During early limb initiation, molecular signals of unknown identity originate from the adjacent somites to induce dorsalization, whereas other signals from the LPM control proximalization (Michaud et al., 1997). In contrast, during mid-to-late limb development, the patterning mechanisms are better characterized. As budding occurs, signals within the limb mesenchyme induce expression of *Wnt-7a* and *Lmx-1* in the dorsal ectoderm and mesenchyme, respectively. To counteract dorsalization, *En-1* is present in the ventral half. The loss of ventralization signals leads to the aberrant expansion of dorsalization signals, and can ultimately lead to limbs exhibiting a double-dorsal phenotype (Loomis et al., 1996). Likewise, a double-ventral phenotype can occur in cases of expanded *En-1* expression (Logan et al., 1997).

1.3.6 Integrated patterning

The signaling mechanisms associated with each axis are not straightforward. In fact, there are numerous examples where signals originating from one axis (*Shh* in the ZPA) can interact with signals from another axis (Scherz et al., 2004). In effect, the promiscuity of these intricate pathways demonstrates the importance not only of the AP, PD and DV axes as separate entities, but also as a well-oiled machine, that signals in an integrated manner, to regulate proper limb morphogenesis.

1.3.7 Mesenchymal differentiation

As specified above, the PZ is the major source of limb progenitors. Once pattern is specified, the mesenchymal cells within the PZ must differentiate into the various components of the limb. While some cells will form bone, muscle and blood vessels, others will die by programmed cell death. My thesis work has emphasized the role of cartilage in retinoid-induced limb dysmorphogenesis; therefore the following section will address aspects of cartilage and bone differentiation.

1.3.8 Cartilage and bone formation

The creation of the limb skeleton can be broken down into two parts: chondrogenesis (cartilage formation) and osteogenesis (bone formation). Specifically, the chondrogenic process consists of stages where cellular condensation and differentiation occur. Prior to limb bud outgrowth, the undifferentiated mesenchymal progenitor cells are separated by an intricate extracellular matrix (ECM) containing glycosaminoglycans and hyaluronan (Olsen et al., 2000). Mesenchymal condensation occurs following the induction of ECM

degradation, thus allowing the cells to form pre-cartilage aggregates. N-CAM and N-cadherin, both cell adhesion molecules, are also induced at this stage, facilitating condensation (Delise and Tuan, 2002).

Once condensation is initiated, specific signals involving SOX proteins are required to drive the differentiation of mesenchymal cells down the chondrocyte lineage. *Sox9* (as well as *L-Sox5* and *Sox6)* are members of the SOX family of transcription factors that are characterized by high-mobility-group (HMG)-box DNA binding domains. In particular, *Sox9* is required for proper chondrocyte differentiation and, in conjunction with *L-Sox5* and *Sox6*, modulates the expression of type II collagen and aggrecan, two major components of the cartilage matrix (Akiyama et al., 2002). *In vivo* genetic studies have demonstrated the inductive nature of Sox9 on immature chondrocyte proliferation as well as the repressive effects on chondrocyte maturation and hypertrophy (Healy et al., 1999). In mice, limb-specific ablation of *Sox9* leads to the absence of limbs and, in humans, mutations in the *Sox9* gene cause skeletal defects associated with campomelic dysplasia (Kanai and Koopman, 1999).

Furthermore, members of the transforming growth factor beta (TGF-β) superfamily of ligands, including bone morphogenetic proteins (BMPs), play numerous roles during chondrogenesis. As their name suggests, BMPs can induce cartilage and bone formation depending on where they are localized. Interestingly, while BMPs expressed in the primordial digit rays cause chondrogenesis, BMP expression (as well as specific BMP receptors) in the interdigital webbing actually induces programmed cell death (Kawakami et al., 1996; Zou et al., 1997). Several

explanations for this observation exist, but it is accepted that the differential expression of BMP receptors in the rays versus the web regions leads to the activation of signals that determine opposing cell fates.

Once the chondrocytes are mature, Indian hedgehog (Ihh), parathyroid hormone (PTH), PTH-related peptide receptor (PTHrP) and RUNX2 work in concert to initiate chondrocyte hypertrophy and provide the scaffold for subsequent trabecular bone formation. As the cells hypertrophy, vascular endothelial growth factor (VEGF) is expressed to induce vascularization and matrix metalloproteinases (MMP) begin to degrade the hypertrophic cartilage matrix. Once blood flow is established, osteoblasts infiltrate the matrix and deposit trabecular bone tissue (containing many components, some of which include hydroxyapatite, type I collagen and osteocalcin). In contrast, chondrocytes that do not hypertrophy comprise the growth plate, the source of proliferating chondrocytes that fuels longitudinal bone growth during post-natal life (Fig. 1.8; reviewed in Goldring et al., 2006).

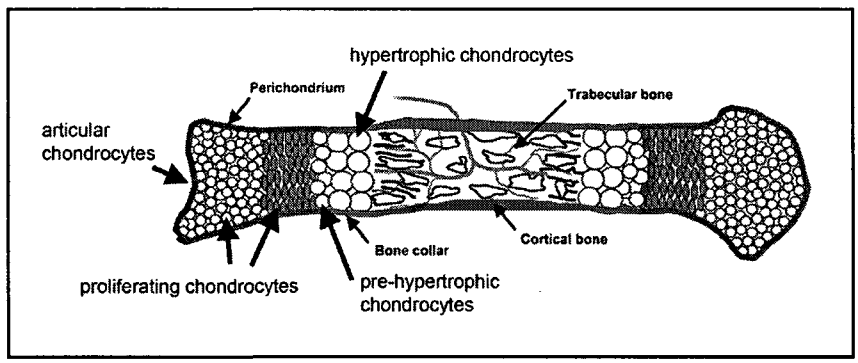

Figure 1.8. Schematic representation of endochondral bone formation in the developing long bone (adapted from Provot and Schipani, 2005).

1.4 Retinoids in the Limb

In the developing limb, all-*trans* RA is produced in areas where RALDH2 activity is high, namely in the LPM and the proximal mesenchyme. As all-*trans* RA is synthesized, it diffuses distally only to be broken down by CYP26B1, the major retinoid metabolizing enzyme expressed in the distal portion of the limb (MacLean et al., 2001). This balance leads to the creation of an all-*trans* RA gradient that has been suggested to control (whether it be directly or indirectly) a multitude of the genes involved in limb development.

As mentioned above, the majority of retinoid activity is mediated through the retinoid receptors. Specifically, pharmacological assessment of retinoid activity has demonstrated that retinoid-binding to RARs (but not RXRs) can elicit biological responses, illustrating the pivotal role in RARs during development. Additionally, pharmacological activation of only RARs led to limb dysmorphogenesis whereas as activation of RXRs did not (Elmazar et al., 1997).

1.4.1 Retinoid receptors in the limb

Retinoid receptors are expressed in a distinct temporal-spatial manner during the development of the limb (Dolle et al., 1989). At the onset of forelimb budding (E9), RARs α and γ are expressed throughout the developing tissue whereas RARβ expression is scarce. At E10, when the autopod/hand plate begins to develop, RARα is expressed in the ectoderm and mesoderm, RARγ only in the mesoderm, and RARβ expression is limited to the proximal mesenchyme. At E12.5, when cells in the web areas begin to apoptose to help sculpt the digits, the expression patterns of the RARs become very distinct. While RARα is expressed ubiquitously in the limb, RARγ

expression is high only in the developing cartilage (it is also expressed in the soft tissue but at lower levels). Adjacent to RARγ expression, RARβ becomes restricted to the interdigital webbing. Finally, when most limb differentiation is completed at E14.5, the majority of RARα expression is restricted to soft tissue, RARγ to the skeletal regions, and RARβ to the interdigital webbing (Fig. 1.9).

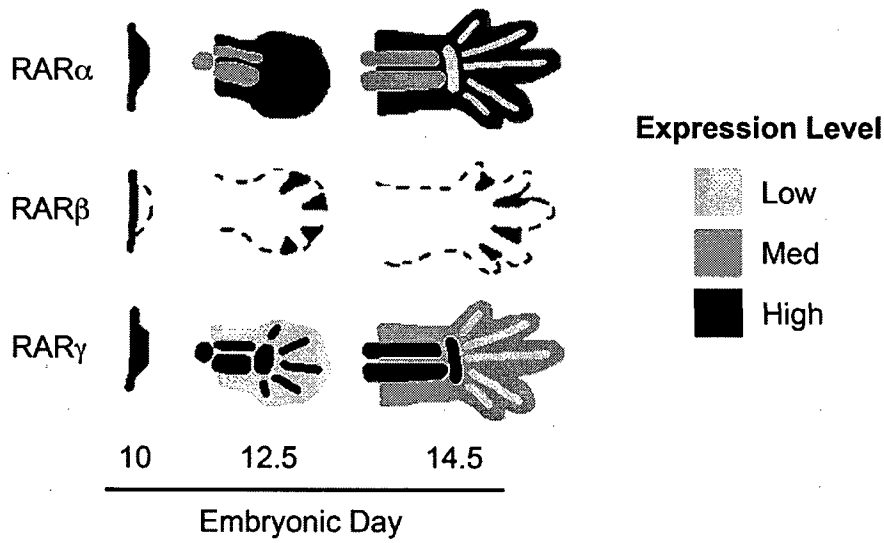

Figure 1.9. The gene expression patterns of RAR subtypes during limb development (adapted from Underhill and Weston, 1998).

RXRs are required for proper retinoid function, but RXR activation alone cannot elicit a retinoid-based biological response. However, RXRs α and β are expressed ubiquitously, in a weak manner, until E16.5 (Dolle et al., 1994). In contrast, at E12.5, RXRγ is specifically localized to regions of the proximal limb where muscle differentiation (myogenesis) is occurring (Mangelsdorf et al., 1992).

Additional work has identified the tissue-specific localization of the various RAR and RXR isoforms (Mollard et al., 2000), illustrating the further complexity that RARs and RXRs confer upon retinoid signaling in the developing limb. In effect, specific spatiotemporal patterning of these RARs and RXRs during limb development suggests that each receptor may play unique roles during embryogenesis.

1.4.2 Retinoid-induced limb dysmorphogenesis

Vitamin A, all-*trans* RA and other retinoids are potent teratogens. Limb defects are often manifested when mid-organogenesis-stage embryos are exposed to aberrant retinoid concentrations. Depending on the dose and timing, all structural elements of the limb can be detrimentally targeted, from the proximal long bones to the distal soft tissue. In rodents, the first publication to illustrate the impact of 'hypervitaminosis A' on limb development used oral administration of vitamin A to mice (Kalter and Warkany, 1961). Dosing animals between GD10.5 and GD12.5 resulted in proximal limb truncations (micromelia) or a complete absence of long bones (phocomelia) as well as marked effects on digit formation.

Subsequently, the teratogenic potential of all-*trans* RA on limb development was also assessed (Kochhar, 1973). *In utero* treatment at GD9 did not result in any observable limb defects, but exposure a half-day later (GD9.5) led to asymmetric forelimb ectrodactyly. At GD10, long bone defects were often observed, and at GD11-11.5, the forelimbs were most sensitive to limb truncation and aberrant digit formation and patterning. Forelimbs were still sensitive to retinoid exposure at GD12.5, but by GD14, limb defects were no longer observed. Hindlimbs were

equally affected, but were delayed by roughly a half-day since hindlimb development occurs 12 to 24 hours after forelimb development.

Apart from the *in vivo* studies, many *in vitro* experiments employing limb bud cell suspensions (micromass cultures) or excised whole limb buds have also been done to demonstrate that limb development is retinoid sensitive (Kwasigroch and Skalko, 1983; Zimmermann and Tsambaos, 1985). These *in vitro* systems have since provided researchers with an optimal means to assess the direct effects of teratogens (whether they be retinoid-based or not) on limb development. In addition to specifying the target organ or system affected, these cultures also allow for the control of dose, exposure and drug kinetics/dynamics, among others. Both *in vivo* and *in vitro* animal models have provided experimental systems by which researchers have assessed aspects of retinoid-induced limb dysmorphogenesis and showed that limbs exposed to exogenous retinoids exhibited limb malformations with variations in location, severity and extent within a phenotypic range that is clearly dependent on dose and time of exposure.

1.4.3 Retinoid-induced limb dysmorphogenesis in humans

In humans, the majority of the data addressing retinoid teratogenicity has been acquired from women of childbearing age who have been prescribed various forms of retinoid-based medications, many of which treat skin diseases. In addition to 13-*cis* RA (isotretinoin), several other aromatic retinoids (etretinate and acitretin) and even all-*trans* RA have been approved for therapeutic and cosmetic uses, respectively. While many of the defects observed in animal models correlated with those seen in human cases, limb defects were seldom seen, especially following 13-

cis RA exposure. In spite of this, several case studies have shown limbs to be affected following isotretinoin exposure (Rizzo et al., 1991). On the contrary, a wider gamut of reductive limb defects have been commonly seen following etretinate and acitretin exposures, with effects ranging from subtle digit patterning defects to cases of severe phocomelia (Geiger et al., 1994; de Die-Smulders et al., 1995).

1.4.4 Retinoid receptors and limb dysmorphogenesis

Because retinoids elicit their biological and teratogenic activity via RAR and RXR receptors, understanding how specific receptors mediate such outcomes is of paramount importance in understanding retinoid action. In fact, the comparison of teratogenic potencies of specific RAR and RXR agonists on limb development has shown that the teratogenic effects are mediated by RARs. Notably, RAR agonists were identified as strong limb teratogens while RXR agonists exhibited no or extremely weak effects on limb outcome (Kochhar et al., 1996). With the advent of RAR subtype-selective agonists, the involvement of each RAR subtype could readily be assessed. Assessment of the teratogenic activity of three compounds (CD336, CD2019 and CD437) selective for RARα, β and γ, respectively, showed the potency of the RARα agonists in inducing limb defects in the offspring of pregnant mice treated on GD11. The agonist for RARβ exhibited an intermediate potency, while limb development was weakly affected following administration of an RARγ agonist (Elmazar et al., 1996). Although RXR agonist administration alone was not teratogenic, co-administration with an RAR-selective agonist potentiated the observed teratogenic effects of the RAR agonist, demonstrating the synergistic effects between RARs and RXRs. Notwithstanding, since this initial study, more

selective and potent retinoid-receptor selective analogs have been developed; comparative analysis of these new analogs may lead to different experimental outcomes. All-*trans* RA-induced teratogenesis in rats was inhibited by co-administration with a RARα antagonist (Eckhardt and Schmitt, 1994), while retinol-induced limb dysmorphogenesis *in vitro* was attenuated by pan-RAR antagonist treatment (Ali-Khan and Hales, 2006b).

In order to further assess the roles of retinoid receptors during development and after an exposure to excess retinoid alike, specific RAR and RXR gene ablation studies have been done. Knockout of single RAR isoforms (α1, β2, γ1) resulted in no observable malformations while individual ablation of complete RAR subtypes (α, β, γ) led to distinct observable phenotypes in various parts of the embryo, yet limb development was only disrupted in a minor fashion (Lohnes et al., 1994). Specifically, RARα and RARγ-null mice exhibited webbed digits (syndactyly) but no skeletal defects; RARβ-null mice were phenotypically identical to wild-type animals. The only RAR knockout (RARKO) mouse to display major limb defects was the $RAR\alpha^{-/-}\gamma^{-/-}$ mouse, where skeletal defects, from the scapula to the digits, were frequently observed. Surprisingly, the presence of one RARα2 allele ($RAR\alpha1^{-/-}\alpha2^{+/-}\gamma^{-/-}$) rescued limb dysmorphogenesis (Lohnes et al., 1994). All in all, the observation of phenotypic aberrations following ablation of two, but not single RARs, indicates the high functional redundancy among these receptors, making the discrimination of each RAR function a difficult task. Furthermore, treatment of single and compound RARKOs with teratogenic doses of all-*trans* RA yielded limbs

exhibiting similar defects to those observed under wild-type treated conditions (with respect to both frequency and severity).

Appendicular skeletons from mice deficient in the various RXR subtypes were not malformed; digit webbing was observed only in RXRα-null mice. However, RXRα-null mice exposed to exogenous all-*trans* RA during mid-organogenesis did not develop limb abnormalities (100% penetrance) and mice heterozygous for RXRα were also not as sensitive to retinoid insult, illustrating a gene dosage effect on limb teratogenesis (Sucov et al., 1995). These results suggest that RXRα is required for the teratogenic signal but is involved through its heterodimerization with the RAR.

1.4.5 Pathogenesis of retinoid-induced limb dysmorphogenesis

There have been many proposed mechanisms by which retinoids can elicit their teratogenic effects on limb development. In such a complex developmental process, a number of targets exist, from cell proliferation and patterning to chondrogenesis and apoptosis. However, in many cases of retinoid-induced limb teratogenicity, aberrant skeletal formation appears to be the most compromised as well as the most observed phenotype (Kwasigroch and Kochhar, 1980). Hence, the following section will specifically address the effects of retinoids on chondrogenesis.

1.4.5.1 Aberrant chondrogenesis as a mechanism of malformation

The timing of retinoid exposure and subsequent limb defects suggests that retinoids can disrupt chondrogenesis (Kochhar and Aydelotte, 1974). In fact, much work has described the detrimental effects of all-*trans* RA directly on cartilage formation. Though, it should be noted that some of the effects that retinoids exert on cartilage are mediated indirectly via the misregulation of other aspects of limb

development such as programmed cell death (Ali-Khan and Hales, 2003) or patterning (Qin et al., 2002).

During early chondrogenesis both *in vivo* and *in vitro* studies have suggested a role for all-*trans* RA in holding the mesenchymal cells in the prechondrogenic condensation stage, preventing matrix synthesis and blocking chondroblast differentiation (Solursh and Meier, 1973). Further work has implicated the aberrant regulation of genes (i.e., *Sox9*) important in chondrogenic condensation as the major mechanism by which retinoids elicit their reductive effects on cartilage growth (Akiyama et al., 2002). Additionally, under conditions where the expression of specific RARs (i.e., RARβ) was enhanced in the limb mesenchyme during organogenesis, chondroprogenitor differentiation was also delayed or completely inhibited (Mendelsohn et al., 1991; Jiang et al., 1994).

Furthermore, micromass cultures containing limb cells over-expressing a human RARα1 (hRARα1) transgene did not differentiate into cartilage, as evidenced by a lack of alcian blue staining when compared to cells with normal endogenous RAR activity (Cash et al., 1997). Of note, whereas there were marked delays in cellular differentiation, cell proliferation was not affected.

In addition to preventing differentiation, there is evidence illustrating the role of all-*trans* RA in the dedifferentiation of round mature chondrocytes to mesenchyme-like chondroprogenitors, which appear flattened and elongated, like fibroblasts (Horton et al., 1987). These altered transitions between various stages are due to the aberrant changes in the synthesis of stage-specific proteins.

Later stages of chondrocyte maturation and endochondral ossification are also susceptible to the detrimental effects of exogenous retinoid exposure. Prehypertrophic chondrocytes cultured *in vitro* and treated with a RAR antagonist failed to express type X collagen (the most predominant protein expressed by cells undergoing hypertrophy) and endochondral ossification was inhibited. Moreover, data suggest that retinoids can upregulate the expression of specific MMPs and collagenases, thus leading to the accelerated breakdown of developing bone tissues (Jimenez et al., 2001).

Ultimately, these changes correspond to the effects of all-*trans* RA on gene transcription, thus leading to the misregulation of crucial chondrogenesis-related molecules (Horton et al., 1987). Identifying and characterizing these altered genes and the associated modes by which retinoids alter them will be crucial for the understanding of retinoid-induced dysmorphogenesis.

1.4.5.2 RARs and chondrogenesis

As mentioned above, various RARs can play important roles in regulating retinoid-induced limb dysmorphogenesis. In terms of specific RARs, in addition to *in vitro* work employing micromass limb cultures, Cash *et al.* conditionally upregulated hRARα in the developing limb under the control of the limb-specific Hoxb-6 promoter fragment (Cash et al., 1997). Their analysis showed high transgene expression within the developing limb bud, which in turn produced skeletal limb defects similar to those observed in situations of embryonic retinoid excess. In addition, pharmacological activation of RARα led to striking phenotypic aberrations

associated with disrupted bone formation, illustrating the importance of RARα activity in retinoid-induced insult.

RARβ, which is expressed in the soft tissue during limb development, has been most commonly associated with the regulation of programmed cell death (Dolle et al., 1989). However, the assessment of mouse RARβ2 promoter activity using an *in vivo* LacZ reporter strain (RARβ2-lacZ fusion transgene) has suggested several possible roles for mouse RARβ2 in limb development (Mendelsohn et al., 1991; Rossant et al., 1991). Notably, exogenous all-*trans* RA exposure in these reporter mice led to a significant upregulation of mRARβ2 expression in the limb, suggesting a link between aberrant retinoid activity and chondrogenic deficit. However, very few direct links between RARβ and cartilage formation have been substantiated.

As for RARγ, its functions during embryogenesis and retinoid teratogenicity have been linked to the patterning and chondrogenesis of the axial, but not the appendicular, skeleton. Specifically, RARγ$^{-/-}$ embryos exposed to teratogenic doses of all-*trans* RA at E8.5 are resistant to lumbarsacral truncations and spina bifida (Lohnes et al., 1993; Iulianella et al., 1999). Additionally, wild-type embryos exposed to all-*trans* RA at E7.5 exhibited various neural tube defects, whereas their RARγ$^{-/-}$ counterparts did not.

From the standpoint of limb development, RARγ ablation did not alter the severity or frequency of all-*trans* RA-induced limb defects when dams were treated on E10.5 or 11.5 (treatment at E12.5 was not assessed; Iulianella and Lohnes, 1997). This observation may be a prime example of receptor redundancy where the presence of the other RARs suffices for the transduction for retinoid teratogenicity.

Despite the functional ambiguity of RARγ during normal and retinoid-induced abnormal limb development, there is ample evidence to suggest otherwise. Namely, during mid-organogenesis (E12.5), RARγ is most highly expressed in areas of cartilage condensation (Ruberte et al., 1990). Given that cartilage and bone are targets that are heavily affected by retinoid insult, the distinct localization of RARγ inherently suggests its involvement in mediating such processes during development and/or teratogenicity.

1.5 Rationale

The main purpose of this thesis was to further explore the molecular mechanisms underlying retinoid-induced limb insult. In so doing, I specifically set out to characterize the roles of RARγ during limb insult.

Congenital limb defects are common structural anomalies affecting approximately 1 out of every 1000 live births and can occur in conjunction with numerous other malformations (Martin et al., 2007). Because limb defects are seldom life threatening, they can lead to socioeconomic and personal burdens. Understanding the molecular and developmental bases of limb defects can help improve the quality of life associated with such disabilities and lead to strategies for birth defect prevention.

Regarding retinoid biology, recent work has shed light on the potential therapeutic importance of RARγ activity. Apart from its high expression in cartilage during embryogenesis, substantial RARγ expression has been localized to keratinocytes, the major component of the epidermis (i.e., skin). As mentioned

above, retinoids are commonly used in the treatment of various skin conditions, but given their high teratogenic index, their use is a major cause of concern among women of childbearing age (Honein et al., 2001). Presently, the synthesis and use of RAR-selective retinoid analogues has been developed with the hope of increasing drug efficacy while decreasing teratogenic potential. Hence, the high expression of RARγ in the skin has provided an impetus to develop RARγ-selective analogues to treat aberrant keratinization (i.e., acne and psoriasis) and skin carcinogenesis (Chen et al., 2004; Clarke et al., 2004).

Thus, the increased clinical use of RARγ-selective compounds is another justification to examine the molecular mechanisms underlying their RARγ-mediated teratogenicity.

1.6 Hypothesis

I hypothesize that RARγ is an important mediator of retinoid-induced limb dysmorphogenesis.

1.6.1 Objective I

Examine how the developing limb bud responds to retinoid-induced limb dysmorphogenesis following the ablation of RARγ.

1.6.2 Objective II

Determine how selective pharmacological activation of RARγ affects limb morphology and the expression of chondrogenesis-related genes.

1.6.3 Objective III

Assess the biological roles of the identified RARγ-responsive candidate genes during limb development.

Chapter 2

Role of retinoid acid receptors alpha1 and gamma in the response of murine limbs to retinol *in vitro*

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Abstract

BACKGROUND: Derivatives of retinol (Vitamin A), commonly referred to as retinoids, signal through retinoic acid and retinoid X receptors (RARs/RXRs) and are essential for normal limb formation. Retinoid imbalances or perturbations in receptor function result in aberrant limb development. To examine the mechanisms underlying retinol-induced limb defects, we determined the responsiveness of limbs from RAR$\alpha1^{-/-}\gamma^{-/-}$ to excess retinol *in vitro*. **METHODS:** RAR$\alpha1^{-/-}\gamma^{+/-}$ mice were bred and embryos recovered at GD12.5; forelimbs were excised and cultured *in vitro* in the presence of *all-trans* retinol acetate (0, 1.25, 12.5 or 62.5 μM) for 6 days. The expression profiles of genes known to affect chondrogenesis (*Sox9* and *Col2a1*) and limb outgrowth (*Meis1*, *Meis2* and *Pbx1a*) were examined by real-time qRT-PCR following retinol exposure for 3 hours. **RESULTS:** Whereas RAR$\alpha1^{-/-}\gamma^{+/+}$ and RAR$\alpha1^{-/-}\gamma^{+/-}$ limbs exhibited deleterious effects on limb outgrowth and chondrogenesis in the presence of exogenous retinol, this outcome was significantly attenuated in RAR$\alpha1^{-/-}\gamma^{-/-}$ limbs. The expressions of *Sox9* and *Col2a1* were significantly decreased in retinol-exposed RAR$\alpha1^{-/-}\gamma^{+/+}$ limbs. In contrast, expression was not altered in limbs from their RAR$\alpha1^{-/-}\gamma^{+/-}$ or RAR$\alpha1^{-/-}\gamma^{-/-}$ littermates. Retinol exposure upregulated the expression of *Meis1* and *Meis2* in RAR$\alpha1^{-/-}\gamma^{+/+}$ limbs; however, in RAR$\alpha1^{-/-}\gamma^{-/-}$ limbs, the expression of both genes was unresponsive to retinol. *Pbx1a* remained unresponsive to retinol treatment in all genotypes. **CONCLUSION:** In the absence of RARα1, RARγ is a functionally important mediator of retinoid-induced limb dysmorphogenesis.

Key words: limb development; chondrogenesis; limb outgrowth; retinoid teratogenesis

Introduction

Vitamin A (retinol) and its derivatives, collectively referred to as retinoids, play key roles in vertebrate development. Many organs, including the developing limb, are dependent on tight control of retinoid concentrations for proper morphogenesis to occur (Ross et al., 2000). Either retinoid deficiency or excess during sensitive windows of development lead to limb dysmorphogenesis, which is commonly characterized by deleterious effects on chondrogenesis (Paulsen et al., 1994) and limb outgrowth (Kwasigroch and Kochhar, 1980).

Retinoid exposure has been implicated in the misregulation of genes required for proper chondrogenesis and outgrowth. Many signaling pathways have been identified as key regulators in both of these aspects of limb development, and several of these have been shown to respond to retinoid signaling. With respect to chondrogenesis, various groups have shown a critical role for the SRY-box containing protein 9 (SOX9) transcription factor in mesenchymal chondroprogenitor condensation (Lefebvre and de Crombrugghe, 1998). Sox9 enhances type II collagen (COL2A1) expression, the main building block of cartilage (Bell et al., 1997). In addition, limb-specific ablation of *Sox9* leads to limbless offspring (Akiyama et al., 2002). Moreover, Sox9 expression is downregulated in micromass limb cell cultures or primary chondrocytes exposed to *all-trans* retinoic acid *in vitro* (Sekiya et al., 2001; Hoffman et al., 2003).

Limb truncation has been linked to the upregulation of genes that are important in proximal-distal limb patterning. Although the apical ectodermal ridge initiates and promotes limb outgrowth, proximally expressed homeodomain

transcription factors, such as the myeloid ecotropic viral insertion site (*Meis*) family members, dictate proximal limb identity and contribute to the rate of limb outgrowth (Capdevila et al., 1999; Mercader et al., 1999; Mercader et al., 2000). Meis proteins can interact with, and induce the nuclear localization of, the homeodomain protein pre-B cell leukemia transcription factor 1 (*Pbx1*), leading to altered gene expression through trimerization with diverse *Hox* family members and contributing to a proximal limb transcription program (Berthelsen et al., 1999). Under normal conditions, endogenous retinoids in the proximal limb bud are believed to control the expression of *Meis1* and *Meis2*. In the presence of excess retinoid, *Meis* members become expressed distally, resulting in limb proximalization and truncation (Mercader et al., 1999; Qin et al., 2002).

Retinoids signal through two members of the nuclear receptor superfamily: retinoic acid receptors (RARα, β and γ), that bind both *all-trans* retinoic acid and 9-*cis* retinoic acid, and retinoid X receptors (RXRα, β and γ) that bind *9-cis* retinoic acid (Chambon, 1994). RAR-RXR heterodimers transduce the retinoid signal through binding to retinoic acid response elements (RAREs) and the ligand-dependent recruitment of co-activators involved in chromatin remodeling (Mangelsdorf et al., 1995; Kastner et al., 1997; Glass and Rosenfeld, 2000). Various N-terminal variant isoforms of both the RARs and the RXRs have been identified (Leid et al., 1992; Mangelsdorf et al., 1992; Lohnes et al., 1995); however, pharmacological manipulation has shown that while RAR activation causes a wide array of teratogenic effects, RXR-selective agonists are non-teratogenic, illustrating

the importance of RARs in mediating limb teratogenesis (Kochhar et al., 1996; Elmazar et al., 1997; Arafa et al., 2000).

As the murine limb develops, the expression patterns of RARs change dynamically in a spatial and temporal manner (Dolle et al., 1989; Weston et al., 2003b). This observation has suggested that the different RARs play specific roles in limb development. However, RAR ablation studies provide evidence for functional receptor redundancy (Chambon, 1994). For instance, while ablation of a single RAR isoform (e.g. RARα1) or certain RAR subtypes (e.g. RARβ) does not affect normal development, complete ablation of other subtypes (RARγ) leads to specific malformations in the axial, but not the appendicular, skeleton (Lohnes et al., 1994; Luo et al., 1995b; Iulianella and Lohnes, 1997). Importantly, only RARαγ double-null mice show severe limb dysmorphogenesis, while expression of RARα2 in RARα1$^{-/-}$γ$^{-/-}$ mice suffices for normal limb development *in vivo* (Lohnes et al., 1994).

The objective of the present study was to assess the role of specific RARs in retinoid-induced limb malformations. To do so, an *in vitro* limb bud culture system was employed to elucidate the impact of compound ablation of RARα1 and γ on the response of limbs to teratogenic concentrations of retinol. We hypothesized that in the absence of RARα1, RARγ is a functionally important mediator of retinoid-induced limb teratogenicity and affects the transcription of genes involved in both chondrogenesis and limb outgrowth. Consistent with this hypothesis, we report that RARα1$^{-/-}$γ$^{-/-}$ limbs are less susceptible to the teratogenic effects of *all-trans* retinol than their littermate controls (RARα1$^{-/-}$γ$^{+/+}$ or RARα1$^{-/-}$γ$^{+/-}$). Thus, RARγ, in the

absence of RARα1, is an important factor in mediating the teratogenic retinoid signal in the limb.

Materials and Methods

Animals and Limb Bud Culture

RARα1$^{-/-}$γ$^{+/-}$ mice in a C57BL/6-129Sv genetic background were crossed to generate RARα1$^{-/-}$γ$^{+/+}$, RARα1$^{-/-}$γ$^{+/-}$ and RARα1$^{-/-}$γ$^{-/-}$ embryos (Lohnes et al., 1994; Mendelsohn et al., 1994). Mice were mated overnight and noon of the day of detection of a vaginal plug was considered gestational day (GD) 0.5. On GD 12.5, pregnant females were sacrificed via cervical dislocation, the embryos were explanted, forelimbs were excised just lateral to the somites and cultured *in vitro* in a chemically defined retinoid-free culture medium as previously described (Kwasigroch et al., 1984; Huang and Hales, 2002; Ali-Khan and Hales, 2003). Because all of the mice lacked RARα1, the embryonic head associated with each set of forelimbs was collected and genotyped for RARγ as previously described (Iulianella et al., 1999). *All-trans* retinol acetate (Sigma, St-Louis, MI) was dissolved in 100% ethanol and added to the culture medium at low (1.25 μM), medium (12.5 μM) and high (62.5 μM) concentrations at the onset of culture. The total volume of ethanol *in vitro* did not exceed 0.5% vol/vol. All animal studies complied with the guidelines set by the Canadian Council on Animal Care.

Limb Morphology and Morphometry

Limbs were cultured *in vitro* for a total of 6 days, with one media change on day 3 (at which time retinol was not added) to replenish the nutrient supply. They were then fixed in Bouin's solution (Harleco, Gibbstown, NJ) overnight, stained with 0.1% toluidine blue (wt/vol 70% ethanol; Fisher Scientific, Montreal, QC, Canada) overnight, dehydrated with a graded ethanol series, cleared and stored in cedarwood oil (Fisher Scientific, Montreal, QC, Canada). Limbs were examined under a dissecting microscope (Wild Heerbrugg 99067, Heerbrugg, Switzerland) and digital images were acquired (JVC GCQX3HD Digital Camera, Japan). Using computer-assisted image analysis software (MCID-7, Image Research Inc., St. Catharine's, ON, Canada), limb surface and relative cartilage (cartilage area/total surface area) were measured as endpoints representing overall growth and chondrogenesis, respectively. The extent and quality of differentiation were assessed using a previously developed Limb Morphogenetic Differentiation Scoring System (Neubert and Barrach, 1977).

Gene Expression Analysis

After 3 hours, each pair of limbs was removed from culture, rinsed with PBS and stored at –20°C in RNAlater RNA Stabilization Reagent (Qiagen, Mississauga, ON, Canada) until further processing. Total RNA from each pair of limbs was extracted using the RNeasy Micro Kit (Qiagen, Mississauga, ON, Canada) and quantified by spectrophotometric UV analysis. RNA was subsequently diluted to a working concentration of 10 ng/μl and Quantitect One-Step SYBR Green RT-PCR

(Qiagen, Mississauga, ON, Canada) was done using the Roche LightCycler according to the manufacturer's instructions (Roche Diagnostics, Laval, QC, Canada). PCR thermal cycling parameters were: 95°C for 15 min (1 cycle) then 94°C for 15 sec, 55°C for 30 sec and 72°C for 20 sec (for 60 cycles). Each group consisted of RNA from 5 pairs of separately cultured limbs, and each sample was measured in duplicate. Embryonic head or hindlimb tissues from phenotypically normal RAR$\alpha 1^{-/-}\gamma^{+/+}$ mice were also collected and RNA extracted to provide 1, 10 and 100 ng/μl RNA stocks used for the generation of standard curves for quantification. The primers used were generated with Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and produced at the Sheldon Biotechnology Centre (McGill University, Montreal, QC, Canada; See Table 1 for sequence information). To normalize the output for each pair of forelimbs, the expression of each gene of interest was divided by β-actin gene expression, a commonly used housekeeping gene whose expression remains unchanged following retinoid exposure. Melting curve analyses were done following each PCR to determine the output and detection quality (i.e., formation of primer-dimers).

Statistical Analysis

All morphology and gene expression data sets were analyzed statistically using SigmaStat 3.0 (Systat Software Inc., Point Richmond, CA). Two-way ANOVA followed by the *post hoc* Holm-Sidak multiple comparison test was used to compare all groups in all genotypes and exposures. The minimum level of significance was $P < .05$ for all tests.

Results

Improved Morphology of RARα1γ$^{-/-}$ Limbs Following Retinol Exposure

To test the effects of exogenous retinoid exposure on RARα1$^{-/-}$γ$^{-/-}$ (hereafter RARα1γ$^{-/-}$) limbs, forelimbs from RARα1γ$^{-/-}$ mice as well as RARα1$^{-/-}$γ$^{+/+}$ (RARα1γ$^{+/+}$) and RARα1$^{-/-}$γ$^{+/-}$ (RARα1γ$^{+/-}$) littermate controls at E12.5 were cultured for 6 days in the absence or presence of various concentrations of retinol.

Limbs from RARα1γ$^{+/+}$ and RARα1γ$^{+/-}$ littermate controls developed normally in the absence of retinol, as shown by proper long-bone and digit formation (Fig. 2.1A and E). After escalating doses of retinol were administered, limb dysmorphogenesis was apparent in a dose-dependent manner, with marked effects on chondrogenesis, outgrowth and patterning (Fig. 2.1A-D and E-H). This effect on limb development was similar in cultured CD-1 and C57BL/6 limbs (data not shown).

RARα1γ$^{-/-}$ limbs, by contrast, did not develop normally in comparison to their littermate controls, as evidenced by stunted long-bone outgrowth (Fig. 2.1A, E and I). In addition, the abnormal development associated with exposure to excess retinol was attenuated, with treated limbs from RARα1γ$^{-/-}$ embryos appearing less susceptible to the teratogenic effects of retinol compared to wild type controls (Fig. 2.1J-L). Long bones were straighter following low dose treatment (Fig. 2.1B, F and J), and after medium dose retinol exposure (Fig. 2.1C, G and K), long-bones and digits were more recognizable and developed than dose-matched RARα1γ$^{+/+}$ or RARα1γ$^{+/-}$ littermates. While RARα1γ$^{-/-}$ limbs exposed to the high dose of retinol appeared highly dysmorphogenic, cartilage structures were still visible, in contrast to either RARα1γ$^{+/+}$ or RARα1γ$^{+/-}$ controls (Fig. 2.1D, H and L).

Computer-assisted analysis was used to measure limb surface area as a marker for overall limb growth. In RARα1γ$^{+/+}$ and RARα1γ$^{+/-}$ controls, exposure to increasing concentrations of retinol led to a decline in limb surface area, with the exception of the RARα1γ$^{+/+}$ limbs exposed to low concentrations of retinol, which exhibited a slight increase in surface area (Fig. 2.2A). In the absence of retinol, the surface area of RARα1γ$^{-/-}$ limbs was significantly decreased compared to littermate controls. Of interest, a significant genotype-specific increase of RARα1γ$^{-/-}$ limb surface area was seen following exposure to the medium concentration of retinol.

We measured the effects on chondrogenesis by measuring the relative cartilage area (cartilage area/limb surface area). RARα1γ$^{+/+}$ and RARα1γ$^{+/-}$ limbs showed significant decreases in chondrogenesis following exposure to medium or high concentrations of retinol compared to genotype-matched control limbs. In RARα1γ$^{-/-}$ limbs a significant decrease was apparent only in cultures exposed to high concentrations of retinol. Compared to RARα1γ$^{+/+}$ and RARα1γ$^{+/-}$ dose-matched controls, cartilage formation in the limbs of RARα1γ$^{-/-}$ compound null mice was less affected by retinol exposure (Fig. 2.2B).

The limbs were subsequently individually scored using the system developed by Neubert and Barrach (1977) to determine effects on limb pattern formation. The scores of control RARα1γ$^{-/-}$ limbs were significantly lower than those of their littermate counterparts. Retinol treatment led to a concentration-dependent decline in limb score in RARα1γ$^{+/+}$ and RARα1γ$^{+/-}$ limbs (Fig. 2.2C). Furthermore, RARα1γ$^{-/-}$ limbs exposed to retinol had higher limb scores than dose-matched RARα1γ$^{+/+}$ and

RARα1γ$^{+/-}$ controls. Thus, RARα1γ$^{-/-}$ limbs were less susceptible to retinol-induced limb dysmorphogenesis.

Effects of Retinol on the Expression of Sox9 and Col2a1 in the Absence of RARα1 and γ

One of the most striking changes in limb development evoked by retinoid excess was a substantial decrease in cartilage formation. To examine possible mechanisms underlying this decrease in chondrogenesis, we investigated the expression profiles of a key regulator of chondrogenesis, Sox9, and a downstream chondrocyte marker, Col2a1 following ablation of RARα1 and γ.

A significant reduction in *Sox9* and *Col2a1* expression was observed in RARα1γ$^{+/+}$ limbs exposed to 12.5 μM retinol (Fig. 2.3A, B). In contrast, retinol treatment of RARα1γ$^{+/-}$ and RARα1γ$^{-/-}$ limbs did not result in any significant changes in gene expression, suggesting that both copies of RARγ may be needed for retinoid-mediated downregulation of *Sox9* and *Col2a1*.

Effects of Retinol on the Expression of Meis and Pbx in the Absence of RARα1 and γ

Because retinol-induced limb truncation was noticeably reduced in the proximal long-bone regions in RARα1γ$^{-/-}$ limbs (Fig. 2.1J, K), we investigated the effects of retinol on *Meis1* and *Meis2* expression in RARα1γ$^{-/-}$ limbs compared to controls. In RARα1γ$^{+/+}$ limbs, the expression of *Meis1* and *Meis2* increased following exposure to 12.5 μM retinol for 3 hours (Fig 2.4A, B). Of interest, in the absence of RARγ, this retinol-dependent increase in *Meis1* and *Meis2* expression was not

observed, suggesting that RARγ (in the absence of RARγ) is necessary for the induction of *Meis1* and *Meis2* by retinol. In RARα1γ$^{+/-}$ limbs, retinol induced an increase in *Meis2,* but not *Meis1*, expression, compared to untreated RARα1γ$^{+/-}$ controls (Fig. 2.4A, B). Expression of the *Meis* binding partner, *Pbx1a*, was not altered by retinol exposure or genotype (Fig. 2.4C).

Discussion

Retinoid receptors not only play crucial roles in the proper development of the embryo, they also mediate retinoid-induced teratogenic events. In this study, we investigated the role of RARγ (in the absence of RARα1) in mediating retinol-induced limb teratogenesis. We demonstrate that *in vitro* cultured limbs from RARα1γ$^{+/+}$ and RARα1γ$^{+/-}$ mice develop normally and are highly susceptible to retinoid exposure. In contrast, there was a remarkable reduction in the sensitivity of RARα1γ$^{-/-}$ limbs to retinoid exposure. To determine an underlying molecular basis for this attenuated teratogenesis, we examined the expression profiles of genes required for proper limb development. In response to retinol exposure, RARα1γ$^{-/-}$, RARα1γ$^{+/+}$ and RARα1γ$^{+/-}$ limbs exhibited different patterns of expression of genes involved in either chondrogenesis (*Sox9* and *Col2a1*) or limb outgrowth (*Meis1/2* and *Pbx1*).

Numerous studies have implicated retinoid signaling in the regulation of chondrogenesis. RAR activity has been closely linked to *Sox9* expression and conditional null mutation studies have shown that Sox9 is essential for chondrogenesis within the limb (Foster et al., 1994; Weston et al., 2000; Akiyama et al., 2002; Weston et al., 2002). *Col2a1* is a gene known to be regulated by Sox9 (Bell et al., 1997). Following retinol exposure, similar decreases in both *Sox9* and *Col2a1* were observed in RARα1γ$^{+/+}$ limbs. The absence of changes in the expression of these genes in RARα1γ$^{+/-}$ and RARα1γ$^{-/-}$ limbs exposed to retinol suggests that RARγ has a role in regulating *Sox9* and *Col2a1* expression under the conditions used here.

The upregulation of *Meis* and *Pbx* members following *in vivo* treatment of GD11 pregnant female mice with 100 mg/kg of all-*trans* retinoic acid has been suggested to underlie retinoid-induced limb truncation (Qin et al., 2002). We observed similar increases in the expression of *Meis1* and *Meis2*, but not *Pbx1a*, in RARα1γ$^{+/+}$ limbs following retinol treatment. Our observation that retinol exposure did not alter *Meis1* or *Meis2* expression in RARα1γ$^{-/-}$ limbs, in which limb truncation was not severe, suggests these genes may be downstream RARγ targets. Moreover, gene dosage is likely to be an important facet of this response, since both copies of RARγ were required for retinol-regulation of *Meis1*. However, it is conceivable that the presence of only 1 copy of the RARγ gene may lead to a delay, rather than an absence of the regulation of downstream genes, becuase no noticeable resistance to retinoid teratogenicity was observed in RARα1γ$^{+/-}$ limbs in culture. Whereas the lack of a response of *Pbx1a* to retinol in RARα1γ$^{-/-}$ and control limbs conflicts with prior work (Qin et al., 2002), it is possible that significant change can be detected only at later time points since additional time may be needed for retinol acetate to be bioactivated (a step that the Qin group circumvented by treating with retinoic acid). RARα1 and γ are important in normal axial patterning. In one study, the ablation of both RARα1 and γ synergistically increased the frequency and severity of axial defects inherent to RARγ null offspring (Lohnes et al., 1994). However, in *in vivo* studies the limbs of mice deficient in either RARα1 or γ developed normally and were sensitive to the teratogenic effects of excess retinoid exposure (Lohnes et al., 1994; Luo et al., 1996; Iulianella and Lohnes, 1997; Subbarayan et al., 1997), while *in vitro* the limbs of RARα1$^{-/-}$γ$^{-/-}$ mice appeared stunted in growth. RARα1γ$^{-/-}$ limbs

may not be able to cope with the stress caused by limb excision or culture. In this regard, RARγ regulates at least one stress response gene, mitogen-activated protein kinase phosphatase 1 (MKP-1), which has a retinoid-dependent anti-apoptotic effect following redox stress (Xu et al., 2002). Alternatively, the excised limbs of compound null mice may behave as retinoid-deficient tissues due to the lack retinoic acid generated by the lateral plate mesoderm and other tissues within the trunk. Previous work showed that targeted disruption of RARα and RARγ in F9 teratocarcinoma stem cells resulted in altered retinoic acid metabolism (Boylan et al., 1995), which suggests that the expression of enzymes required for the conversion of retinol into retinoic acid may be dependent on the presence of these RAR isoforms. This possibility is consistent with the less-disrupted RARα1$\gamma^{-/-}$ limbs in the presence of low levels of retinol.

To date, only RXRα null mice have been associated with a resistance to retinoid-induced limb defects *in vivo* (Sucov et al., 1995). Here, we demonstrate that RARγ, in the absence of α1, plays a role in transducing retinoid-induced limb dysmorphogenesis, and that these effects correlate with RARγ-dependent regulation of players in chondrogenesis (*Sox9* and *Col2a1*) and limb outgrowth (*Meis1* and *Meis2*).

Acknowledgements

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Tables

Table 2.1

Genes, Accession Numbers, Primer IDs, Primer Sequences and Amplicon Lengths

Gene	Accession #	Primer ID	Sequence (5' -> 3')	Amplicon Length (bp)
β-actin	NM_007393	β-actinL β-actinR	GCT CTT TTC CAG CCT TCC TT AGG TCT TTA CGG ATG TCA ACG	101
Meis1	NM_010789	Meis1L Meis1R	CTC TCT CTC CTC TCT CTC CCT CTT GCA GTT TTT CCC ATC CTT CC	121
Meis2	U57343	Meis2L Meis2R	CCT CAA CGC ACA TAC ACA CA GCT CCT TTT TAT CCC CCA AG	101
Pbx1a	NM_183355	Pbx1aL Pbx1aR	CGG AAG AGA CGG AAT TTC AA CCG CAC TTC TTG GCT AAC TC	119
Sox9	NM_011448	Sox9L Sox9R	TAT GTG GAT GTG TGC GTG TG CCA GCC ACA GCA GTG AGT AA	137
Col2a1	NM_031163	Col2a1L Col2a1R	GAA GGT GCT CAA GGT TCT CG CTT TGG CTC CAG GAA TAC CA	103

Figures and Legends

Figure 2.1. The effect of retinol treatment on RARα1γ$^{+/+}$, RARα1γ$^{+/-}$ and RARα1γ$^{-/-}$ limbs following *in vitro* limb bud culture. Limbs were excised from mice on GD 12.5 and cultured for 6 days in the presence of 1.25, 12.5 or 62.5 μM *all-trans* retinol acetate. Cartilage was stained with 0.01% toluidine blue. (**A - H**) RARα1γ$^{+/+}$ and RARα1γ$^{+/+}$ limbs developed normally under control conditions and showed dose-dependent and detrimental effects on limb growth, chondrogenesis and patterning following escalating doses of retinol. (**I - L**) Although proper limb growth was not seen in α1γ$^{-/-}$ limbs under control conditions, these limbs were less susceptible to the teratogenic effects of retinol exposure than those of their littermates. Scale bar = 1mm.

Figure 2.1

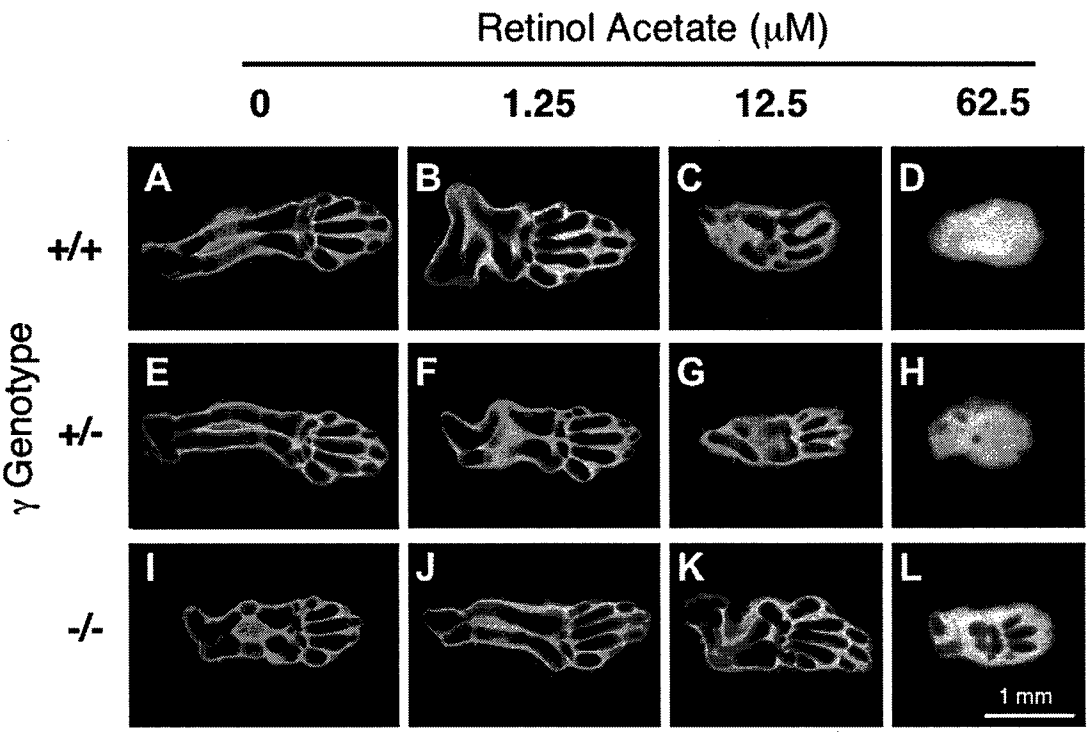

Retinol Acetate (μM)
0
1.25
12.5
62.5
γ Genotype
+/+
+/-
-/-
A
B
C
D
E
F
G
H
I
J
K
L
1 mm

Figure 2.2. Effects of retinol on limb growth, chondrogenesis and differentiation/patterning *in vitro*. (**A**) Limb surface area, a surrogate marker for limb growth, was significantly decreased in untreated RARα1γ$^{-/-}$ limbs and significantly increased following exposure to 12.5 μM retinol when compared to RARα1γ$^{+/+}$ and RARα1γ$^{+/-}$ controls. (**B**) The percentage (%) of cartilage was quantified by calculating the ratio between the area of toluidine blue-stained cartilage and total surface area. Specific differences occurred in 12.5 μM and 62.5 μM retinol-treated limbs (i.e.,, RARα1γ$^{-/-}$ samples displayed greater cartilage formation compared to dose-matched genotypes). (**C**) Limb morphogenetic differentiation score measured the amount and quality of the cartilage anlagen development. Limb scores were reduced in RARα1γ$^{-/-}$ limbs under control conditions compared to those of littermate controls, indicating impaired development *in vitro*. RARα1γ$^{-/-}$ limbs showed less disruption of development when treated with 1.25, 12.5 and 62.5 μM retinol, as indicated, when compared to exposure-matched controls. Statistics were analyzed by 2-way ANOVA and the *post hoc* Holm-Sidak multiple comparison test (n = 10 – 35). *Significant increase, $P < .05$, vs. dose-matched genotypes; **significant decrease, $P < .05$, vs. dose-matched RARα1γ$^{+/+}$ genotype; $^{\#}$significantly different, $P < .01$, vs. genotype-matched controls.

Figure 2.2

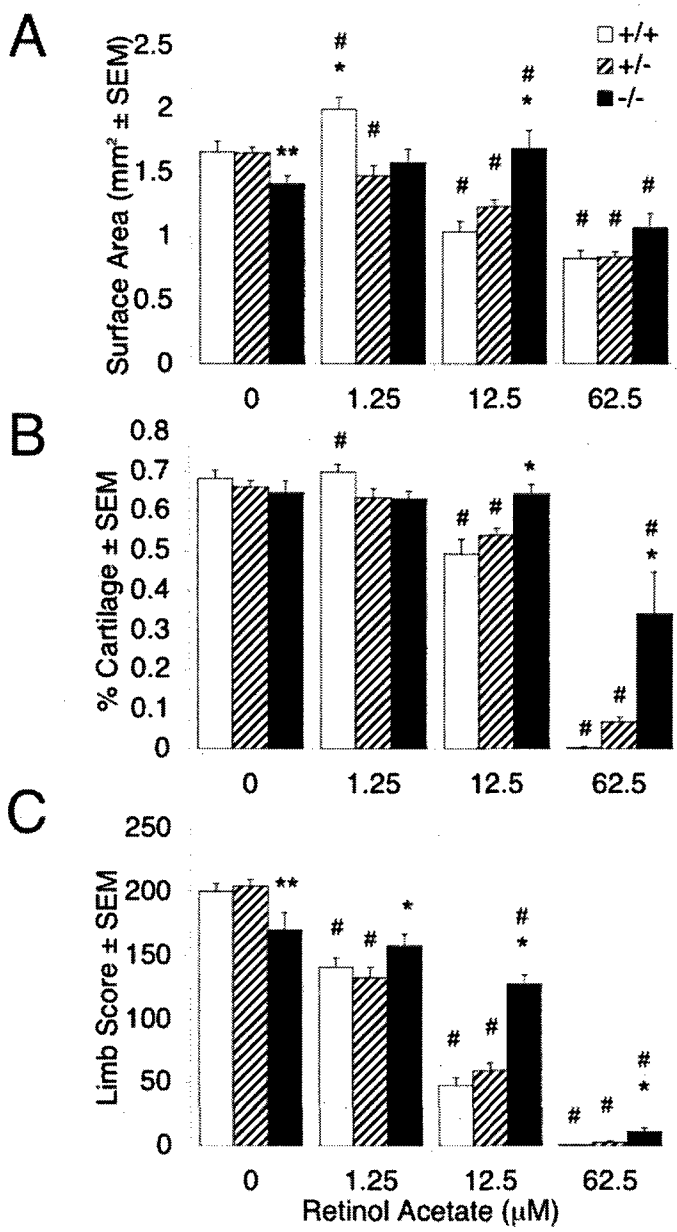

A
B
C
Surface Area (mm² ± SEM)
% Cartilage ± SEM
Limb Score ± SEM
+/+
+/-
-/-
0
1.25
12.5
62.5
Retinol Acetate (μM)

Figure 2.3. Effect of retinol treatment on the expression of chondrogenesis-related genes. Limbs were cultured *in vitro* for 3 hours in the absence or presence of 12.5 μM retinol. (**A**) *Sox9* expression was reduced following retinol treatment in RARα1γ$^{+/+}$ limbs, but was not affected in RARα1γ$^{+/-}$ and RARα1γ$^{-/-}$ limbs. (**B**) *Col2a1* gene expression paralleled *Sox9* expression with marked effects elicited by retinol treatment in RARα1γ$^{+/+}$, but not RARα1γ$^{+/-}$ or RARα1γ$^{-/-}$ limbs. Each bar represents the mean gene expression of separately conducted limb cultures (n = 5) normalized against β-actin expression. Statistics were analyzed by 2-way ANOVA and the *post hoc* Holm-Sidak multiple comparison test. *Significant decrease, $P < .05$, vs. genotype-matched control limbs; $^{\#}$significant decrease, $P < 0.05$, vs. dose-matched RARα1γ$^{+/+}$ limbs.

Figure 2.3

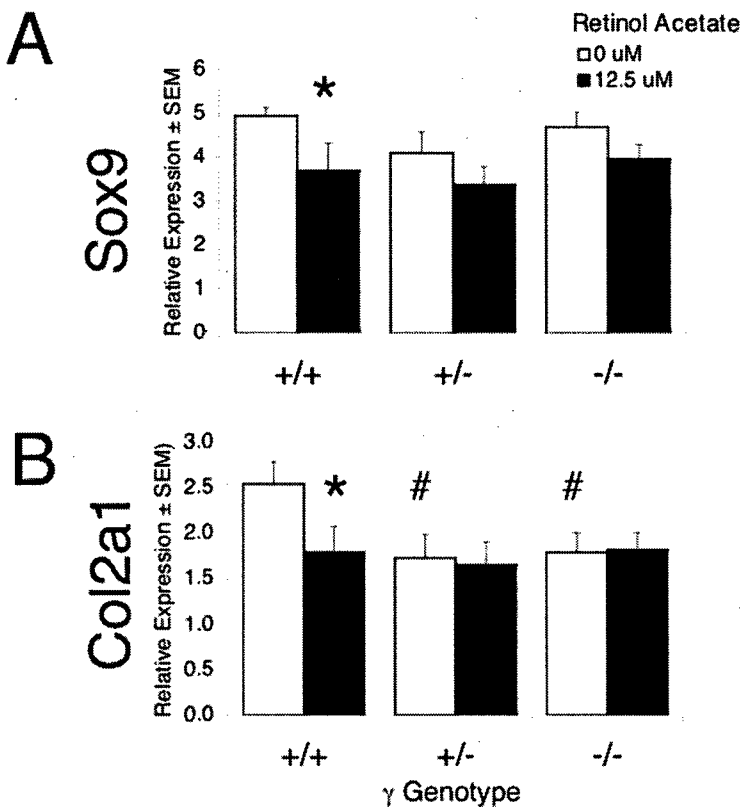
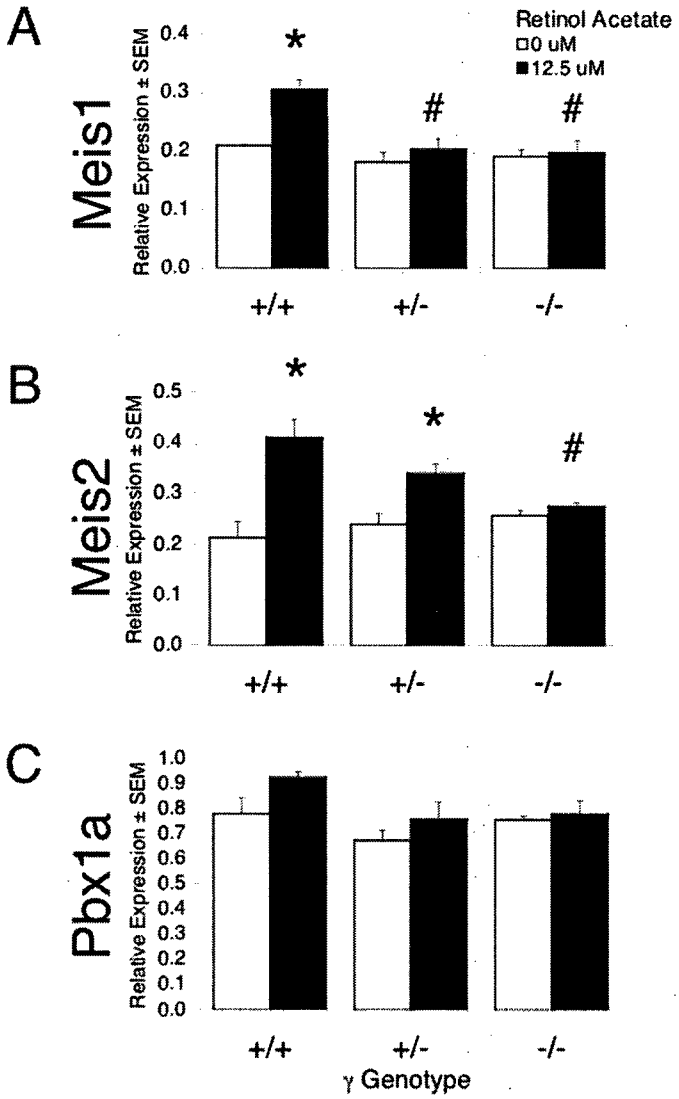

A
Sox9
Relative Expression ± SEM
Retinol Acetate
0 uM
12.5 uM
*
+/+
+/-
-/-
B
Col2a1
Relative Expression ± SEM)
*
#
#
+/+
+/-
-/-
γ Genotype

Figure 2.4. Changes in the expression of genes involved in proximo-distal limb development following retinol treatment. Limbs were cultured *in vitro* for 3 hours in the absence or presence of 12.5 μM retinol then RNA extracted for qRT-PCR analysis. (**A**) *Meis1* gene expression increased following retinol treatment in RARα1γ$^{+/+}$ limbs, but was unchanged in RARα1γ$^{+/-}$ and RARα1γ$^{-/-}$ limbs. (**B**) *Meis2* gene expression increased following retinol treatment in both RARα1γ$^{+/+}$ and RARα1γ$^{+/-}$ limbs, but not in RARα1γ$^{-/-}$ limbs. (**C**) *Pbx1a* gene expression was unaffected with respect to dose or genotype. Each bar represents the mean gene expression of separately conducted limb cultures (n = 5) normalized against β-actin expression. Statistics were analyzed by 2-way ANOVA and the *post hoc* Holm-Sidak multiple comparison test. *Significant increase, $P < .05$, vs. genotype-matched control limbs; $^{\#}$significant decrease, $P < .05$, vs. dose-matched RARα1γ$^{+/+}$ limbs.

Figure 2.4


A
Meis1
Relative Expression ± SEM
0.4
0.3
0.2
0.1
0.0
*
#
#
Retinol Acetate
□0 uM
■12.5 uM
+/+
+/-
-/-
B
Meis2
Relative Expression ± SEM
0.5
0.4
0.3
0.2
0.1
0.0
*
*
#
+/+
+/-
-/-
C
Pbx1a
Relative Expression ± SEM
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
+/+
+/-
-/-
γ Genotype

Connecting Text for Chapter 2 to 3

Following ablation of RARγ, the data presented in Chapter 2 demonstrate the importance of RARγ in mediating retinoid insult. Because RARγ-null mice were shown to be equally sensitive to retinoid-induced limb defects *in vivo* as a phenomenon attributed to the redundancy of RARs, our model employed RARγ-null limbs under and RARα1-null background to account for the redundant effects that RARα1 may contribute in the case of the $RAR\alpha^{+/+}\gamma^{-/-}$ limbs. In spite of this, the following issue has been raised: does RARα1 actually play a compensatory role in the absence of RARγ or is it the other way around?

Therefore, to (1) complement our observations following RARγ-deficiency in Chapter 2 and (2) definitively single out RARγ function, BMS-189961, a RARγ-selective pharmacological agonist, was used in Chapter 3 to further assess the particular and specific roles of RARγ in mediating retinoid-induced limb dysmorphogenesis.

Chapter 3

Retinoic Acid Receptor Gamma-induced Misregulation of Chondrogenesis in the Murine Limb Bud *In Vitro*

Eugene Galdones and Barbara F. Hales

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Abstract

Vitamin A derivatives modulate gene expression through retinoic acid and rexinoid receptor (RAR/RXR) heterodimers and are indispensable for limb development. Of particular interest, RARγ is highly expressed in cartilage, a target affected following retinoid-induced limb insult. The goal of this study was to examine how selective activation of RARγ affects limb development. Forelimbs from E12.5 CD-1 mice were cultured for 6 days in the presence of all-*trans* RA (pan-RAR agonist; 0.1 or 1.0 μM) or BMS-189961 (BMS961, RARγ-selective agonist; 0.01 or 0.1 μM) and limb morphology assessed. Untreated limbs developed normal cartilage elements whereas pan-RAR or RARγ agonist-treated limbs exhibited reductive effects on chondrogenesis. Retinoid activity was assessed using RAREβ2 (Retinoic acid response element β2)-lacZ reporter limbs; after 3 h of treatment, both drugs increased retinoid activity proximally. To elucidate the expression profiles of a subset of genes important for development, limbs were cultured for 3 h and cRNA hybridized to osteogenesis-focused microarrays. Two genes, matrix GLA protein (*Mgp*; chondrogenesis inhibitor) and growth differentiation factor-10 (*Gdf10*/*Bmp3b*) were induced by RA and BMS-189961. Real-time PCR was done to validate our results and whole mount *in situ* hybridizations against *Mgp* and *Gdf10* localized their upregulation to areas of cartilage and programmed cell death, respectively. Thus, our results illustrate the importance of RARγ in mediating the retinoid-induced upregulation of *Mgp* and *Gdf10*; determining their roles in chondrogenesis and cell death will help further unravel mechanisms underlying retinoid teratogenicity.

Keywords: Matrix GLA protein, growth differentiation factor 10, retinoic acid receptor gamma, retinoic acid, limb development, chondrogenesis

Introduction

All-*trans* retinoic acid (RA), the most bioactive metabolite of vitamin A (retinol), is a potent morphogen that plays crucial roles during embryo development (Kochhar, 1967). Retinoid concentrations are tightly regulated during embryogenesis. Both deficiency (Hale, 1930) and excess can cause malformations of various embryonic structures in a time- and dose-dependent manner (Collins and Mao, 1999).

Limb development is an orchestrated process; a multitude of signals converge that specify and sculpt the heterogeneous (i.e., bone, muscle, skin) limb structure. Retinoids actively regulate various signaling pathways during normal limb morphogenesis, and it is no surprise that retinoid imbalances alter such developmentally-important processes (Romand et al., 2004). Additionally, the molecular mechanisms responsible for patterning the limb bear striking similarities to various other organ systems, making the limb an excellent model to help decipher how teratogens elicit their detrimental effects on organogenesis (Yamada, 2005).

As the limb develops, mesenchymal cells are recruited, migrate, and condense, initiating chondrogenesis, the earliest phase in skeletal development. These mesenchymal condensations differentiate and give rise to cartilaginous anlagen that eventually yield the ossified elements that comprise the skeleton (Olsen et al., 2000). Previous studies have demonstrated that excess retinoid disrupts chondrogenesis (Kochhar, 1973; Kwasigroch and Kochhar, 1980). Although these effects on cartilage formation have been attributed to aberrant proliferation, differentiation and apoptosis (Zakeri and Ahuja, 1994; Ali-Khan and Hales, 2006a),

many of the genes and their underlying downstream mechanisms that regulate the retinoid-induced reduction in chondrogenesis are still unresolved.

Retinoids elicit their biological effects by regulating gene transcription. In the presence of ligand, retinoic acid:rexinoid nuclear receptor (RAR:RXR) heterodimers bind to retinoic acid response elements (RARE) and recruit and activate transcriptional machinery (Bastien and Rochette-Egly, 2004). Various RAR and RXR isotypes (and variant isoforms) have been identified (α, β, γ) yet only pharmacological activation of RARs, and not RXRs, causes teratogenic effects, illustrating the importance of RARs in mediating the teratogenic signal in the developing limb (Arafa et al., 2000). Expression of RARs in the limb is temporally and spatially dynamic. During limb outgrowth, while RARα is expressed ubiquitously and RARβ is restricted to the webbing, the cartilage tissue which is highly sensitive to retinoid insult, expresses RARγ (Dolle et al., 1989).

Single RAR isoform ablation studies have uncovered the importance of RARγ in controlling axial development and patterning, but receptor redundancy has been linked to the lack of effect seen in the appendicular skeletons of RARγ-deficient mice (Chambon, 1994; Lohnes et al., 1995). Notwithstanding, previous work in our lab has shown that limbs deficient in RARγ (on an α1-null background) were less susceptible to retinoid-induced limb dysmorphogenesis. Chondrogenesis and overall limb growth in these transgenic mice were vastly improved following retinoid insult, when compared to their retinoid-treated wildtype counterparts, indicating the importance of RARγ in mediating limb teratogenesis (Ch. 2; Galdones et al., 2006).

Contrary to RARγ ablation, the objective of the present study was to determine the outcomes of aberrant RARγ activation on limb morphogenesis. To do so, an *in vitro* limb bud culture system was employed (Kwasigroch and Skalko, 1983) and aberrant RARγ activation was induced pharmacologically using BMS-189961 (BMS961), a highly RARγ-selective agonist (Klaholz et al., 2000). Given the predominantly high expression of RARγ in cartilage during limb development, we hypothesized that retinoid-induced limb dysmorphogenesis is mediated by a RARγ-dependent misregulation of genes important for proper chondrogenesis.

From a pharmacological standpoint, RARγ is an extensively pursued drug target for the treatment of several medical conditions including polycystic acne, psoriasis, diabetes and various cancers (Johnson and Chandraratna, 1999). Unfortunately, such treatments are contraindicated for women of childbearing age. Understanding the modes of RARγ-mediated toxicity during development will effectively aid in the development of safer, less teratogenic retinoid analogs.

In this study, we demonstrate the teratogenic action of a RARγ-selective agonist on the developing limb. Retinoid-responsive reporter mice (RARβ2-lacZ) were employed to assess the location and extent of retinoid activity following activation of RARγ (Rossant et al., 1991). Gene expression analysis using chondrogenesis-focused microarrays has identified two RARγ-responsive genes (*Mgp* [matrix GLA protein] and *Gdf10* [growth differentiation factor-10]) that may have novel uncharacterized roles in transducing the teratogenic signal in the limb. Furthermore, *in situ* hybridization (ISH) has localized *Mgp* to areas of developing

cartilage and *Gdf10* to areas of programmed cell death in the limb, suggesting independent functions for each of these candidate genes.

Materials and Methods

Limb Bud Culture and Drug Treatments

Pregnant gestational day (GD) 12 CD-1 mice were euthanized, embryos were explanted and forelimbs were excised just lateral to the somites and cultured *in vitro* in a chemically-defined culture medium, as previously described (Ali-Khan and Hales, 2003). All-*trans* retinoic acid (RA; Sigma, St. Louis, MI) and BMS-189961, a RARγ-selective agonist (BMS961; a gift from Bristol-Myers Squibb, Wallingford, CT), were dissolved in 100% EtOH. Limbs were exposed to low and high concentrations of RA (0.1 and 1.0 μM) or BMS961 (0.01 and 0.1 μM) at the onset of culture. All animal studies complied with the guidelines established by the Canadian Council on Animal Care.

Limb Morphology

Limbs were cultured for 6 days, with one change of the medium that was not supplemented with RA or BMS961, on day 3, as previously described (Galdones et al., 2006). Briefly, limbs were fixed and stained with 0.1% toluidine blue (Fisher Scientific, Montreal, Canada) and examined under a dissecting microscope (Wild Heerbrugg 99067; Wild Leitz, Ottawa, Canada) and photographs acquired with a JVC digital camera (JVC GCQX3HD, Tokyo, Japan). The extent of proper limb development was quantified using a limb morphogenetic scoring system (Neubert

and Barrach, 1977). Five separate replicates were completed and examined for morphological changes or markers.

RAREβ2-hsp68lacZ Reporter Mice and Staining

To examine the extent and localization of retinoid activity, RAREβ2-hsp68*lacZ* double-transgenic males were mated with wildtype CD-1 females to produce single copy transgene offspring. These embryos were explanted at E12.5 and limbs cultured for 3 and 12 h as described above, in the absence or presence of RA or BMS961. Tissues were fixed immediately with 0.25% glutaraldehyde then stained with X-gal for 2-4 h, as previously described (Rossant et al., 1991). Limbs were rinsed in PBS then visualized under a dissecting microscope. Results were acquired from three independent limb culture experiments.

RNA Extraction

After 3 h *in vitro*, each group of six to eight forelimbs was removed from culture, rinsed with PBS and stored at -80°C in RNAlater RNA Stabilization Reagent (Qiagen, Mississauga, Canada) until further processing. Total RNA was extracted using the RNeasy Micro Kit (Qiagen) and quality and purity/quantity assessed by agarose gel electrophoresis and spectrophotometric UV analysis in 10mM Tris, pH 8.0 buffer, respectively. Five separate independent cultures of each treatment group were completed and used for microarray and quantitative RT-PCR analysis.

cRNA Synthesis and Hybridization

Starting with 2 μg of total mRNA, the TrueLabeling-AMP™ 2.0 Kit (Superarray Bioscience, Frederick, MD) was used to produce, amplify and biotinylate antisense cRNA for hybridization following the manufacturer's guidelines. The cRNA product was purified with ArrayGrade™ cRNA Cleanup (SuperArray Bioscience) and then hybridized overnight to Mouse Oligo GEArray® Osteogenesis Microarrays (OMM-026, SuperArray Bioscience), using 3 μg of cRNA per array and following the manufacturer's suggested HybTube Standard Protocol. The following day, the membranes were stained with an alkaline-phosphatase-conjugated streptavidin antibody (1:8000, SuperArray Bioscience) and the chemiluminescence signal was developed using the CDP-Star® Kit (SuperArray Bioscience). Five arrays, each from independent limb cultures, were completed for each treatment group.

Data Acquisition and Gene Expression Analysis

The array images were recorded using X-ray film, digitized using a desktop scanner, saved as 16-bit TIFF files and uploaded onto GEArray Expression Analysis Suite (GEASuite, SuperArray Bioscience). Array alignment and raw intensity quantification was done using GEASuite, then the data were imported into Agilent GeneSpring 7.3 GX (Agilent Technologies, Palo Alto, CA) where background correction and all further analysis was completed. For each individual array, the gene detection threshold was set at a raw signal intensity of 2X the background intensity; to minimize array-to-array variation, expression values were normalized to the median of all measurements on that individual array. Upon identification of the uniquely and

commonly expressed genes in each group, statistical comparisons between control and RA or BMS961 treated groups were done using Student's *t*-tests ($p \leq 0.05$). Genes were only included in the significantly regulated group of genes when significance was detected in at least three out of five replicates, and the changes were all in the same direction (i.e., either all up- or all downregulated).

Quantitative Real-time PCR

RNA was diluted to a final working solution of 10 ng/μl and Quantitect One-Step SYBR Green RT-PCR (Qiagen) was done using the Roche LightCycler (Roche Diagnostics, Laval, Canada). The quantitative real-time PCR cycling parameters were as follows: 95°C for 15 min (1 cycle) and then 94°C for 15 sec, 55°C for 30 sec, and 72°C for 20 sec (for 50 cycles) and each sample was measured in duplicate. Embryonic head tissue was also collected, and RNA extracted to provide 1, 10, and 50 ng/μl RNA stocks used to generate standard curves for quantification. The primers were generated with Primer3 software (http://frodo.wi.mit.edu) and produced by Alpha DNA (Montreal, Canada; see Table 1 for sequence information). To normalize the output, the expression of each gene of interest was divided by 18S gene expression, a commonly used housekeeping gene whose expression remains unchanged in murine limbs following retinoid exposure (Ali-Khan and Hales, 2006a). Melting-curve analyses were done following each PCR to determine the output and detection quality (i.e., the formation of primer-dimers). Each treatment consisted of RNA from four separate culture experiments and each sample was measured in duplicate.

Whole Mount ISH

Limbs cultured for 3, 6 or 12 h were rinsed with PBS containing 0.1% Tween 20 (PBT) and fixed overnight at 4°C in 4% paraformaldehyde. The following day, the limbs were dehydrated through a methanol/PBT gradient and stored at -20°C until further use. For whole mount in situ hybridization (ISH), a DIG RNA labeling kit (Roche Diagostics) was used to synthesize *Mgp* and *Gdf10* antisense probes from plasmids kindly provided by G. Karsenty (Columbia University, New York, NY) and S.-J. Lee (Johns Hopkins University, Baltimore, MD), respectively. Whole mount ISH was done as previously described (Decimo et al., 1995). After hybridization, limbs were rinsed in PBT and photographed under a dissecting stereomicroscope (Wild Leitz). Three separate whole mount ISH experiments were done for each gene of interest.

Results

Aberrant Activation of RARγ is Teratogenic to the Developing Limb

Because retinoid insult in the limb is attenuated in the absence of RARγ (Galdones et al., 2006), our first goal was to determine if and how aberrant RARγ activation affects limb development. *In vitro* cultured murine limbs were treated with either RA (pan-RAR agonist) or BMS961 (RARγ-selective agonist) at E12.5. The treatment of limbs with either drug resulted in dose-dependent detrimental effects on gross morphology after culture for 6 days, with marked effects on chondrogenesis, as evidenced by decreases in toluidine blue stained cartilage (Fig. 3.1A). After exposure to low concentrations of RA or BMS961 (RA: 0.1 μM, BMS961: 0.01 μM), the zeugopod (long bones) appeared growth retarded whereas the autopod (paw) remained intact. However, at high exposures (RA: 1.0 μM, BMS961: 0.1 μM) both limb regions were equally undifferentiated.

A limb scoring system was used to determine the quality and extent of morphogenetic differentiation of the cartilage elements that comprise the forming limb after exposure to the pan- or RARγ-selective agonist (Neubert and Barrach, 1977). In accordance with the gross morphology, limb scores were compromised following treatment with either the pan- or RARγ-selective agonist (Fig. 3.1B).

RARγ-selective Agonism Induces Retinoid Activity in RAREβ2-hsp68lacZ Reporter Limbs

To determine the amount and distribution of retinoid activity in limbs following RA or BMS961 exposure, we treated transgenic limbs from a retinoid reporter strain

containing a transgene under the control of the retinoid sensitive RARβ2 response element (Rossant et al., 1991). In the absence of retinoid, the *lacZ* transgene was strongly expressed in the interdigital zones and observable along the axes of the forming long bones after 3 h *in vitro* (Fig. 3.2). With the addition of 0.1 or 1.0 μM RA for 3 h, transgene expression was strongly up-regulated in the proximal mesenchyme, necrotic zones, dorsal ectoderm and interdigital webbing. At 3 h, BMS961 treatment (0.01 or 0.1 μM) increased transgene expression in the proximal mesenchyme as well, but only in a modest fashion; transgene expression in the webbing was affected similarly in BMS961 and RA-treated limbs.

After 12 h *in vitro*, vehicle-treated limbs exhibited minimal transgene expression. In contrast, both pan-RAR and RARγ-selective activation induced restricted transgene expression in the zone of polarizing activity, the limb signaling center responsible for anterior-posterior patterning (Tickle, 2006). Additionally, while RA did not induce transgene expression in the interdigital zones at this later time point, BMS961 exposure resulted in a faint induction of staining in the webbing. Thus, because maximal effects on *lacZ* transgene expression were observed after 3 h *in vitro*, subsequent experiments were done at that early timepoint.

Changes in Gene Expression Induced by Retinoic Acid or BMS961 Treatment

It is clear that retinoids have detrimental effects on chondrogenesis (Jiang et al., 1995) yet the various mechanisms underlying this process are still unresolved. We adopted a gene array approach to assess how the regulation of chondrogenesis-related genes in the limb was affected following pan-RAR or RARγ-selective insult.

Limbs were treated for 3 h with either RA or BMS961 and RNA was hybridized to Mouse Oligo Osteogenesis Microarrays from GEArray® containing approximately 100 genes important in various aspects of cartilage and bone development.

In comparing the vehicle-treated limbs to those treated with 0.1 μM or 1.0 μM RA, 55 genes were commonly expressed in all groups (Supplementary Table S3.1). Four genes were expressed uniquely under control conditions while one gene was expressed only after exposure to 1.0 μM RA (Fig. 3.3A). Only two genes were expressed in two out of three treatment groups (0 and 0.1 μM; genes listed in Fig. 3.3A). Limbs treated with BMS961 exhibited a similar outcome, with the majority of genes commonly expressed between all groups (58; Supplementary Table S3.2) although one gene was expressed solely after exposure to low concentrations; 4 genes were expressed in both control and 0.01 μM BMS961-treated limbs, but not in 0.1 μM exposed limbs (genes listed in Fig. 3.3B). All uniquely expressed genes were only expressed at minimal baseline levels, therefore our analysis was limited to the groups of commonly expressed genes.

Activation of RARγ is Implicated in the Misregulation of Matrix Gla Protein (Mgp) and Growth Differentiation Factor-10 (Gdf10) Gene Expression

To identify genes important in mediating retinoid-induced limb teratogenesis, the lists of commonly expressed genes in all RA or BMS961 treatment groups were analyzed for statistical significance ($P < .05$) using Agilent GeneSpring 7.3 GX software. Comparison between vehicle and the two RA-treated groups identified 9 genes that were significantly up- or downregulated by treatment (Table 3.2). Our

work identified a subset of genes that were significantly changed (either up- or downregulated) following RA exposure (Table 3.2). Several of the identified genes are related to bone morphogenetic protein/transforming growth factor-β (BMP/TGF-β) signaling (*Smad2*, *Bmp4* and *Gdf10*) and components of cartilage and extracellular matrix (*Col11a1*, *Spp1* and *Dcn*), while others are linked to matrix breakdown (*Ctsk*), oxidative stress (*Nfkb1*) and regulation of chondrogenesis (*Mgp*).

When comparing the control versus the two BMS961-treated groups, only 2 genes were identified as being up-regulated by RARγ activation (Table 3.2). The lists of significantly altered genes were compared; both of the genes altered by RARγ-selective activation were also misregulated by RA exposure (Fig. 3.3C). These genes were *Mgp*, a potent inhibitor of chondrogenesis and osteogenesis (Price and Williamson, 1985) and *Gdf10*, a bone morphogenetic protein of uncharacterized function in the developing limb (Cunningham et al., 1995).

Array Verification by qRT-PCR

We used qRT-PCR to assess the gene expression profiles of the two RARγ-responsive genes, *Mgp* and *Gdf10*. After culture for 3 h, the expression of both *Mgp* and *Gdf10* was significantly up-regulated by exposure to low or high concentrations of RA as well as the high concentration of BMS961; the expression of these genes was not responsive to the low concentration of BMS961 (Fig. 3.4A and B). Interestingly, after 12 h in culture, the expression of both genes did not differ from control in the RA or BMS961 exposed limbs, illustrating that the effect of the retinoids on the expression of these genes in the cultured limbs is transient; indeed,

the expression of *Gdf10* was significantly down-regulated in 0.1 μM RA-treated limbs at 12 h.

In addition, to further validate the results from the array experiments, we selected two genes that were pan-RAR, but not RARγ-responsive. *Dcn* (decorin), a proteoglycan associated with collagen (Scott and Orford, 1981) and *Ctsk* (cathepsin K), a cysteine protease expressed in bone (Garnero et al., 1998) were both downregulated by exposure to 1.0 μM RA for 3 h (Fig. 3.4C and D). As anticipated from the array data, exposure to either 0.01 or 0.1 μM BMS961 had no effect on the expression of these genes in limbs for 3 h *in vitro*.

Distribution of Mgp and Gdf-10 Gene Transcripts Following pan-RAR and RARγ-Selective Activation

Whole mount *in situ* hybridizations were performed to determine the distribution of *Mgp* and *Gdf10*, the gene transcripts misregulated by RARγ activation. Vehicle-treated limbs exhibited very little *Mgp* staining at 3 h; *Mgp* expression was induced by RA in a concentration-dependent manner. The induction of *Mgp* expression was restricted to the forming long bones at low RA concentrations and was extended distally into the rays at high RA levels. *Mgp* expression was up-regulated strongly by BMS961 in the forming long bones, as well as the proximal mesenchyme, but was not induced in the digits by exposure to either concentration of BMS961 (Fig. 3.5A). At 6 h, *Mgp* transcripts were detected in the long bones in vehicle exposed limbs; exposure to either RA further upregulated *Mgp* expression at this time point while BMS961 did not. Interestingly, by 12 h *in vitro*, little *Mgp*

expression was found in any treatment group, echoing the results seen by qRT-PCR where a transient decrease in expression was observed (Fig. 3.4A).

Localization of the expression of *Gdf10* was distinct from that of *Mgp*. G*df10* expression was faintly observable in the interdigital webbing under control conditions at 3 h (Fig. 3.5B). Ectopic expression was induced by exposure to either the low or high concentration of RA and shown to be highly localized to the interdigital webbing as well as the anterior and posterior necrotic zones, all areas that are pre-destined to apoptose and help sculpt the developing limb. In addition, high concentrations of RA induced *Gdf10* transcripts in the proximal regions of the forming digits. BMS961 treatment resulted in similar expression patterns, albeit not as intense as their RA-treated counterparts. At 6 h, when control limbs expressed very little *Gdf10*, RA and BMS961 induced ectopic expression in the regions of programmed cell death and proximal rays. By 12 h *in vitro*, in RA-exposed limbs only faint staining was observable in the digits; however, BMS961-treated limbs still expressed low levels of *Gdf10* in the webbing, necrotic zones and proximal rays (Fig. 3.5B).

Discussion

Although the effects of excess retinoids on development have been described, the mechanisms by which normal limb chondrogenesis and patterning is disrupted, and specifically the roles of distinct RARs, are not completely understood. Given that RARγ is highly expressed in the forming cartilage (Dolle et al., 1989), we hypothesized that RARγ mediates retinoid-induced limb dysmorphogenesis. Specifically, the goal of this study was to examine how pharmacological activation of RARγ affects limb chondrogenesis.

Our results show that treatment of limbs with either RA (pan-RAR agonist) or BMS961 (RARγ-selective agonist) *in vitro* is detrimental to limb chondrogenesis and growth. Microarray-based expression analysis of chondrogenesis-related genes identified a group of RA-responsive genes and two RARγ-responsive genes (*Mgp* and *Gdf10*). The localization of up-regulated *Mgp* transcripts to chondrocytes, and those of *Gdf10* to interdigital webbing/necrotic zones, suggests that they play novel roles during retinoid-induced limb dysmorphogenesis; namely the misregulation of chondrogenesis and programmed cell death, respectively.

RARγ-induced Limb Defects

Both pan-RAR and RARγ-selective activation is detrimental to limb morphogenesis, significantly affecting the proper chondrogenesis of long bones, carpalia and digits (Fig. 3.1). Specifically, while RA-treated limbs exhibited marked reductive effects on all cartilage elements at 0.1 and 1.0 μM RA concentrations, BMS961 elicited phenotypically similar effects at concentrations that were 10-fold

less (0.01 and 0.1 μM). While there is evidence that BMS961 can exhibit weak affinity for RARβ, the effective concentration (EC_{50}) at which BMS961 binds RARβ is 3-fold higher than our highest treatment (Klaholz et al., 2000), suggesting that BMS961 is indeed acting as a RARγ-selective agonist in our limb bud cultures.

Retinoid Activity

The pattern of retinoid-induced lacZ upregulation in areas of endogenous RARβ expression has been observed previously (Rossant et al., 1991). Given that transgene expression is driven by three copies of the highly retinoid-responsive response element of RARβ2 (RAREβ2), the specific sequence of RARβ2 may not have a strong binding affinity for RARγ. Indeed, RARγ-associated retinoid activity is most likely higher than observed, because RARγ-selective activation induced gene expression changes in areas (i.e., chondrocytes) where no lacZ transgene expression was detected (Fig. 3.5A).

Pan-RAR Responsive Genes

Several groups, including our own, have studied global gene expression changes in the limb following retinoid excess (Qin et al., 2002; Ali-Khan and Hales, 2006a). To refine our search, and because chondrogenesis is severely affected by exogenous retinoid exposure, we assessed the expression changes of genes known to participate in aspects of cartilage and bone development.

Interestingly, the direction in which the genes were regulated was mixed; contrary to previous work illustrating the positive uni-directionality of the gene

changes following exogenous retinol (vitamin A) exposure (Ali-Khan and Hales, 2006a), a discrepancy that may be explained by the kinetics of the drug used. The immediate bioactivity of RA treatment leads to direct effects on their respective targets whereas retinol must be activated into RA, thus it would be of particular interest to assess retinol-induced gene changes following timepoints later than 3 h *in vitro*.

Classically, in the absence of ligand, RARs remain associated with RAREs and transcriptionally repress target genes; however, once a ligand is bound, the RARs change conformation, release their associated co-repressors and in turn recruit co-activators and the transcriptional machinery that drive gene expression (Weston et al., 2003a). In our study, while several genes were up-regulated by RA treatment (*Mgp*, *Gdf10, Spp1* and *Bmp4*), others (*Ctsk, Col11a1, Nfkb1, Smad2* and *Dcn*) were significantly down-regulated. How this downregulation occurs is not well understood, yet at least two explanations exist: (1) RA may up-regulate an intermediate gene upstream, which in turn inhibits the transcription of the observed target gene, or (2) the liganded RAR/RXR heterodimers may bind negative response elements (NRE) that repress rather than activate transcription. These NREs have been identified upstream of several retinoid-responsive genes, including MGP (Kirfel et al., 1997). However, since we have observed an upregulation of *Mgp* in our system, the activity of the NRE must be cell and tissue dependent.

We propose that these RA-responsive genes may play important novel roles in mediating retinoid-induced limb dysmorphogenesis. Since the scope of this study was focused on the RARγ-mediated regulation of gene expression, future studies will

be required to determine how the misregulation of these newly identified RA-responsive genes affect limb morphogenesis.

RARγ Responsive Genes

In contrast to limbs treated with RA, BMS961-treated limbs exhibited a significant upregulation of only two genes: *Mgp* and *Gdf10* (Fig. 3.3C). Whole mount *in situ* hybridizations were performed to determine the extent and localization of gene upregulation; *Mgp* was expressed and significantly up-regulated in chondrocytes (Fig. 3.5A). Of interest, *Gdf10* upregulation was limited to the interdigital webbing and the necrotic zones (Fig. 3.5B). These differential locations of expression suggest that each of these genes performs unique functions following retinoid-induced misregulation.

Inhibition of Chondrogenesis by Mgp

MGP, a γ-carboxyglutamic acid-rich, vitamin K-dependent protein (Price and Williamson, 1985), has been characterized as a potent inhibitor of chondrocyte maturation and mineralization (Yagami et al., 1999). Mice lacking *Mgp* are viable, but exhibit spontaneous calcification of arteries and cartilage (Luo et al., 1997). Our array experiments and follow-up qRT-PCR analysis show that *Mgp* expression is significantly up-regulated by RA and BMS961. These results are consistent with those of Cancela and Price (1992) who identified a putative RARE upstream of *Mgp* bearing a striking resemblance to the RAREβ2 (de The et al., 1990) and showed the RA-dependent upregulation of *Mgp* in various human tissues, including

chondrocytes (Cancela and Price, 1992). However, whereas the RARγ-selective agonist did significantly up-regulate *Mgp*, it did not do so to the same extent as RA (Fig. 3.4A), suggesting that other RARs (α and β) may participate in its complete transcriptional regulation.

Although *Mgp* transcripts were not detected by whole mount ISH in untreated limbs, RA and BMS961-treated limbs aberrantly expressed *Mgp* in areas of chondrocyte development. Because chondrogenesis is a major target in retinoid-induced limb dysmorphogenesis, the distribution of *Mgp* transcripts may correlate with the reductive effects associated with retinoid insult. This is the first report implicating *Mgp* in a teratological context. MGP has been identified as playing a role as a functional inhibitor of BMP-2 and BMP-4 function (Yao et al., 2007). Given that regulated BMPs are required for proper limb development, further work is needed to determine whether *Mgp* is indispensable for mediating specific retinoid-induced aberrations during development.

Gdf10 and Programmed Cell Death

GDF10 is a member of the TGFβ superfamily and is highly related to BMP-3 (Cunningham et al., 1995), hence *Gdf10* is also commonly referred to as BMP-3b. Expression of *Gdf10* during organogenesis is highly localized in uterus, adipose and brain and less so in bone; ablation of *Gdf10* resulted in no apparent effects on developmental outcome (Zhao et al., 1999). Many TGFβ superfamily members have been linked to various aspects of limb development: BMP-2, -4 and -7, the most highly characterized, play crucial roles in precartilaginous and chondrocyte

differentiation, apical ectodermal ridge-dependent limb outgrowth, anterior-posterior patterning and interdigital cell death (Kawakami et al., 1996), while TGFβ and GDF5 have been implicated in joint development (Storm and Kingsley, 1999). *Gdf10* function in the limb has not been previously characterized (Zhao et al., 1999). Experiments targeting osteoblast function *in vitro* have recently revealed that GDF10 can inhibit BMP-2-dependent alkaline phosphatase activity, hence illustrating the negative regulation of GDF10 on osteogenesis (Hino et al., 1999). We are the first to show that *Gdf10* can be regulated by retinoids and that its upregulation is localized specifically to domains of programmed cell death in the limb, namely the webbing and necrotic zones (Fig. 3.5B). We postulate that *Gdf10* plays an integral role in regulating the apoptosis associated with retinoid teratogenicity (Ali-Khan and Hales, 2003).

Summary

These results show the importance of RARγ agonists in mediating retinoid-induced limb dysmorphogenesis. Our chondrogenesis-focused gene expression analysis has identified a handful of retinoid-regulated candidate genes that may play novel roles in limb dysmorphogenesis. Notably, our work is the first indication that *Mgp* and *Gdf10* are significantly (albeit, transiently) up-regulated following pan-RAR or RARγ-selective activation. The identification of these candidate genes and their association with RARγ will help to unravel new mechanisms underlying retinoid-induced insult during development.

Acknowledgements

We should like to thank Bristol Myers Squibb for providing us with the RARγ-selective agonist, BMS-189961 and Dr. Gerard Karsenty and Dr. Se-Jin Lee for the *Mgp* and *Gdf10* cDNA probes, respectively. Also, we are grateful to Dr. Glenn Maclean for his technical advice. This work was supported by the Canadian Institutes for Health Research.

Tables

Table 3.1

Primer sequences used for quantitative RT-PCR.

Name	Symbol	Genbank No.	Left Primer	Right Primer
18S RNA	*Rn18s*	X00686	AAA CGG CTA CCA CAT CCA AG	CCT CCA ATG GAT CCT CGT TA
Matrix gla protein	*Mgp*	NM_008597	GCG AAG AAA CAG TCA TTT GGT	TCA ACC CGC AGA AGG AAG
Growth differentiation factor 10	*Gdf10*	NM_145741	ATG CCC AGA ATT TCC ACA AG	AAG TCC AGC ACC TGA GAG GA
Cathepsin K	*Ctsk*	NM_007802	GAA CGA GAA AGC CCT GAA GA	CAC ACC TCT GCT GTA AAA CTG G
Decorin	*Dcn*	NM_007833	GTC TGG CCA ATG TTC CTC AT	AGG TCA TTT TGC CCA ACT GC

Table 3.2

List of genes significantly up- or down-regulated following RA or BMS961 treatment

0 vs 0.1 μM RA			Relative Intensity			
Gene	Symbol	Genbank	0 μM	0.1 μM	Direction	*P*
Matrix gla protein	*Mgp*	NM_008597	1.84	2.52	↑	0.049
Growth differentiation factor 10	*Gdf10*	NM_145741	1.43	2.01	↑	0.009

0 vs 1.0 μM RA			Relative Intensity			
Gene	Symbol	Genbank	0 μM	1.0 μM	Direction	*P*
Cathepsin K	*Ctsk*	NM_007802	1.18	0.90	↓	0.046
Type XI (1a) collagen	*Coll11a1*	NM_007729	1.41	1.01	↓	0.042
Nuclear factor of kappa light chain gene enhancer in b cells 1	*Nfkb1*	NM_008689	1.62	1.41	↓	0.039
Secreted phosphoprotein 1	*Spp1*	NM_009263	0.88	1.11	↑	0.022
Growth differentiation factor 10	*Gdf10*	NM_145741	1.43	2.08	↑	0.015
MAD homolog 2	*Smad2*	NM_010754	1.42	1.24	↓	0.007
Decorin	*Dcn*	NM_007833	1.68	1.13	↓	0.005

0.1 vs 1.0 μM RA			Relative Intensity			
Gene	Symbol	Genbank	0.1μM	1.0 μM	Direction	*P*
Bone morphogenetic protein 4	*Bmp4*	NM_007554	1.07	1.30	↑	0.043
Decorin	*Dcn*	NM_007833	1.69	1.13	↓	0.014

0 vs 0.01 μM BMS961			Relative Intensity			
Gene	Symbol	Genbank	0 μM	0.01 μM	Direction	*P*
Matrix gla protein	*Mgp*	NM_008597	1.84	2.33	↑	0.008

0 vs 0.1 μM BMS961			Relative Intensity			
Gene	Symbol	Genbank	0 μM	0.1 μM	Direction	*P*
Growth differentiation factor 10	*Gdf10*	NM_145741	1.43	1.80	↑	0.023

Figures and Legends

Figure 3.1. The effects of pan-RAR and RARγ-selective activation on limbs following 6 days in culture. Limbs were collected from CD-1 mice at embryonic day 12.5 and cultured *in vitro* for 6 days in the presence or absence of pan-RAR agonist, all-*trans* retinoic acid (0.1, 1.0 μM) or RARγ-selective agonist, BMS961 (0.01, 0.1 μM). (**A**) Limbs were stained with 0.1% toluidine blue (w/v 70% ethanol). Both RA and BMS961 treatment result in marked effects on chondrogenesis, namely long bone outgrowth and digit formation. (**B**) The limb morphogenetic scores measured the extent of differentiation and chondrogenesis following treatment.

Figure 3.1

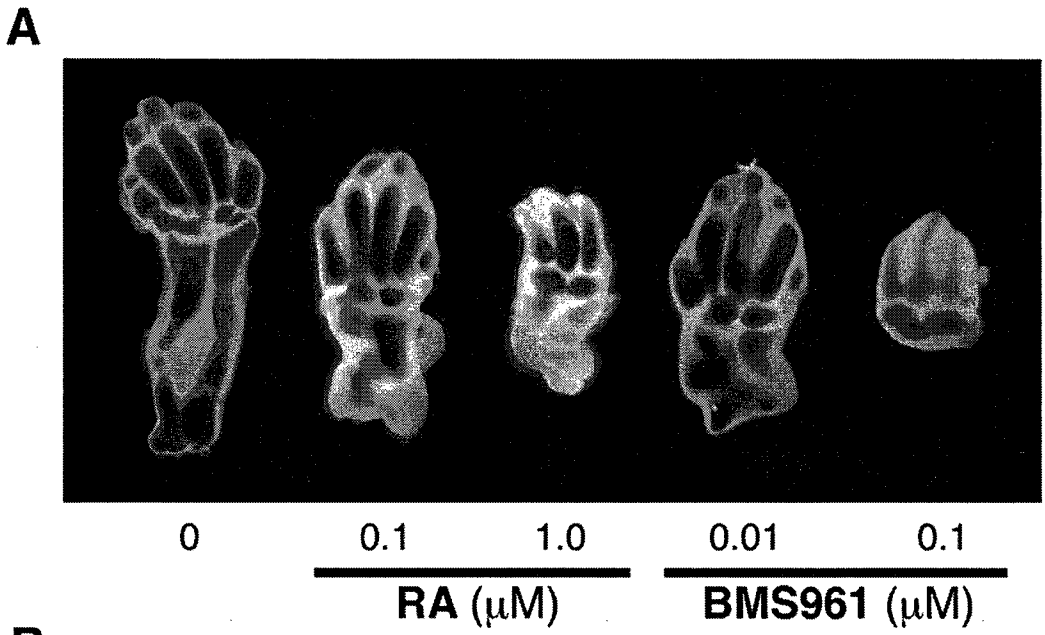

A
0
0.1
1.0
0.01
0.1
RA (μM)
BMS961 (μM)
B
Limb Score ± SEM
250
200
150
100
50
0
*
*
*
*
0
0.1
1.0
0.01
0.1
RA (μM)
BMS961 (μM)

Figure 3.2. Localization of retinoic acid receptor activity, as measured using the RAREβ2-hsp68*lacZ* retinoic-responsive reporter mice.

Figure 3.2

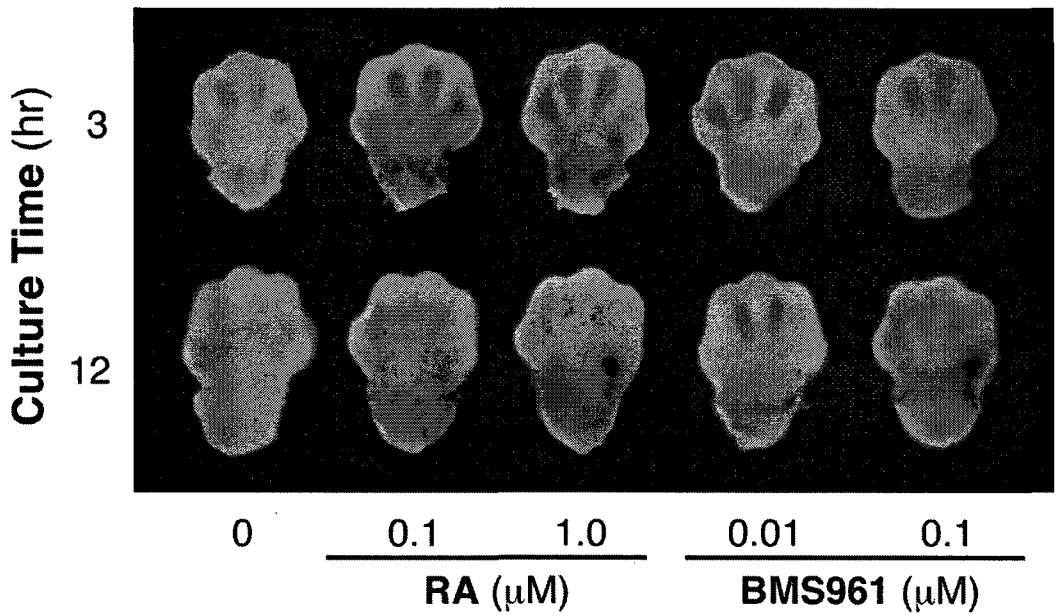


Figure 3.3. Venn diagrams of gene expression. (**A**) The numbers of genes that were uniquely and commonly expressed following exposure to 0, 0.1 or 1.0 μM RA for 3 h. (**B**) The numbers of genes that were uniquely and commonly expressed following exposure to 0, 0.01 or 0.1 μM BMS961 for 3 h. (**C**) Genes that were significantly up or down-regulated when comparing control-treated limbs to those treated with either low and high RA and/or BMS961 treatment.

Figure 3.3

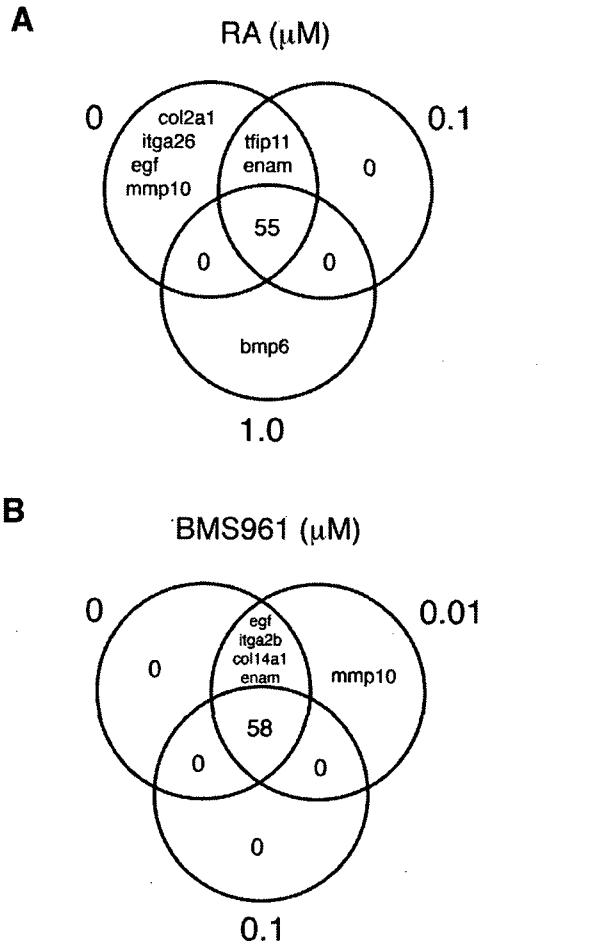

A
RA (μM)
0
0.1
1.0
col2a1
itga26
egf
mmp10
tfip11
enam
0
55
0
0
bmp6
B
BMS961 (μM)
0
0.01
0.1
0
egf
itga2b
col14a1
enam
mmp10
58
0
0
0
C
RA
BMS961
ctsk
col11a1
nfkb1
spp1
smad2
dcn
bmp4
mgp
gdf10
0

Figure 3.4. qRT-PCR validation of the changes in expression of genes responsive to pan-RAR and RARγ-selective activation. Limbs were cultured *in vitro* for 3 (*open bars*) or 12 (*closed bars*) h in the absence or presence of RA (0.1, 1.0 μM) or BMS961 (0.01, 0.1 μM). (**A**) *Mgp* gene expression is up-regulated by RA and BMS961 treatment after 3 h in culture but returns to baseline expression levels by 12 h. (**B**) *Gdf10* expression is up-regulated in response to RA and BMS961 treatment following 3 h, but not 12 h *in vitro*. (**C**) *Dcn* and (**D**) *Ctsk* are both down-regulated by RA but not BMS961 after 3 h in culture. Each bar represents the mean gene expression of 4 separately conducted limb cultures normalized against 18S mRNA expression. Statistical analysis was done using 2-way ANOVA and the Holm-Sidak post-hoc multiple-comparison test. *Significant increase, $P < 0.05$; #Significant decrease, $P < 0.05$.

Figure 3.4

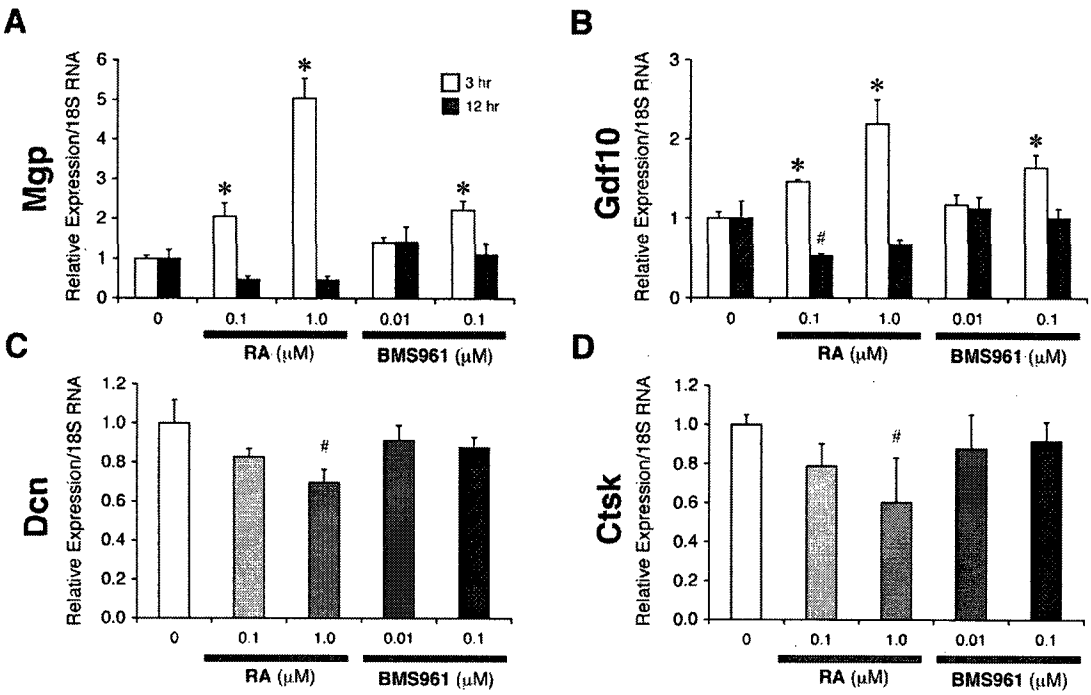
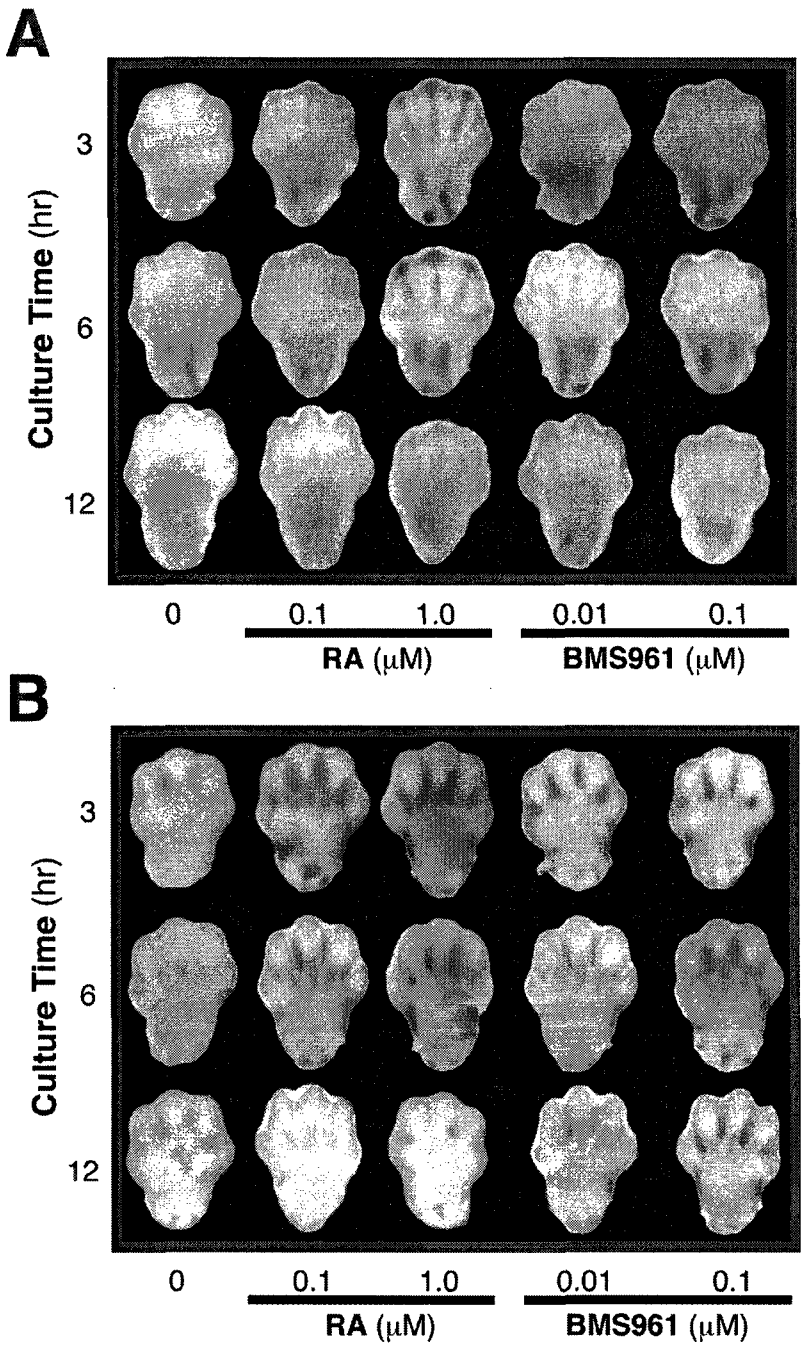

A
Mgp
Relative Expression/18S RNA
3 hr
12 hr
0
0.1
1.0
0.01
0.1
RA (μM)
BMS961 (μM)
B
Gdf10
Relative Expression/18S RNA
C
Dcn
Relative Expression/18S RNA
D
Ctsk
Relative Expression/18S RNA

Figure 3.5. Localization of RARγ-responsive gene transcripts following pan-RAR or RARγ-selective activation. Whole mount *in situ* hybridization of (**A**) *Mgp* and (**B**) *Gdf10* in E12.5 limbs following exposure to RA or BMS961 for 3, 6 and 12 h *in vitro*.

Figure 3.5


A
Culture Time (hr)
3
6
12
0
0.1
1.0
0.01
0.1
RA (μM)
BMS961 (μM)
B
Culture Time (hr)
3
6
12
0
0.1
1.0
0.01
0.1
RA (μM)
BMS961 (μM)

Supplementary Tables

Supplementary Table 3.1

	Relative Intensity				
Symbol	0 μM	0.1μM	1.0 μM	Genbank	Description
Twist1	2.65	3.37	2.79	NM_011658	Twist gene homolog 1 (Drosophila)
Cdh11	2.58	3.25	2.71	NM_009866	Cadherin 11
Col5a1	2.58	3.24	2.74	NM_015734	Procollagen, type V, alpha 1
Fn1	2.57	3.17	2.67	NM_010233	Fibronectin 1
Gapdh	2.60	3.27	2.78	NM_008084	Glyceraldehyde-3-phosphate dehydrogenase
Vcam1	2.62	3.28	2.77	NM_011693	Vascular cell adhesion molecule 1
Tgfb3	2.55	3.18	2.76	NM_009368	Transforming growth factor, beta 3
Sox9	2.56	3.20	2.06	NM_011448	SRY-box containing gene 9
Sparc	2.54	3.15	2.55	NM_009242	Secreted acidic cysteine rich glycoprotein
Bmp7	2.32	3.12	2.76	NM_007557	Bone morphogenetic protein 7
Col1a2	2.39	2.68	2.06	NM_007743	Procollagen, type I, alpha 2
Bgn	2.54	3.09	2.59	NM_007542	Biglycan
Serpinh1	2.33	2.43	2.37	NM_009825	Serine (or cysteine) proteinase inhibitor, clade H, member 1
Pdgfa	2.48	2.90	2.46	NM_008808	Platelet derived growth factor, alpha
Twist2	2.35	2.96	2.74	NM_007855	Twist homolog 2 (Drosophila)
Vegfb	2.45	2.89	2.30	NM_011697	Vascular endothelial growth factor B
Col15a1	2.35	2.69	2.26	NM_009928	Procollagen, type XV
Col3a1	2.07	2.21	1.69	NM_009930	Procollagen, type III, alpha 1
Col4a1	2.36	2.81	2.36	NM_009931	Procollagen, type IV, alpha 1
Anxa5	2.31	2.71	2.40	NM_009673	Annexin A5
Igf1r	2.34	2.35	2.12	NM_010513	Insulin-like growth factor I receptor
Gdf10	1.43	2.01	2.08	NM_145741	Growth differentiation factor 10
Tgfb2	2.12	2.33	1.82	NM_009367	Transforming growth factor, beta 2
Mglap	1.84	2.52	2.58	NM_008597	Matrix gamma-carboxyglutamate (gla) protein
Col9a3	2.24	2.47	1.95	NM_009936	Procollagen, type IX, alpha 3
Mmp2	2.10	2.36	2.05	NM_008610	Matrix metalloproteinase 2
Smad1	1.77	1.68	1.92	NM_008539	MAD homolog 1 (Drosophila)
Bmpr2	1.86	2.03	1.86	NM_007561	Bone morphogenic protein receptor, type II (serine/threonine kinase)
Scarb1	1.94	1.93	1.76	NM_016741	Scavenger receptor class B, member 1
Dspp	1.68	1.84	1.98	NM_010080	Dentin sialophosphoprotein
Col11a1	1.41	1.12	1.01	NM_007729	Procollagen, type XI, alpha 1
Col18a1	1.64	1.78	1.53	NM_009929	Procollagen, type XVIII, alpha 1
Col1a1	1.39	1.35	1.24	NM_007742	Procollagen, type I, alpha 1
Col6a1	1.56	1.58	1.35	NM_009933	Procollagen, type VI, alpha 1
Nfkb1	1.62	1.48	1.41	NM_008689	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)
Smad3	1.30	1.24	1.28	NM_016769	MAD homolog 3 (Drosophila)
Csf3	1.42	1.53	1.63	NM_009971	Colony stimulating factor 3 (granulocyte)
Col4a2	1.41	1.43	1.32	NM_009932	Procollagen, type IV, alpha 2
Smad2	1.42	1.32	1.24	NM_010754	MAD homolog 2 (Drosophila)
Dcn	1.68	1.69	1.13	NM_007833	Decorin
Ahsg	1.38	1.27	1.37	NM_013465	Alpha-2-HS-glycoprotein
Bmp3	1.28	1.36	1.47	NM_173404	Bone morphogenetic protein 3
Itgb1	1.32	1.35	1.25	NM_010578	Integrin beta 1 (fibronectin receptor beta)
Bmp2	1.31	1.42	1.37	NM_007553	Bone morphogenetic protein 2
Tgfbr2	1.03	0.92	0.92	NM_009371	Transforming growth factor, beta receptor II
Sost	1.04	1.06	1.38	NM_024449	Sclerostin

	Relative Intensity				
Symbol	0 μM	0.1μM	1.0 μM	Genbank	Description
Ctsk	1.18	1.13	0.90	NM_007802	Cathepsin K
Vegfa	1.10	1.06	1.19	NM_009505	Vascular endothelial growth factor A
Bmp4	1.10	1.07	1.30	NM_007554	Bone morphogenetic protein 4
Col14a1	0.97	0.93	0.90	NM_181277	Procollagen, type XIV, alpha 1
Vegfc	1.01	1.09	0.95	NM_009506	Vascular endothelial growth factor C
Col4a5	0.95	0.93	0.88	NM_007736	Procollagen, type IV, alpha 5
Tuft1	1.13	1.17	1.09	NM_011656	Tuftelin 1
Col10a1	1.01	0.97	1.01	NM_009925	Procollagen, type X, alpha 1
Spp1	0.88	0.92	1.11	NM_009263	Secreted phosphoprotein 1

Supplementary Table 2

	Relative Intensity				
Symbol	0 μM	0.01 μM	0.1 μM	Genbank	Description
Vcam1	2.62	3.09	2.52	NM_011693	Vascular cell adhesion molecule 1
Twist1	2.65	3.14	2.54	NM_011658	Twist gene homolog 1 (Drosophila)
Cdh11	2.58	3.10	2.49	NM_009866	Cadherin 11
Col5a1	2.58	3.07	2.50	NM_015734	Procollagen, type V, alpha 1
Gapdh	2.60	3.11	2.50	NM_008084	Glyceraldehyde-3-phosphate dehydrogenase
Fn1	2.57	3.04	2.46	NM_010233	Fibronectin 1
Tgfb3	2.55	2.94	2.47	NM_009368	Transforming growth factor, beta 3
Bgn	2.54	3.00	2.40	NM_007542	Biglycan
Sparc	2.54	2.98	2.45	NM_009242	Secreted acidic cysteine rich glycoprotein
Serpinh1	2.33	2.48	2.23	NM_009825	Serine (or cysteine) proteinase inhibitor, clade H, member 1
Pdgfa	2.48	2.84	2.40	NM_008808	Platelet derived growth factor, alpha
Col1a2	2.39	2.65	2.15	NM_007743	Procollagen, type I, alpha 2
Vegfb	2.45	2.72	2.32	NM_011697	Vascular endothelial growth factor B
Igf1r	2.34	2.41	2.21	NM_010513	Insulin-like growth factor I receptor
Sox9	2.56	2.91	2.40	NM_011448	SRY-box containing gene 9
Tgfb2	2.12	2.07	2.04	NM_009367	Transforming growth factor, beta 2
Twist2	2.35	2.71	2.46	NM_007855	Twist homolog 2 (Drosophila)
Anxa5	2.31	2.69	2.22	NM_009673	Annexin A5
Col15a1	2.35	2.53	2.05	NM_009928	Procollagen, type XV
Col3a1	2.07	2.26	1.92	NM_009930	Procollagen, type III, alpha 1
Col4a1	2.36	2.55	2.10	NM_009931	Procollagen, type IV, alpha 1
Bmp7	2.32	2.86	2.37	NM_007557	Bone morphogenetic protein 7
Mmp2	2.10	2.17	2.06	NM_008610	Matrix metalloproteinase 2
Col9a3	2.24	2.39	2.00	NM_009936	Procollagen, type IX, alpha 3
Scarb1	1.94	1.83	1.82	NM_016741	Scavenger receptor class B, member 1
Bmpr2	1.86	1.90	1.63	NM_007561	Bone morphogenic protein receptor, type II (serine/threonine kinase)
Smad1	1.77	1.69	1.72	NM_008539	MAD homolog 1 (Drosophila)
Nfkb1	1.62	1.43	1.51	NM_008689	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)
Mglap	1.84	2.33	2.20	NM_008597	Matrix gamma-carboxyglutamate (gla) protein
Col11a1	1.41	1.41	1.16	NM_007729	Procollagen, type XI, alpha 1
Col1a1	1.39	1.49	1.36	NM_007742	Procollagen, type I, alpha 1
Gdf10	1.43	1.72	1.80	NM_145741	Growth differentiation factor 10
Smad3	1.30	1.24	1.32	NM_016769	MAD homolog 3 (Drosophila)
Dspp	1.68	1.63	1.44	NM_010080	Dentin sialophosphoprotein
Col18a1	1.64	1.55	1.45	NM_009929	Procollagen, type XVIII, alpha 1
Dcn	1.68	1.85	1.54	NM_007833	Decorin
Col6a1	1.56	1.67	1.56	NM_009933	Procollagen, type VI, alpha 1
Itgb1	1.32	1.19	1.25	NM_010578	Integrin beta 1 (fibronectin receptor beta)
Smad2	1.42	1.38	1.39	NM_010754	MAD homolog 2 (Drosophila)
Col4a2	1.41	1.32	1.14	NM_009932	Procollagen, type IV, alpha 2
Csf3	1.42	1.50	1.35	NM_009971	Colony stimulating factor 3 (granulocyte)
Bmp2	1.31	1.23	1.23	NM_007553	Bone morphogenetic protein 2
Ahsg	1.38	1.32	1.35	NM_013465	Alpha-2-HS-glycoprotein
Col4a5	0.95	0.98	0.93	NM_007736	Procollagen, type IV, alpha 5
Ctsk	1.18	1.11	1.02	NM_007802	Cathepsin K
Tfip11	0.96	0.94	0.95	NM_018783	Tuftelin interacting protein 11
Tgfbr2	1.03	0.96	0.98	NM_009371	Transforming growth factor, beta receptor II
Bmp4	1.10	1.14	1.14	NM_007554	Bone morphogenetic protein 4

	Relative Intensity				
Symbol	0 μM	0.01 μM	0.1 μM	Genbank	Description
Sost	1.04	0.89	0.89	NM_024449	Sclerostin
Col10a1	1.01	1.11	1.07	NM_009925	Procollagen, type X, alpha 1
Tuft1	1.13	1.24	1.20	NM_011656	Tuftelin 1
Bmp3	1.28	1.44	1.27	NM_173404	Bone morphogenetic protein 3
Spp1	0.88	0.96	0.92	NM_009263	Secreted phosphoprotein 1
Vegfa	1.10	0.97	1.12	NM_009505	Vascular endothelial growth factor A
Vegfc	1.01	1.07	1.09	NM_009506	Vascular endothelial growth factor C
Col2a1	0.90	0.92	0.95	NM_031163	Procollagen, type II, alpha 1
Tgfb1	0.89	0.99	1.02	NM_011577	Transforming growth factor, beta 1

Connecting text for Chapter 3 to 4

In Chapter 3, pharmacological activation of RARγ was shown to be highly teratogenic to developing limbs. Additionally, microarray analysis of approximately 100 chondrogenesis- and osteogenesis-related genes was performed to identify the potential mechanisms by which aberrant RARγ activation may exert a detrimental effect on limb morphogenesis. Matrix GLA protein (*Mgp)* was identified as one of two RARγ-responsive genes that was significantly upregulated following BMS-189961 treatment.

Through the warfarin-induced inhibition of MGP activity *in vitro*, the data presented in Chapter 4 have focused on elucidating the potential importance of MGP in mediating normal and retinoid-induced abnormal limb development.

Chapter 4

The Role of Matrix GLA Protein in Retinoid-induced Limb Dysmorphogenesis

Eugene Galdones and Barbara F. Hales

Manuscript in preparation.

Abstract

Vitamin A (Retinol) and its retinoid derivatives are indispensable for limb morphogenesis. Imbalances in retinoid activity during limb development cause reductive malformations targeting limb cartilage. Previous work examining the molecular mediators underlying retinoid-induced limb dysmorphogenesis identified Matrix GLA protein (*Mgp*; a potent inhibitor of bone development) as a gene that was significantly upregulated following retinoid excess.

The goal of this study was to determine the biological importance of MGP in mediating retinoid-induced limb insult. Mid-organogenesis stage murine limb buds (gestational day 12.5) were cultured and co-treated with all-*trans* retinoic acid and warfarin, a potent inhibitor of MGP activity. After 6 d *in vitro*, we show that the inhibition of Mgp activity results in the significant attenuation of retinoid-induced limb insult when compared to warfarin-untreated controls. However, examination of potential downstream targets of MGP results in no observable warfarin-induced changes in protein expression of SOX9 or SMAD1/5/8 phosphorylation following retinoid excess.

Although the molecular mechanisms downstream of MGP activity remain elusive, our results illustrate the potential involvement of MGP in mediating retinoid-induced limb dysmorphogenesis.

Introduction

Retinoids (vitamin A and its derivatives) are involved in the control and maintenance of many physiological processes. All-*trans* retinoic acid (RA) is the most bioactive retinoid metabolite and exerts the majority of its physiological effects through two classes of nuclear receptors, retinoic acid receptors (RAR) and retinoid X receptors (RXR; Leid et al., 1992). RAR/RXR heterodimers act as nuclear transcription factors that bind chromatin and regulate gene expression (Bastien and Rochette-Egly, 2004). Retinoid concentrations must be tightly regulated; retinoid deficiency and excess can both lead to developmental malformations (Geelen, 1979).

Chondrogenesis, otherwise defined as the formation of cartilage, can be highly affected by retinoid imbalances (Underhill and Weston, 1998). At the onset of chondrogenesis, expression of SRY-related HMG-box gene 9 (*Sox9*) is required to initiate the condensation of mesenchymal cells into chondrocytes. *Sox9* is co-expressed with type II collagen (the major component of cartilage; Bell et al., 1997) and regulates its expression. Conditional ablation of *Sox9* in the limb results in the complete absence of limb cartilage elements (Akiyama et al., 2002).

Other molecules, including bone morphogenetic proteins (BMPs), partake in the specification of mesenchymal cell fate. As their name suggests, BMPs can stimulate bone and cartilage development by inducing the step-wise maturation of cartilage into bone (reviewed in Wu et al., 2007). BMP-induced cell signaling is characterized by the phosphorylation of cytoplasmic Smad proteins (pSMAD1, 5 and 8; pSMAD1/5/8). Once phosphorylated, Smads accumulate in the nucleus, where

they regulate transcription of target genes. As such, C-terminal Smad phosphorylation can be used as a surrogate marker for BMP activity (Whitman, 1998).

The molecular mechanisms underlying retinoid-induced teratogenicity in the developing limb have not been fully characterized. The embryonic limb is a well-characterized model of chondrogenesis and exposure to excess all-*trans* RA during mid-organogenesis leads to reductive malformations affecting limb cartilage (Kochhar, 1973; Tickle, 1987). Our group is actively interested in identifying the modes by which retinoids elicit their detrimental effects on chondrogenesis using a limb bud culture model. Exposure of the developing limb to exogenous all-*trans* RA disturbs the regulation of expression of a number of genes (Qin et al., 2002; Ali-Khan and Hales, 2006a). Whole mount *in situ* hybridization identified matrix γ-carboxyglutamic acid (GLA) protein (MGP) as a gene of particular interest because it was significantly upregulated by all-*trans* RA treatment in areas of cartilage development in limbs (Ch. 3; Galdones and Hales, 2008).

MGP is a vitamin K-dependent (VKD) protein that must be γ-carboxylated at specific GLA residues in order to exhibit biological activity. Proper developmental expression of MGP is crucial; ablation of MGP in mice leads to extensive arterial calcification and abnormal skeleton formation (Luo et al., 1997). Human mutations of *MGP* have been linked to Singleton-Merten and Keutel syndromes, diseases that are characterized by abnormal arterial and ectopic cartilage calcification, respectively (Gay and Kuhn, 1976; Munroe et al., 1999). Furthermore, recent work has identified MGP as a negative regulator of chondrocyte maturation, the process

that precedes bone mineralization (Yagami et al., 1999). Additionally, it has been demonstrated that MGP binds BMPs *in vitro* and effectively inhibits their biological activities (Yao et al., 2008).

The purpose of this study was to determine the functional involvement of MGP in retinoid-induced limb dysmorphogenesis. We hypothesize that the aberrant upregulation of MGP plays an important role in transducing the retinoid-induced teratogenic effects observed in the limb. To test this hypothesis we have examined how inhibition of MGP function affects the response of limbs to exogenous all-*trans* RA exposure. To inhibit MGP activity, limbs were co-treated with warfarin, a classic vitamin K antagonist that shares a common ring structure with vitamin K. Warfarin inhibits vitamin K epoxide reductase (VKOR), preventing this enzyme from recycling oxidized vitamin K into its reduced form following the γ-carboxylation of target proteins (Furie and Furie, 1988; Berkner, 2008). Chronic administration of warfarin to rats decreased levels of bone Gla protein, causing excessive mineralization characterized by complete fusion of the proximal tibial growth plate and cessation of longitudinal growth (Price et al., 1982).

Our results demonstrate that warfarin significantly attenuates retinoid-induced limb dysmorphogenesis, implicating γ-carboxylated MGP in mediating retinoid teratogenicity. To investigate the downstream targets of MGP, we assessed the expression profiles of two chondrogenesis-related proteins, SOX9 and pSMAD1/5/8. All-*trans* RA did not affect SOX9 expression; in contrast, pSMAD1/5/8 activation was upregulated. Interestingly, none of the effects of all-*trans* RA were altered by

warfarin co-treatment. Further studies are required to elucidate the mechanisms by which MGP plays a role in retinoid-induced limb dysmorphology.

Materials and Methods

In vitro limb bud culture

Pregnant gestation day (GD) 12.5 CD-1 mice were euthanized, embryos were explanted and forelimbs were excised just lateral to the somites and cultured *in vitro* in a chemically-defined culture medium, as previously described (Ali-Khan and Hales, 2003). All-*trans* retinoic acid (RA; Sigma, St. Louis, MI) and 3-(α-acetonylbenzyl)-4-hydroxycoumarin sodium salt (warfarin; Sigma) were dissolved in 100% ethanol and distilled water, respectively. Limbs were exposed to low or high (0.1 or 1.0 μM) concentrations of all-*trans* RA and/or a fixed concentration (3 μg/ml) of warfarin at the onset of culture. A dose-finding study (0, 3, 15 and 30 μg/ml) was done to determine the optimal concentrations of warfarin for our system. Since the lowest and highest concentrations (3 and 30 μg/ml) of warfarin tested resulted in equivalent effects on limb morphology, the lowest concentration was used to minimize any non-specific effects that may be associated with warfarin treatment (Supplemental Fig. S4.1). All animal studies complied with the guidelines established by the Canadian Council on Animal Care.

Limb morphology

Limbs were cultured for 6 days, with one change of the culture medium that was not supplemented with RA or warfarin on day 3, as previously described (Ch. 3;

Galdones and Hales, 2008). On day 6 limbs were submerged in Bouin's fixative for 3 h, then stained with 0.1% toluidine blue overnight (Fisher Scientific, Montreal, Canada), examined under a dissecting microscope (Wild Heerbrugg 99067; Wild Leitz, Ottawa, Canada), and photographed with a digital camera (JVC, Tokyo, Japan). The extent of proper limb development was quantified using a limb morphogenetic scoring system (Neubert and Barrach, 1977). Morphometric analysis of limb surface area was done using computer-assisted image analysis (MCID-8, Image Research Inc., St. Catharine's, Canada). Three separate replicates were completed and examined for morphological and morphometric changes.

Western blot analysis

Limbs (8-12 per group) treated with all-*trans* RA and/or warfarin were cultured for 3, 6 or 12 h then flash frozen in liquid nitrogen and stored at -80°C. To prepare whole limb protein lysates, limbs were thawed on ice and briefly sonicated in RIPA buffer (25 mM Tris [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail (Active Motif, Carlsbad, CA). The lysates were cleared by centrifugation and samples quantified using a standard spectrophotometric assay (Bio-Rad Laboratories, Mississauga, Canada). Protein (10 μg/sample) was denatured by heating to 95°C for 5 min, electrophoresed on 12.5% denaturing acrylamide gels (Matsudaira and Burgess, 1978) and then transferred onto PVDF membranes (GE Healthcare Inc., Buckinghamshire, United Kingdom).

PVDF membranes were first blocked in 5% non-fat milk in TBS-T (137 mM NaC1, 20 mM Tris [pH 7.4], 0.1% Tween 20) for 1 h at room temperature and then incubated with primary antibody diluted in 3% non-fat milk in TBS-T overnight at 4°C. The primary antibodies and their working concentrations were as follows: SOX9 (sc-17340, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-SMAD1/5/8 (#9511, 1:1000; Cell Signaling, Danvers, MA) and ACTB (sc-1616, 1:5000, Santa Cruz Biotechnology). Donkey anti-goat (sc-2020, 1:10000, Santa Cruz Biotechnology) or donkey anti-rabbit (NA9340, 1:10000, GE Healthcare Inc.) secondary antibodies conjugated to horseradish peroxidase were used.

Western blots were visualized with the Enhanced Chemiluminescence Plus Kit and Amersham Hyperfilm™ ECL (GE Healthcare Inc.). Quantification of the Western blot data was done using a ChemiImager 4000 and AlphaEase 5.5b software (Alpha Innotech, San Leadro, CA). Three replicates were done for each experiment; values represent the mean ± SEM.

Statistical Analysis

The data from all morphology/morphometry and western blot studies were analyzed using SigmaStat (Systat Software Inc., San Jose, CA). Two-way analysis of variance (ANOVA) was done to compare the untreated and warfarin treated groups, followed by a *post-hoc* Holm-Sidak multiple comparison test to compare all groups. The *a priori* level of significance was $P < .05$.

Results

Inhibition of γ-carboxylation attenuates retinoid-induced limb dysmorphogenesis

Murine embryonic limb buds were cultured for 6 days *in vitro* in the presence or absence of all-*trans* RA. Limbs were stained with toluidene blue to visualize the effects of drug exposure on the cartilage anlagen. Under control conditions, the limb cartilage elements (long bones, carpalia and digits) were clearly recognizable (Fig. 4.1A). Limbs treated with warfarin alone (3, 15 or 30 μg/ml) also developed normally (Supplementary Fig. S4.1, Fig. 4.1A), with no observable effects on morphology.

Limbs exposed to low or high all-*trans* RA exhibited concentration-dependent reductions in limb morphology. These reductions were characterized by limb truncation due to the presence of less differentiated or unrecognizable cartilage elements (Fig. 4.1A). Strikingly, the limb morphology of tissues exposed to low all-*trans* RA and warfarin was vastly improved, in many cases, resembling the morphology of control limbs (Fig. 4.1A). Warfarin co-treatment also significantly improved the morphology of limbs exposed to high concentrations of all-*trans* RA. Long bones and digits were more recognizable when compared to their RA dose-matched counterparts in the absence of warfarin, but complete rescue of retinoid-induced dysmorphogenesis was not observed.

The amount and extent of limb differentiation were quantified using a limb scoring system (Fig. 4.1B; Neubert and Barrach, 1977); limb surface area was measured morphometrically as a surrogate marker for overall limb growth (Fig. 4.1C; Galdones et al., 2006). Limb scores and surface areas of warfarin-treated limbs in the absence of all-*trans* RA were indistinguishable from untreated controls (Fig. 4.1B

and C). There was a significant reversal of the RA-mediated effects on limb differentiation and growth in when limbs were co-cultured with warfarin when compared to their dose-matched counterparts in the absence of warfarin.

Putative downstream targets of all-trans RA and warfarin action on limbs

Next we assessed the effects of all-*trans* RA, in the absence or presence of warfarin, on the expression of proteins that play fundamental roles in chondrogenesis, with the goal of identifying the mechanism(s) by which warfarin attenuated retinoid-induced insult in the developing limb.

SOX9, a key regulator of mesenchymal condensation, was the first protein assessed. At all time points, SOX9 protein expression remained unchanged following all-*trans* RA and/or warfarin treatment (Fig. 4.2A and B).

Next, we examined the activation of pSMAD1/5/8 signaling as a surrogate marker for BMP activity. Control tissues contained very little pSMAD1/5/8; there was a significant increase in pSMAD1/5/8 expression in low (2.4 fold) and high (4.5-fold) all-*trans* RA-exposed limbs after 3 h *in vitro* (Fig. 4.2A). Warfarin-treated dose-matched counterparts exhibited comparable increases in pSMAD1/5/8 expression (3- and 4.9-fold). After 6 h *in vitro*, the significant phosphorylation of pSMAD1/5/8 persisted only in limbs exposed to high all-*trans* RA-treatment, as evidenced by 3.5-fold (warfarin untreated) and 3.4-fold (warfarin-treated) increases (Fig. 4.2C). At 12 h, SMAD1/5/8 phosphorylation status returned to baseline.

Discussion

Although retinoid teratogenicity has been widely studied, both *in vitro* and *in vivo*, the downstream mechanisms that mediate retinoid-induced insult have yet to be fully resolved. The attenuation of retinoid teratogenicity in the limb has been demonstrated following genetic and molecular interventions targeting retinoid receptor function (Sucov et al., 1995; Iulianella et al., 1999; Ali-Khan and Hales, 2006b); however, we are the first to demonstrate the active attenuation of all-*trans* RA-mediated dysmorphogensis in the developing limb by warfarin. Warfarin alone (3 – 30 μg/ml) did not affect limb morphology (Supplementary Fig. S4.1). Nevertheless, *in vivo* evidence and human case studies have shown that warfarin itself is teratogenic during early pregnancy and can lead to a variety of nasal and skeletal (including the limb) defects as well as mental deficits (Fourie and Hay, 1975). Warfarin dysmorphology is observed after exposures during early gestation, before coagulation factors are present in the embryo, suggesting that inhibition of other vitamin K dependent GLA proteins are responsible for these effects.

Vitamin K-dependent γ-carboxylation of distinct residues on MGP is required for its anti-chondrogenic and -osteogenic functions (Price and Williamson, 1985). *In vivo* and *in vitro* warfarin treatment has been used to elucidate the roles of MGP in controlling not only cartilage mineralization but vessel and tooth mineralization as well (Danziger, 2008). However, because warfarin directly affects γ-carboxylase enzyme activity (rather than direct effect on MGP itself), one cannot exclude the participation of other GLA proteins that may be expressed in the limb.

MGP, first isolated from bone, is a potent inhibitor of mineralization of various tissues, including bone, tooth and blood vessels (Luo et al., 1995a; Luo et al., 1997). Although MGP is retinoid-responsive (Cancela et al., 1990; Cancela and Price, 1992), its participation in mediating retinoid-regulated processes during normal and abnormal development has not been well explored. Using a chondrogenesis-based microarray approach, we identified MGP as a potential mediator of the reductive effects underlying retinoid insult in the developing limb cartilage. Using *In situ* hybridization, we demonstrated an all-*trans* RA-induced upregulation of *Mgp* expression in areas of developing cartilage as early as 3 h post-treatment (Ch.3; Galdones and Hales, 2008). Interestingly, MGP-deficient mice exhibit inappropriate calcification of various cartilages, including the growth plate, which leads to short stature, osteopenia and fractures (Luo et al., 1997). Elucidation of the extent to which MGP-ablation affects retinoid-insulted limb dysmorphogenesis would provide further insight regarding the involvement of MGP in mediating the teratogenicity of retinoids.

Sox9 and retinoid activity

Given the marked improvement in cartilage formation following warfarin co-treatment, we examined the molecular basis underlying this striking morphological observation. No significant changes were detected in protein concentrations of Sox9, a key molecule in early chondrogenesis, following all-*trans* RA or warfarin treatment (Fig. 4.2A and B). In a previous study, decreases in *Sox9* transcript were observed following all-*trans* retinol exposure in the developing limb (Ch. 2; Galdones et al.,

2006). As well, SOX9 activity (and cartilage production) was significantly increased in micromass limb cultures following exposure RAR antagonist treatment (Weston et al., 2002), thus signifying a regulatory link between SOX9 and retinoid activity.

The observation that all-*trans* RA did not affect SOX9 protein concentrations in these experiments may be due to temporal changes in the regulation of this protein during limb development. Chondrogenesis in the murine embryonic forelimb begins at approximately E10.5. Sox9 is responsible for the induction of mesenchymal differentiation into chondrocytes (Healy et al., 1999). By E12.5, cartilage production has progressed considerably and the majority of the Sox9 effects on cartilage initiation may be complete. Hence, the examination of proteins directly downstream of SOX9 (i.e., type II collagen or aggrecan) and/or proteins expressed at later stages of bone development, such as type X collagen during chondrocyte hypertrophy (a process that is actively occurring at E12.5) may provide more time-dependent information regarding the effects of retinoids on SOX9 function.

Effects of retinoids on BMP function during limb development

BMPs play vital roles during limb development because their manipulation can compromise many aspects of morphology. Specifically, studies employing dominant-negative and constitutively active BMP receptors led to the inhibition and induction of cartilage formation, respectively (Zimmerman et al., 1996). Moreover, modifications in Noggin activity, a potent endogenous inhibitor of BMP activity, also disrupt the morphogenesis of various limb cartilage elements (Brunet et al., 1998;

Capdevila and Johnson, 1998). Retinoids have been shown to regulate BMP signaling and the activity of both BMP and RA has been linked to the control of the size and shape of the developing limb anlagen (Weston et al., 2000). Our results examining BMP activity (through SMAD1/5/8 phosphorylation) clearly validate the retinoid-induced regulation of BMP activity as evidenced by the concentration-dependent upregulation of pSMAD1/5/8 activity following excess all-*trans* RA exposure (Fig. 4.2A and C).

Despite the fact that there has been recent *in vitro* data supporting the physical interactions between BMP-2 and/or BMP-4 and γ-carboxylated MGP in endothelial cells (Yao et al., 2008), inhibition of MGP activity did not alter Smad phosphorylation. This observation suggests that the MGP/BMP interaction may not occur in limb cartilage or (2) is not detectable due to the cell heterogeneity in the cultured limbs.

Summary

This study provides evidence that γ-carboxylated MGP plays an important role in mediating retinoid teratology. Notably, striking improvements in chondrogenesis and overall morphology were observed in cultured limbs exposed to excess all-*trans* RA and warfarin, inhibiting vitamin K dependent γ-carboxylation of MGP. Although warfarin co-treatment attenuated retinoid-induced insult in the limb, offsetting retinoid toxicity during pregnancy with warfarin would be unwarranted, given the warfarin-induced embryopathies that may result. Notwithstanding, our observations emphasize the necessity to further unravel the involvement of γ-carboxylation status

in chondrogenesis and retinoid teratology, such that safer methods for birth defect prevention may be developed.

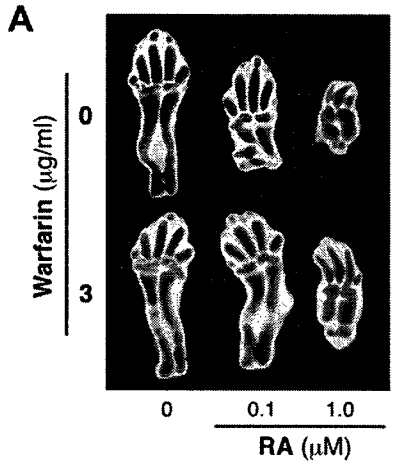
Acknowledgements

We thank Dr. Daniel Bernard for his generosity and insight. This work was supported by the Canadian Institutes for Health Research.

Figures and Legends

Figure 4.1. The effect of warfarin on all-*trans* RA co-treated limbs after 6 days *in vitro*. Limbs were collected from embryonic day 12.5 CD-1 mouse embryos and cultured for 6 days in the absence or presence of all-*trans* RA (0.1 and 1.0 μM) and/or warfarin (3 μg/ml). (**A**) Limbs were stained with 0.1% toluidene blue (w/v 70% ethanol). A concentration-dependent increase in limb dysmorphogenesis was observed in limbs treated with all-*trans* RA alone. Warfarin co-treatment resulted in a significant attenuation in retinoid-mediated limb dysmorphology. (**B** and **C**) The limb morphogenetic scores and morphometric limb surface area measurements quantified the quality and extent of limb differentiation and morphogenesis, respectively. Three independent limb cultures (n = 3) were done; each roller bottle contained 8-12 limbs. Statistical analysis was done using a two-way ANOVA followed by a Holm-Sidak *post-hoc* multiple comparison test; $*P < .01$, significant increase when compared to warfarin-untreated dose-matched limbs.

Figure 4.1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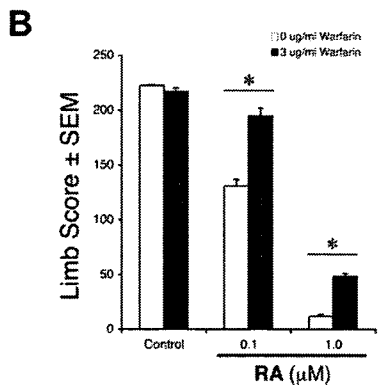
0
3
0
0.1
1.0
RA (μM)


B
0 ug/ml Warfarin
3 ug/ml Warfarin
Limb Score ± SEM
250
200
150
100
50
0
*
*
Control
0.1
1.0
RA (μM)

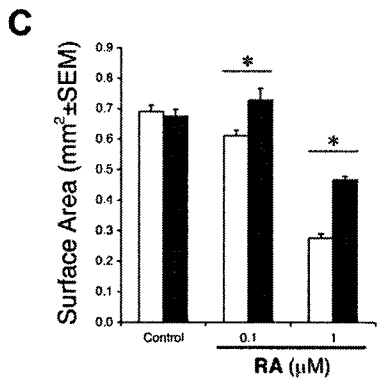
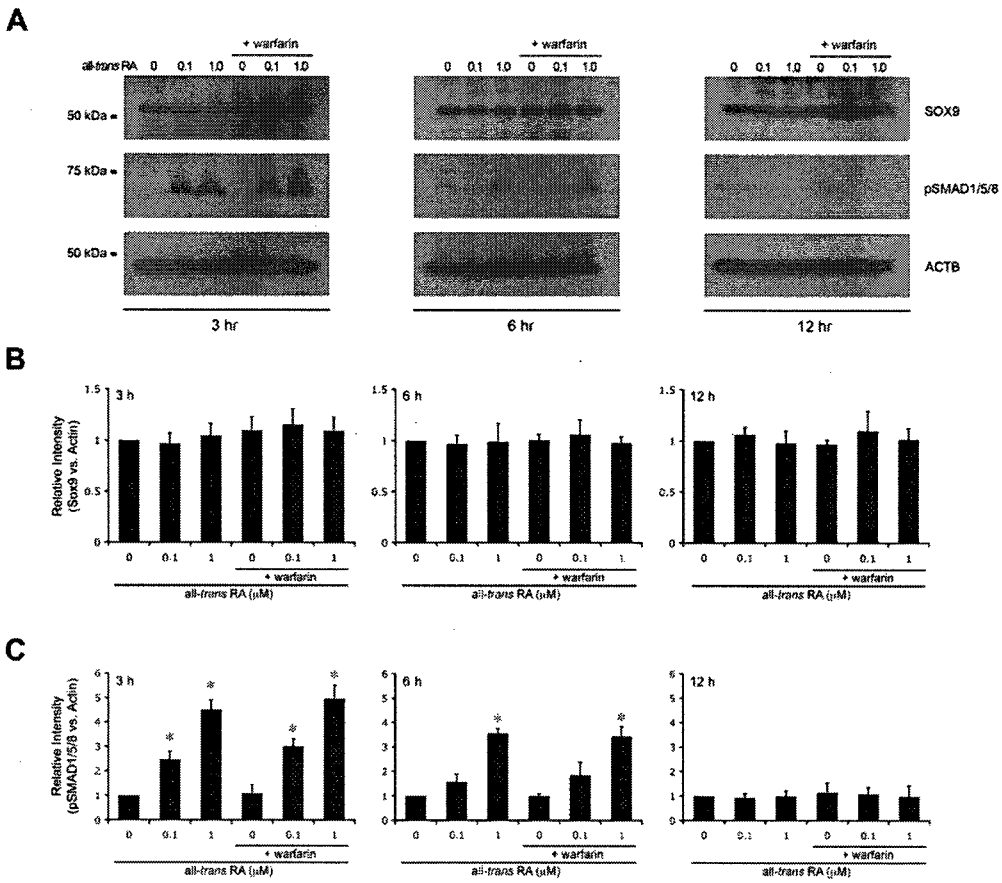

C
Surface Area (mm²±SEM)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
Control
0.1
1
RA (μM)

Figure 4.2. Western blot analysis of chondrogenesis-related proteins in E12.5 limbs following all-*trans* RA and warfarin co-treatment. Limbs were cultured for 3, 6 or 12 h, then whole limb lysates were collected and run on 12.5% SDS-PAGE. (**A**) Representative western blots illustrating the effects of all-*trans* RA (with or without warfarin co-treatment) on the expression of SOX9 and phospho-SMAD1/5/8. Actin was used as a loading control. (**B** and **C**) Densitometric quantification of the western blots for SOX9 and pSMAD1/5/8, respectively, after 3, 6 or 12 h *in vitro*. The relative band intensities were measured against that of actin expression. The bars represent the relative fold-change in band intensity (± SEM) when normalized to 1 (i.e., the band density observed in warfarin-untreated/all-*trans* RA-untreated control limbs). Statistical analysis (two-way ANOVA and Holm-Sidak MCT) was done on relative band intensities (n = 3 separate western blot experiments) that were normalized to 1; $*P < .05$, significant increase in protein expression when compared to their all-*trans* RA dose-matched warfarin-untreated counterparts.

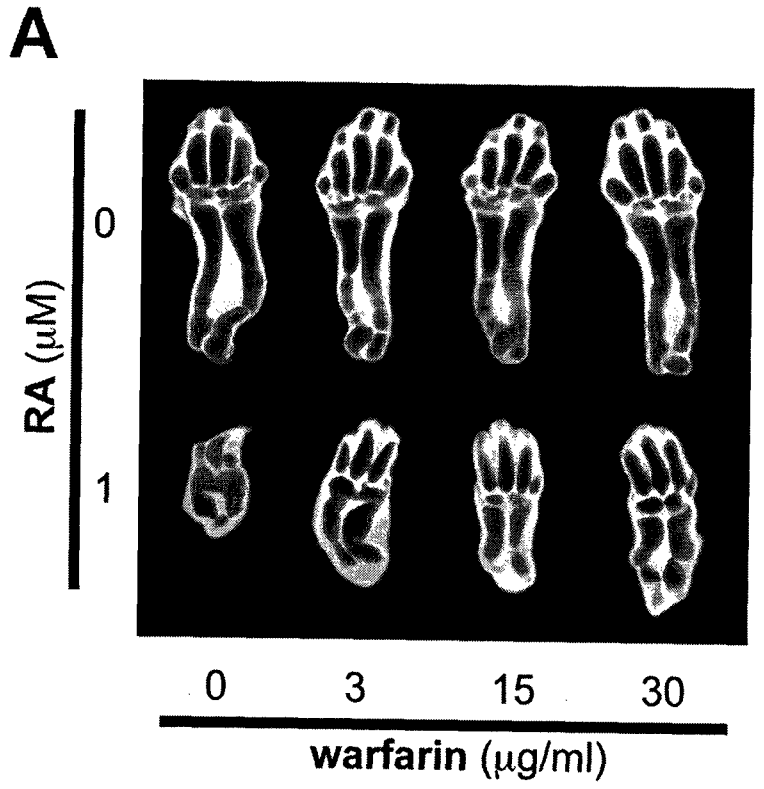
Figure 4.2


A
+ warfarin
all-trans RA 0 0.1 1.0 0 0.1 1.0
50 kDa
75 kDa
50 kDa
SOX9
pSMAD1/5/8
ACTB
3 hr
6 hr
12 hr
B
3 h
6 h
12 h
Relative Intensity (Sox9 vs. Actin)
all-trans RA (μM)
+ warfarin
C
Relative Intensity (pSMAD1/5/8 vs. Actin)
all-trans RA (μM)
+ warfarin

Supplementary Figures

Supplementary Fig. S4.1. The effect of increasing concentrations of warfarin on limbs exposed to 1.0 μM (high) all-*trans* RA. Limbs were cultured for 6 days *in vitro* in the absence or presence of 1.0 μM all-*trans* RA in conjunction with escalating concentrations of warfarin (3, 15 and 30 μg/ml). (**A**) Limb stained with 0.1% toluidene blue illustrate that increasing concentrations of warfarin co-treatment is not detrimental to control limbs, but can significantly improve the morphology of high all-*trans* RA treated limbs. (**B**) Quantification of the limb morphology results by limb morphogenetic scoring. Statistical analysis was done using a two-way ANOVA followed by a Holm-Sidak *post-hoc* multiple comparison test; $^{*}P < .05$, significant warfarin-dependent increase when compared to warfarin-untreated/all-*trans* RA-treated tissues. All retinoid-treated limbs exhibited a significant decrease in limb score and surface area ($P < 0.05$) when compared to warfarin dose-matched all-*trans* RA untreated controls; for clarity, these observations are not included in the histograms.

Supplementary Fig. S4.1



Connecting text for Chapter 4 to 5

Mgp and *Gdf10* were identified as two RARγ-responsive genes that were significantly upregulated following the pharmacological induction of RARγ activity. Whereas Chapter 4 assessed the biological importance of *Mgp*, Chapter 5 explored the elusive role of *Gdf10* in the embryonic limb.

As observed in Chapter 3, *Gdf10* expression was significantly upregulated in the interdigital webbing following excess all-*trans* RA and BM961 insult. To partially mimick the retinoid-induced upregulation of *Gdf10* in the limb, human recombinant GDF10-soaked beads were implanted into developing limb buds then cultured *in vitro*. This ectopic overexpression of GDF10 was performed to determine the roles of GDF10 excess on limb morphology, in hopes of potentially identifying its development biological role during retinoid-induced limb insult.

Chapter 5

The Ectopic Overexpression of Growth Differentiation Factor-10 Disrupts Limb Development *In Vitro*

Eugene Galdones and Barbara F. Hales

Manuscript in preparation.

Abstract

Bone morphogenetic proteins (BMP) are important for limb development, yet the roles of some BMP family members during limb morphogenesis remain elusive. Retinoids (derivatives of vitamin A) are potent modulators of BMP activity and must be properly regulated as the limb develops. Excess retinoid exposure during embryogenesis is detrimental to the limb. Moreover, much work has implicated the misregulation of BMP activity in mediating various aspects of retinoid-induced limb dysmorphogenesis.

We have recently identified growth differentiation factor-10 (Gdf10/BMP3b) as a gene that is significantly upregulated following retinoid insult, yet its involvement in limb morphogenesis has not been characterized. The goal of this study was to determine the biological role of GDF10 in the limb. To do so, recombinant human GDF10 (rhGDF10)-soaked beads were implanted into E12.5 murine limb buds and cultured for 6 d *in vitro*. We provide evidence that ectopic overexpression of rhGDF10 in the distal limb regions (especially in the interdigital web) can lead to the significant malformation of proximal cartilage elements. This work highlights the importance of properly regulated concentrations of GDF10 during limb development.

Introduction

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily of growth factors that play crucial roles during embryo development (Hogan, 1996). As their name suggests, BMPs were initially shown to induce bone formation, yet exhibit a multiplicity of functions that span beyond osteogenesis (reviewed in Wozney et al., 1988; Wu et al., 2007). BMPs are produced as precursor proteins that require cleavage into a mature domain and homo- or heterodimerize (Reddi, 1997). To date, there are more than 20 BMP-related molecules that have been identified (Miyazono et al., 2005). Classically, BMPs elicit their biological activity by binding to type I and type II serine-threonine kinase heterotetramers that subsequently phosphorylate BMP-specific Smad signaling pathways, thus leading to gene regulation (Derynck and Zhang, 2003).

All-*trans* retinoic acid (RA) is a bioactive retinoid (i.e., vitamin A derivative) that plays an important role in the developing embryo. Through nuclear retinoic acid and rexinoid receptor heterodimers, all-*trans* RA modulates the transcriptional activity of target genes (Leid et al., 1992). Retinoid imbalances during embryonic life have been associated with disturbances in the regulation of gene expression, ultimately leading to abnormal development. Additionally, much work has studied the relationship between retinoid activity and the misregulation of several BMP family members (Weston et al., 2000; Hoffman et al., 2006).

The development of the embryonic limb is a widely accepted experimental model of morphogenesis (Cohn and Tickle, 1996). The limb is a heterogeneous tissue composed of many cell types destined for various cell fates (i.e., bone, muscle

or skin). During early-to-mid organogenesis, the developing embryonic limb is particularly susceptible to retinoid-induced insult. Although the effects characterizing retinoid-mediated limb insult have been attributed to marked disruptions in key developmental programs such as chondrogenesis, patterning and programmed cell death, many of the players controlling these processes have not been fully elucidated (Kochhar, 1973; Jiang et al., 1995; Ali-Khan and Hales, 2003).

Studies in our lab have focused on elucidating the mechanisms underlying retinoid-induced limb dysmorphogenesis. Genome-wide and chondrogenesis-focused microarray analyses were done previously in our lab to identify genes that mediate retinoid-induced pathogenesis (Ch. 3; Ali-Khan and Hales, 2006a; Galdones and Hales, 2008). In particular, we have identified growth differentiation factor-10 (GDF10; also known as BMP3b) to be significantly upregulated in the developing limb following excess all-*trans* RA exposure, suggesting its involvement during retinoid-induced insult.

GDF10 (and its most highly related family member, BMP3/BMP3a) represents a structurally different subgroup of BMPs. GDF10 was first isolated from the adult rat femur and subsequently demonstrated to be expressed in various other adult tissues such as the cerebellum, lung, aorta and gonads (Cunningham et al., 1995; Takao et al., 1996). GDF10 is present during mouse embryogenesis as of embryonic day (E) 8.5 and expressed in developing skeletal structures at E12.5 and E14.5. Strikingly, although GDF10 is highly localized to specific cell populations (such as the developing cartilage) during embryogenesis, Gdf10-null mice appear indistinguishable from their wild-type counterparts (Zhao et al., 1999). This

observation has been attributed to the functional redundancy of BMP3, that can compensate in the absence of GDF10. Mammalian GDF10 function *in vivo* has not been well-characterized but *in vitro* assessment of GDF10 in cultured osteoblasts demonstrated its inhibitory effect on bone development (Hino et al., 1999). Furthermore, work in *Xenopus* embryos has implicated GDF10 function as a dorsalizing morphogen, affecting head formation (Hino et al., 2003).

Following retinoid excess, a significant downregulation of *Gdf10* transcript was observed in rat primary calvarial osteoblasts (Hino et al., 1999). In contrast, we have demonstrated a significant retinoid-induced upregulation in *Gdf10* expression in murine limb buds. Of particular interest, *Gdf10* overexpression was localized to limb areas destined for programmed cell death (i.e., interdigital and necrotic zones; Ch. 3; Galdones and Hales, 2008); interestingly, *Gdf10* expression was weakly upregulated in developing bone, where *Gdf10* was previously documented to be abundant (Takao et al., 1996).

Ultimately, the biological function of GDF10 in the developing limb has not been characterized. Given that retinoid excess can misregulate *Gdf10* expression, we hypothesize that aberrant GDF10 activity may mediate the detrimental effects of retinoids on limb morphology. To address our hypothesis, we implanted mid-organogenesis-stage murine limbs with recombinant human GDF10 (rhGDF10)-soaked beads and cultured them *in vitro* to assess how limb morphology was affected by ectopic overexpression of GDF10.

Materials and Methods

Affi-Gel® bead preparation

Affi-Gel® Blue beads (100-200 mesh, Bio-Rad Laboratories, Mississauga, Canada) were rinsed and centrifuged three times in 1X phosphate-buffered saline (PBS) then bead suspensions were aliquoted in distinct 20 μl volumes onto a Petri dish, kept uncovered and dried under a fume hood. Once dried, 25 μl of recombinant human G GDF10 (rhGDF10; 50 μg/ml in 1X PBS/0.1% bovine serum albumin [BSA]; R&D Systems, Minneapolis, MN) or vehicle alone (1X PBS/0.1% BSA) was applied to the beads and allowed to soak for 1 h at 4°C.

In vitro limb bud culture

Pregnant gestation day (GD) 12.5 CD-1 mice were euthanized, embryos were explanted and the forelimbs were excised just lateral to the somites and placed in Hank's buffered salt solution (Invitrogen, Burlington, Canada). rhGDF10 or vehicle-soaked beads were surgically implanted in one of two locations in the developing limb: (1) the distal interdigital web between digits II and III, or (2) directly within digit III. Following bead implantation, limbs were cultured *in vitro* in a chemically-defined culture medium (BGJb, Invitrogen), as previously described (Ch. 3; Galdones and Hales, 2008). All animal studies complied with the guidelines established by the Canadian Council on Animal Care.

Limb morphology

Limbs were cultured for 6 d, with one change of the culture medium on the morning

of day 3. Briefly, limbs were fixed with Bouin's for 3 hr then stained with 0.1% toluidine blue in 70% ethanol overnight (Fisher Scientific, Montreal, Canada), dehydrated with ascending ethanol concentrations and cleared with cedarwood oil (Fisher Scientific). Limbs were examined under a dissecting microscope (Wild Heerbrugg 99067; Wild Leitz, Ottawa, Canada) and photographs acquired with a JVC digital camera (JVC GCQX3HD, Tokyo, Japan). The extent of limb differentiation was quantified using a limb morphogenetic scoring system (Neubert and Barrach, 1977). Limb scores from zeugopod (long bones) and autopod (carpalia and digits) cartilaginous elements were quantified to differentiate the proximal versus distal effects of bead implantation. Morphometric analysis of limb surface area was also done to assess proper limb growth using computer-assisted image analysis (MCID-8, Image Research Inc., St. Catharine's, Canada).

Statistical analysis

All data were analyzed using SigmaStat (Systat Software Inc., San Jose, CA). Two-way analysis of variance (ANOVA) followed by *post-hoc* Holm-Sidak multiple comparison tests were done to compare all groups. The *a priori* level of significance was $P < .05$.

Results

Ectopic overexpression of rhGDF10

After 6 days *in vitro*, untreated control limbs developed normal and fully recognizable digits, carpalia and long bone elements (Fig. 5.1A). Limbs implanted in either the web or the ray with PBS (vehicle)-soaked beads also developed normally. Following the implantation of rhGDF10-soaked beads into the ray, the majority of limbs (14/20) exhibited normal limb morphogenesis, although proximal long bone development was decreased in several cases (6/20). Strikingly, rhGDF10-bead implantation into the web resulted in the frequent severe decreases in the differentiation of proximal long bones (14/18; Fig. 5.1A).

The quality of limb morphogenesis of proximal and distal limb structures was also quantified. Proximal and distal limb scores from PBS-implanted limbs did not differ from the controls. However, limbs implanted with rhGDF10 in the ray had modestly lower scores in proximal elements while rhGDF10 in the web affected distal limb elements in a minor fashion. In contrast, limbs implanted with rhGDF10 in the web exhibited significant decreases in limb score (Fig. 5.1B).

Limb surface area measurements were also used to quantify overall limb growth. Following implantation of Gdf10 in the ray, limb surface areas remained unchanged relative to un-implanted or PBS-implanted limbs. However, while PBS-soaked beads implanted in the web did not affect limb surface area, surface area was significantly reduced by rhGDF10-bead web implantation (Fig. 5.1C).

Discussion

BMPs comprise a particular subgroup of the TGF-β superfamily of ligands; their biological activities must be tightly regulated because they play integral roles in a multitude of developmental processes. Specifically, BMPs play well-established roles in the control of cartilage and bone formation (Wu et al., 2007).

Imbalances in retinoid activity during development affect chondrogenesis throughout the embryo. Moreover, BMPs are not only regulated by retinoids but also implicated in transduction of retinoid teratogenicity. The abnormal and retinoid-induced aberrant functions of several TGF-β superfamily ligands (i.e., BMP-2, -4 and -7 and GDF5) in the developing limb have been well characterized (reviewed in Wan and Cao, 2005); however, the functions of several other TGF-β family members remain elusive.

Previous work from our laboratory identified *Gdf10* as a retinoid-responsive gene that was significantly upregulated in the limb following retinoid excess (Ch. 3; Galdones and Hales, 2008). *Gdf10* is a structurally distinct member of the BMP family and its precise biological activity is not clear. In this study, we implanted rhGDF10-soaked beads into the limb to examine how ectopic rhGDF10 overexpression affected limb morphogenesis. We showed that ectopic overexpression of rhGDF10 at E12.5 resulted in the truncation of the proximal long bones after 6 d *in vitro*. Although long bone defects were observed following bead implantation into the ray, their frequency and severity were substantially increased when rhGDF10-beads were implanted into the web (Fig. 5.1A). Our results are the

first to illustrate a potential role for GDF10 during retinoid-induced limb dysmorphogenesis.

The location of bead implantation

Our results have shown a location-dependent effect of rhGDF10-bead implantation on limb development. Whereas the implantation of beads within the ray and web both led to significant changes in limb morphology, the implantation into the webbing led to more frequent and severe long bone malformations.

This observation suggests that GDF10 may be more bioactive in specific areas on the limb. Since GDF10 elicits the majority of its biological activity by binding TGF-β/activin receptors (Daluiski et al., 2001), the spatial expression and localization of these receptors in the limb tissue may explain the differential biological responses observed. This phenomenon has been shown to occur with specific BMP receptor (BMPR) isoforms, where their expression in the digital rays and web differs greatly. Briefly, it was shown that the expression of BMPR-1B in the ray allowed the induction of osteogenesis, while the expression of BMPR-1A in the web permitted apoptosis (Kawakami et al., 1996). Interestingly, the expression of specific activin receptors (ActRIIa and ActRIIb) was spatially-regulated in the chick limb (Merino et al., 1999b). More work addressing the interactions between GDF10 and its receptor(s) will be required to further understand the mechanisms underlying the GDF10-induced limb dysmorphogenesis.

Non-local effects of rhGDF10

Previously documented bead implantation experiments in the limb (and other tissues) using other BMPs or TGF-β family members resulted in local effects on

cartilage morphology or apoptosis. In particular, ectopic application of BMP4 resulted in the local upregulation of cartilage formation (Hoffman et al., 2006) or local aberrant programmed cell death in the limb (Ganan et al., 1996). Conversely, following rhGDF10-bead implantation into the distal limb, no local effects on morphogenesis were observed. The morphogenesis of digit III was not affected by rhGDF10. Although apoptosis was not directly assessed, rhGdf0 overexpression did not appear to affect the soft tissue at the implantation site (between digits II and III) either.

Importantly, the distal ectopic misexpression of GDF10 in our system resulted in marked effects on proximal structures, indicating that GDF10 may elicit its effects on limb development in an as-of-yet uncharacterized manner. GDF10 has been shown not to exhibit any osteogenic activity (Takao et al., 1996), perhaps explaining the lack of any observable local effects.

However, recent work has demonstrated the importance of GDF10 function on embryonic head patterning (Hino et al., 2003). Examining how GDF10 regulates limb patterning will be instrumental in understanding the non-local effects of GDF10 on proximal limb patterning.

Summary

Taken together, the limb dysmorphogenesis induced by ectopic overexpression of rhGDF10 illustrates the potential involvement of GDF10 during limb development. Examining the molecular mechanisms underlying the non-local

long bone dysmorphogenesis will provide further clues regarding GDF10 function during normal and abnormal limb development.

Acknowledgements

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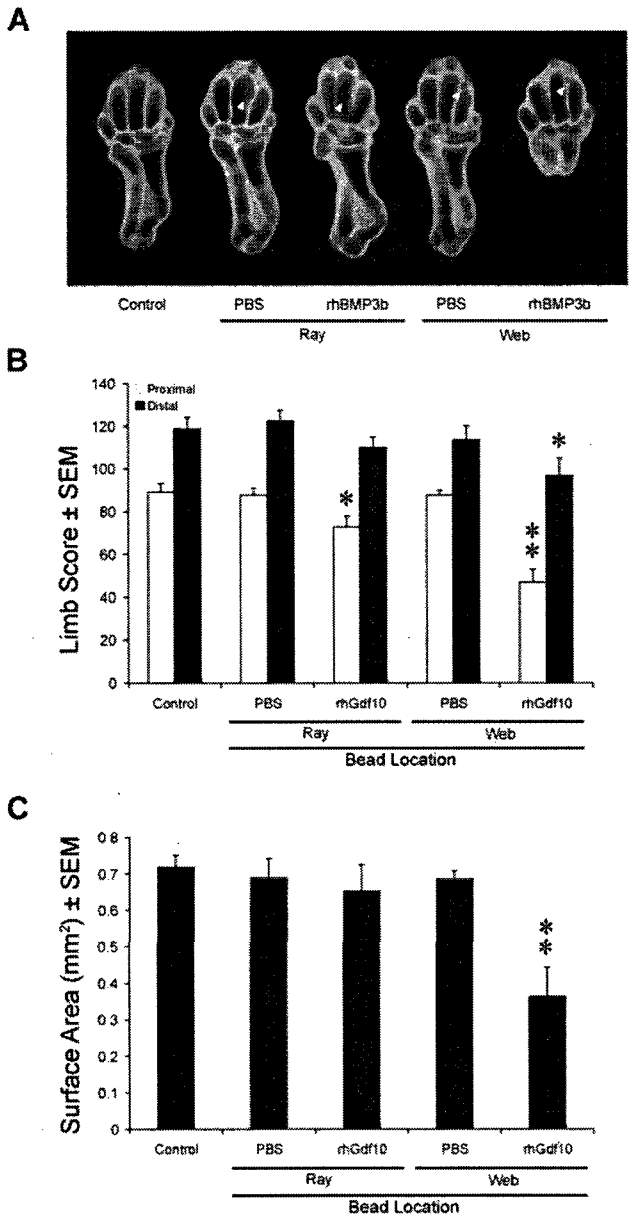
Figures and Legends

Fig. 5.1. Ectopic overexpression of rhGDF10 in the proximal interdigital web leads to the dysmorphogenesis of proximal long bone structures. E12.5 murine limb buds were implanted with either PBS- or rhGDF10-soaked beads and cultured for 6 d *in vitro*. (**A**) Limbs stained with 0.1% toluidene blue illustrate the dysmorphogenesis of the long bones induced by rhGDF10-bead implantation within the web, but not the ray. Arrowheads indicate bead implantation sites. (**B**) Proximal (handplate and carpalia) and distal (radius and ulna) limb morphogenetic scores quantifying the quality of limb morphogenesis. (**C**) Whole limb surface area measurements quantifying the extent of overall limb development. Three independent culture experiments were done (n = 3); each bottle contained 6-10 limbs. Statistical analysis was done using two-way ANOVA and the Holm-Sidak multiple comparison test; *Significant decrease, $P < 0.05$, **significant decrease, $P < 0.01$.

Figure 5.1


A
Control
PBS
rhBMP3b
PBS
rhBMP3b
Ray
Web
B
Proximal
Distal
Limb Score ± SEM
140
120
100
80
60
40
20
0
Control
PBS
rhGdf10
PBS
rhGdf10
Ray
Web
Bead Location
C
Surface Area (mm²) ± SEM
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
PBS
rhGdf10
PBS
rhGdf10
Ray
Web
Bead Location

Chapter 6

Discussion

6.1 The importance of RARγ

In 1962, the first retinoid-based therapeutic compound (tretinoin) was introduced to fight acne. Since then, a plethora of retinoid analogs have been developed to intervene with other dermatological conditions from the cosmetic photodamage associated with aging to more severe cases of skin inflammation such as acne and psoriasis (reviewed in Katsambas and Dessinioti, 2008). Although proven to be effective in the treatment of these conditions, the teratogenicity associated with exogenous retinoid exposure still remains a major issue. In effect, it is paramount that safer retinoid therapies with reduced or abolished retinoid toxicity be developed. As such, one approach used to develop safer retinoids has been to synthesize RAR-selective analogues. In contrast to the early generation retinoid compounds that bind and activate all RAR isoforms, selective RAR activation can elicit therapeutic activity in tissues where the particular RAR in question is functionally expressed, while preventing the activation of the RARs that are not involved, in hopes of minimizing drug toxicity.

RARγ is abundantly expressed in the skin and has provided researchers with a druggable target in the treatment of the aforementioned skin pathologies. The clinical significance of RARγ illustrates the need to understand its role not only during normal development, but following retinoid excess as well.

RARγ has also been linked to various types of cancer (skin, breast and lung, just to name a few; Brtko, 2007; Fenaux et al., 2007). RARγ-selective analogues have also been used to induce the differentiation or the programmed cell death of transformed cells (Vivat-Hannah and Zusi, 2005). With the advent of new retinoid-

based compounds, understanding their modes of action will greatly help in the design of safer, less-teratogenic compounds.

As such, my work has highlighted the importance of RARγ in retinoid-induced teratogenesis and has particularly focused on the mechanisms of action of RARγ following retinoid exposure. Using the embryonic limb as a surrogate model of organogenesis, my work has contributed towards identifying potential molecular mechanisms by which dysmorphogenesis may occur.

This chapter will (1) illustrate how my work has implicated RARγ in mediating retinoid insult; (2) propose further avenues of research that will advance our understanding of RARγ in the limb; and (3) explore the broader impact of my work as it pertains to the complex field of retinoid teratology.

6.2 Linking RARγ function and limb dysmorphogenesis

The developing limb bud is particularly sensitive to imbalances in retinoid concentrations, illustrating the importance of vitamin A and its metabolites during embryo development. Following retinoid excess during organogenesis, the limb can exhibit a multitude of reductive malformations, many of which are characterized by notable effects on limb chondrogenesis, an observation shown by many groups, including our own (Jiang et al., 1995; Ali-Khan and Hales, 2003; Zhou and Kochhar, 2004). Specifically, the differentiation of all cartilage elements (long bones, carpalia and digits) can be disrupted by retinoid excess. The principal aim of this thesis was to help unravel the mechanisms underlying retinoid-induced teratogenesis in the developing limb.

RARs play integral roles in mediating the transcriptional effects often associated with retinoid activity. During embryonic limb development, the RARs are expressed in specific and discrete locations. Whereas RARα and RARβ transcripts are localized to soft tissue and areas of programmed cell death, respectively, RARγ expression is most highly observed in areas of cartilage formation (Dolle et al., 1989). Given our interest in relationship between retinoids and chondrogenesis in the developing limb as well as the corresponding abundant expression of a particular RAR isoform, RARγ, in highly retinoid-susceptible limb cartilage, we hypothesized that RARγ is crucially involved in the pathogenesis of retinoid-induced limb defects.

Chapters 2 and 3 directly assessed the functional roles of RARγ in mediating retinoid teratogenicity whereas Chapters 4 and 5 explored the functional roles of two genes identified to be regulated by RARγ.

In the first study (Chapter 2), I described how limbs respond to retinoid insult following the genetic ablation of RARγ. The key observation was the attenuation of retinoid-induced limb insult in the absence of RARγ (under an RARα1-null background). Additionally, chondrogenesis (*Sox9* and *Col2a1*) and patterning (*Meis1* and *Meis2*) genes normally misregulated by excess retinoid exposure remained unresponsive in the absence of RARγ. These data emphasized the importance of RARγ in (1) the misregulation of crucial developmental genes and, (2) more importantly, mediating retinoid-induced limb dysmorphogenesis.

To complement the previous observations, the experiments in Chapter 3 examined how the pharmacological activation of RARγ affected not only limb morphology, but the transcriptional signals that control limb chondrogenesis as well.

Limbs treated with either all-*trans* RA (pan-RAR agonist) or BMS-189961 (RARγ-selective agonist) displayed significant retardations in overall limb growth and cartilage formation. Chondrogenesis-focused microarray analysis was also done to uncover the potential gene targets misregulated by RARγ activity. Two genes, *Mgp* and *Gdf10,* were identified as RARγ-responsive genes that may be involved in retinoid-induced limb dysmorphogenesis. Whole mount ISH for *Mgp* and *Gdf10* revealed distinct expression patterns; *Mgp* was localized in the developing cartilage whereas *Gdf10* was highly expressed in areas destined for programmed cell death. Accordingly, this study (1) highlighted the specific involvement of aberrant RARγ activity during bouts of retinoid excess and (2) identified two RARγ-responsive genes that may influence the retinoid-induced teratogenic outcomes in the limb.

To further understand the role of MGP in the limb, the experiments in Chapter 4 assessed how inhibition of MGP activity affected retinoid-induced limb dysmorphogenesis. MGP activity was partially inhibited by warfarin treatment, a commonly used pharmacological inhibitor of Mgp activity (by preventing the γ-carboxylation of functional GLA residues). Limbs treated with all-*trans* RA and warfarin exhibited a significant attenuation in retinoid-induced limb insult when compared to limbs treated with RA alone. The warfarin-induced improvements in limb morphology were characterized by more differentiated and recognizable cartilage limb elements, highlighting the importance of the RARγ-responsive *Mgp* gene activity in mediating retinoid pathogenesis in the limb.

Finally, Chapter 5 addressed the biological functionality of the other identified RARγ-responsive gene, *Gdf10*. To simulate the aberrant retinoid-induced

upregulation of *Gdf10*, limb buds were implanted with rhGDF10-soaked beads to ectopically overexpress Gdf10 in the distal limb regions. Ectopic misexpression within the distal interdigital web (between digits II and III), but not within the distal digital ray (digit III), resulted in striking deleterious effects on proximal limb morphology. Given the spatial specificity (web vs. ray) and non-local (proximal) modes of GDF10 action, the data illustrated the potential role and ability of GDF10 to mediate limb dysmorphogenesis.

Taken together, these four independent yet complementary approaches have clearly implicated RARγ activity in transducing the teratogenic effects of retinoids on the developing limb. I have shown that retinoid-induced limb dysmorphogenesis could be significantly altered following either the genetic ablation or pharmacological selective activation of RARγ. Additionally, I have not only identified candidate genes regulated by RARγ but have also proposed several mechanisms by which RARγ may mediate the retinoid-induced reductive effects on limb chondrogenesis.

6.3 Future directions

6.3.1 RARγ and chondrogenesis

Using an *in vitro* limb culture system, we were able to directly assess the effects of retinoid insult on limb morphology. At the onset of culture, E12.5 embryonic murine limb bud cartilage exists in an early state of condensation. Limb outgrowth is well underway and the locations of the presumptive long bones and digits are taking shape. After 6 days in culture, all the long bones, carpalia and digits have substantially differentiated and are quite recognizable. Following retinoid insult,

limbs exhibit notable reductions in cartilage formation. To determine the involvement of RARγ in the retinoid-mediated effects on chondrogenesis, limbs deficient in RARγ were cultured *in vitro* (Chapter 2) and shown to be less-sensitive to retinoid insult. Conversely, wild-type limbs treated with a RARγ-selective agonist led to marked deleterious effects on cartilage (Chapter 3). Collectively, these data have linked RARγ function to the misregulation of limb chondrogenesis.

The whole organ culture is an excellent tool to assess overall dysmorphology (with a particular interest in cartilage differentiation), yet the limb is a heterogeneous system comprised of various tissues. To focus on the direct effects of RARγ on cartilage, limbs could be subjected to trypsin treatment and primary limb micromass cultures prepared (Ahrens et al., 1977). Additionally, the ADTC5 chondrogenic clonal cell line isolated from mouse embryonal carcinoma cells could provide an even more homogeneous experimental model (Atsumi et al., 1990). Both experimental systems have been shown to be excellent models for chondrocyte differentiation and maturation and often used to facilitate the elucidation and characterization of molecular signals misregulated by retinoid imbalances (Jiang et al., 1995; Hoffman et al., 2003). However, it should be noted that even though these two-dimensional systems may provide a more chondrogenic population of cells, their findings cannot substitute for the morphological information gathered through three-dimensional organ culture or *in vivo* analysis (to be addressed later).

Notwithstanding, application of the RARγ-selective agonist to either chondrogenic cell culture could provide more specific molecular insight as to what RARγ may be doing to alter chondrogenic processes. As in Chapter 3, a

chondrogenesis-focused microarray analysis would likely identify a more extensive subset of RARγ-misregulated genes in either cell culture model.

Consequently, upon identifying potential candidate genes, assessing their importance in transducing the retinoid-mediated effects on chondrogenesis would be the next step towards unraveling the importance RARγ. The use of RNA interference (RNAi) would be an efficient and potentially high-throughput means to interfere with the expression of these candidate genes (Pushparaj et al., 2008). RARγ-selective agonist treatment of cells altered by gene-specific knockdown may determine the necessity of distinct gene products (and their associated signaling pathways) in mediating the reductive effects on cartilage. Attenuation in retinoid-dependent decreases in cartilage formation and maturation following the knockdown of particular genes could provide potential experimental leads. Importantly, the experimental follow-up of candidate genes in whole organ culture (and ultimately *in vivo*) would be required to determine their biologically relevant effects on limb morphology prior to and following retinoid insult.

To complement the pharmacological activation of RARγ (Chapter 3), constitutively-active RARγ could be transfected into cells or tissues to determine how cartilage is affected. RNAi specifically targeting RARγ expression would also create another model of RARγ-deficiency. Should RAR redundancy play a limiting role, combinatorial selective knockdown of other RAR subtypes and isoforms would be critical in characterizing this process. Hence, the data generated by RARγ gene knockdown could be correlated to the results that we have presented in this thesis, further strengthening our understanding of RARγ function.

6.3.2 RARγ and the regulation of gene expression

Our work has implicated RARγ in the misregulation of chondrogenesis-related and unrelated genes alike. In identifying these particular players downstream of RARγ activation, understanding the mechanisms underlying the observed gene misregulation is equally as important as the identification of the genes themselves.

The gene expression analysis in Chapters 2 and 3 illustrated that the misregulation of RARγ activity could lead to a gene-dependent up- or down-regulation of expression. Classically (as mentioned in Chapter 1), upon RAR activation, co-factors are recruited to unwind chromatin, making the accessibility of transcriptional machinery possible, thus leading to the misregulation of gene expression (reviewed in Bastien and Rochette-Egly, 2004). In contrast, as was seen in the case of *Sox9* and *Col2a1* (Chapter 2), the induction of retinoid activity (in the presence of RARγ) led to transcriptional repression rather than activation. Understanding the biochemistry of RARγ action would help elucidate how RARγ could lead to very different transcriptional outcomes.

Specifically, ascertaining the upstream and/or downstream regulatory regions that bind RARγ may prove fruitful in explaining the opposing directional changes due to excess retinoid exposure. A "promoter-bashing" deletion analysis of the particular genes of interest might provide insight as to which *cis*-regulatory regions (i.e., response elements) may control transcriptional activity. In the case of genes misregulated in the upward direction, previous work suggests that RARγ associates with retinoic acid response elements (RARE) and leads to the recruitment of transcriptional machinery (Leid et al., 1992; Mangelsdorf et al., 1995). In contrast, in

the case of genes that are misregulated in the downward direction (i.e., *Sox9*), their regulatory regions may contain negative response elements (NRE) that upon ligand binding maintain chromatin in its repressive state. Upon identification of these novel NREs, electrophoretic mobility-shift assays (EMSA) and chromatin immunoprecipitation (ChIP) could be performed to substantiate the physical interactions between RARγ and these negative regulatory elements. NREs have already been identified upstream of a handful of genes including human *MGP* (Kirfel et al., 1997), rat *Oxt* (oxytocin; Lipkin et al., 1992) and murine *Oct4* (octamer-4; Schoorlemmer et al., 1994). Since there are a plethora of genes that are downregulated by exogenous RAR activation, many NREs have yet to characterized. With the identification of these novel regulatory elements and the subsequent characterization of their importance may eventually shift the biochemical paradigms associated with the regulation of retinoid activity.

6.3.3 From gene expression to protein function

As mentioned above, the induction of retinoid activity directly leads to the regulation of gene transcription. In this thesis, particular attention has been placed on identifying the genes misregulated by RARγ. As measured by qRT-PCR in *in vitro* cultured limb tissue, RARγ ablation was shown to dampen the misregulation of *Sox9* and *Col2a1* following retinoid insult while selective activation of RARγ resulted in the upregulation and identification of *Mgp* and *Gdf10*, two RARγ-responsive genes. In either case, RARγ was shown to be clearly associated (albeit not necessarily indispensable) for their retinoid-mediated misregulation.

In Chapter 3, whole-mount ISH was used to localize *Mgp* and *Gdf10* transcripts in the developing limb. After 3 and 6 h *in vitro*, *Mgp* was strictly localized to areas of developing cartilage whereas *Gdf10* was strongly expressed in domains containing soft tissue (i.e., proximal interdigital webbing and necrotic zones) and weakly expressed in digits. Although whole mount ISH has been a well-accepted tool among developmental biologists to localize transcript and an effective means to generate clues regarding gene function, examining the functional proteins would solidify the relationship between location and function. Therefore, assessing the localization of the candidates of interest would be the next logical step in further understanding their roles in RARγ-mediated limb dysmorphogenesis.

Immunohistochemical or immunofluorescent analysis of fixed histological sections or whole mount preparations would be paramount in visualizing the domains of protein localization. In addition, confocal-based image analysis of immunofluorescently-stained and cleared limbs (to render them translucent) would allow for the high-resolution characterization of protein localization at cellular and subcellular levels following retinoid-induced limb insult. This technology that has already been developed and used for the imaging of whole organogenesis-stage embryos (Zucker et al., 1998).

6.3.4 RARγ and matrix GLA protein

With the identification of the RARγ-responsive gene *Mgp* in Chapter 3, the work in Chapter 4 set out to further characterize the importance of this candidate gene in mediating retinoid-induced limb dysmorphogenesis. Strikingly, I have

demonstrated that inhibition of MGP activity by warfarin treatment was able to significantly attenuate the retinoid-induced insult commonly observed in warfarin-untreated limbs.

Warfarin indirectly inhibits the vitamin K-dependent γ-carboxylation of MGP by disrupting VKOR activity, the enzyme responsible for the recycling of vitamin K from its inactive reduced form into its oxidized active form (Furie and Furie, 1988). However, there are various other GLA proteins (including a group of clotting factors) whose function can be affected by warfarin exposure.

With respect to developing bone, osteocalcin (OC) is a GLA protein that is solely expressed in late-stage osteoblasts (Hauschka et al., 1975) and has been shown to be upregulated by all-*trans* RA (Oliva et al., 1993; Thaweboon et al., 2005). Characterizing OC expression in the absence and presence of excess retinoid will be required to determine its involvement in the warfarin-induced rescue of retinoid-mediated limb insult.

To directly determine the biological functionality of MGP, the development of a biologically-active recombinant MGP would allow for ectopic overexpression. In chick, ectopic transfection of an RCAS-MGP plasmid in the limb has been done, which resulted in a marked inhibition of bone formation (Yagami et al., 1999). Modulation of MGP expression/activity via limb-specific gene ablation (Logan et al., 2002) or RNA interference (reviewed in Krull, 2004) would also provide researchers with more specific experimental models targeting of MGP function and would eliminate the legitimate concerns regarding the MGP-independent actions of warfarin (Price, 1989).

Furthermore, several groups have designed novel conformational-specific antibodies against carboxylated and undercarboxylated MGP protein to assess its γ-carboxylation status (Sweatt et al., 2003; Schurgers et al., 2005). Applying this technology to our limb culture system may help answer if in fact warfarin exposure affects MGP conformation (and thus function) during *in vitro* limb development.

Finally, to definitively implicate RARγ in MGP-mediated limb dysmorphogenesis, it would be invaluable to determine whether warfarin is able to rescue limb dysmorphogenesis (as in Chapter 4) following RARγ-selective agonist exposure. Should warfarin-induced rescue be observed, this would provide irrefutable evidence linking RARγ activity to MGP-mediated limb dysmorphogenesis following retinoid insult.

6.3.5 RARγ and growth differentiation factor-10

Chapter 5 examined the ability of GDF10 to alter embryonic limb development. Ectopic overexpression of rhGDF10 in the interdigital webbing resulted in marked detrimental effects on the morphogenesis of the developing long bones. This observation suggested the potential importance of GDF10 in the control of limb patterning and/or chondrogenesis. Understanding how the distal ectopic application of rhGDF10 could affect development of proximal structures will be integral in elucidating the functions of Gdf10 in normal and retinoid-induced limb dysmorphogenesis.

Even though the Affi-Gel Blue beads are soaked with 50 μg/ml of rhGDF10, the exact concentration of protein within each bead following implantation as well as the stability and bioactivity of rhGDF10 once implanted are unknown.

Immunohistochemical analysis of human Gdf10 at multiple time points following the onset of culture would provide information regarding (1) the diffusability of rhGDF10 and (2) the kinetics of rhGDF10 as culture progression occurs. Perhaps at E12.5 (time 0; when the bead is initially implanted), the bead resides in relatively close proximity to the proximal limb tissues, an area of the limb that may be temporally susceptible to rhGDF10 overexpression. Additionally, the absence of any effects on the developing handplate may be due to the biological inactivity of rhGDF10 at later time points (when handplate development may be more susceptible to imbalances in rhGDF10 activity). Bead implantation at earlier (E10.5-11.5) and later time points (E13.5-14.5) would help unravel whether the proximal effects are related to the time of rhGDF10 exposure or otherwise.

Alternatively, given previous evidence implicating GDF10 in the regulation of patterning (Hino et al. 2003), examining if and how GDF10 may influence the signals controlling proximal limb development may explain the proximal truncations observed in Chapter 5. In particular, examining how various Hox genes, including *Meis1* and *Meis2*, respond to ectopic rhGDF10 overexpression may provide potential clues as to mechanisms underlying the proximal dysmorphogenesis.

Another striking observation was the location-dependent effects of bead implantation on limb dysmorphology. Whereas beads implanted in the webbing resulted in marked effects on the proximal cartilage anlagen, implantation into the

digital rays led to minor and less frequent effects on the developing handplate with not observable proximal dysmorphogenesis. Determining how Gdf10 activity is differentially mediated in the different limb domains should clarify our observations. Particularly, specific BMP receptor (BMPR) isoforms, have been shown to be differentially expressed in the web and rays (Kawakami et al., 1996). Whereas the BMPR-1B subtype in the ray appear to account for the chondrogenic effects of BMPs, BMPR-1A subtype expressed in the interdigital webbing leads to the induction of cell death pathways (Zou et al., 1997; Merino et al., 1999a). Because Gdf10 can classically bind to TGF-β and activin receptors (structurally related to BMPR), the characterization of their localization and activation in the limb following GDF10 overexpression may prove fruitful in the elucidation of the effects on limb phenotype (Daluiski et al., 2001; Allendorph et al., 2007).

Finally, in contrast to its overexpression, *Gdf10*-null mice have previous been generated and develop normally (Zhao et al., 1999). Consequently, these mice provide an excellent model to examine how GDF10-deficiency affects pan-RAR or RARγ-selective activation *in vitro* and will provide answers regarding the involvement of Gdf10 in transducing the retinoid/RARγ-mediated limb insult.

6.3.6 *In vivo* assessment of RARγ

In vitro systems are approachable methodologies that have facilitated the study of many, if not all, physiological, developmental and toxicological processes. They are tools that are oftentimes used to dissect signaling pathways as well as provide alternative, more humane models to tackle very daunting biological

questions. However, correlating our *in vitro* results with those observed *in vivo* will further support and enhance the biological significance of our work.

With respect to retinoid biology, there is a wide array of evidence exploring the *in vivo* roles of many of the proteins that lay within the retinoid axis, from retinoid receptors to putative or well-characterized downstream targets of these receptors (Lohnes et al., 1994; Mendelsohn et al., 1994). RAR gene ablation studies have been done to examine the roles of RARγ during *in utero* and postnatal development. Additionally, these studies were taken one step further by examining how RARγ-deficiency could affect the processes underlying retinoid-induced dysmorphogenesis. It was shown that while RARγ-null mice exposed to teratogenic concentrations of all-*trans* RA *in vivo* were equally sensitive to limb insult (Iulianella and Lohnes, 1997). Conversely, the all-*trans* RA-induced homeotic transformations within the caudal vertebrae frequently observed in wild-type mice were prevented following RARγ ablation. Effectively, the absence of any observed rescue in the developing limb was attributed to the functional redundancy among RAR family members (Chambon, 1994). To address this functional redundancy, in Chapter 2, we examined the susceptibility of RARγ-null limbs (under and RARα1-null background, thus removing the most ubiquitously-expressed receptor that may compensate for the absence of RARγ). Briefly, contrary to RARα1γ$^{+/+}$ and RARα1γ$^{+/-}$ limbs that developed normally and were highly susceptible to retinoid excess, retinoid-induced limb dysmorphogenesis was drastically attenuated in RARα1γ-/- limbs. Additionally, the mere fact that RARα1γ-null limbs did not grow normally *in*

vitro clearly illustrated the importance of these particular receptors (and their endogenous activity) during normal and abnormal limb development alike.

In spite of this, there are other genetic *in vivo* manipulations that could further unravel the importance of RARγ in retinoid insult. For instance, to compliment our RARγ ablation and pharmacological ablation studies, transgenic mice could be genetically-engineered to express a constitutively active RARγ transgene, not unlike what Cash and colleagues have done to assess the role of RARα in the modulation of chondrogenesis (Cash et al., 1997). The transgene could be ubiquitously expressed to determine how RARγ affects all embryonic tissues. Alternatively, transgene expression could also be regulated by a limb-specific promoter fragment, allowing for the ectopic expression of the fusion receptor in the limb, our particular tissue of interest. Whereas Cash and colleagues have used the murine *Hoxb-6* (homeobox protein 2.2) promoter fragment to control their constitutively active RARα protein, the use of the *Prx1* (paired-related homeobox gene 1) enhancer would be another viable candidate for the limb-specific induction of the RARγ transgene (Martin and Olson, 2000). The *Prx1* enhancer has successfully been used to drive the expression of Cre recombinase in transgenic mice; recombinase activity was first observed at E9.5, at the onset of limb outgrowth (Logan et al., 2002). To date, these *Prx1*-Cre mice have been the most-commonly used genetic model for the limb-specific knockout of many genes, highlighting the specificity of *Prx1* in the presumptive and developing limb bud.

Conversely, there have been several mouse models that were developed to mimic retinoid excess. As described in Chapter 1, the amount of retinoid production

is counterbalanced by its subsequent degradation by various catabolizing enzymes such as cytochrome P450 (CYPs) enzymes. CYP26A1 and CYP26B1 are two well-established enzymes that can efficiently degrade all-*trans* RA, with the later isoform being the predominant player in retinoid catabolism in the developing limb (Abu-Abed et al., 1998; MacLean et al., 2001). Several groups have genetically knocked-out *Cyp26b1* function during embryogenesis, therefore shifting the retinoid balance towards production, thus leading to retinoid excess *in vivo* (Yashiro et al., 2004; MacLean et al., 2007). In effect, both groups have shown that *Cyp26b1*-null mice exhibit malformations associated with retinoid teratogenicity, one of which includes limb dysmorphogenesis (Yashiro et al., 2004).

Importantly, Petkovich and colleagues have recently co-ablated the *Cyp26b1* and *RARγ* gene and have successfully shown that RARγ-deficiency could significantly rescue the limb insult associated with ectopic levels/domains of all-*trans* RA following *Cyp26b1* ablation, further substantiating the importance of RARγ in mediating retinoid-induced limb dysmorphogenesis (Pennimpede and Petkovich, personal communication and unpublished results). Alternatively, the genes encoding *Cyp26b1* and *RARγ* could both be flanked with loxP sites and selectively-ablated in the developing limb via incorporation of the previously mentioned *Prx1*-Cre mice (Nagy, 2000; Logan et al., 2002). These experiments would provide an excellent *in vivo* model for the study of RARγ function during retinoid excess. In addition, should a mouse expressing Cre-recombinase activity specifically in limb cartilage be generated, RARγ could be conditionally ablated in chondrocytes rather than in the generalized limb mesenchyme as seen in instances of Prx1-Cre crosses.

Additionally, the use of RNAi in whole organ and embryo culture as well as *in utero* has been gathering recent attention. The use of RNAi has been well characterized in cell culture, yet recent work has adapted gene knockdown to target organs in culture (Davies et al., 2004), cultured embryos (Calegari et al., 2004) as well as developing embryos *in utero* (Bai et al., 2003). Applying gene knockdown technology *in utero* to embryos destined to be exposed to excess retinoid would provide us with a way to not only validate the results in the culture dish or bottle, but also offer a less-cumbersome and more affordable method to tackle gene silencing, contrary the high-cost and time-consuming methods associated with genome-wide or conditional gene knockout as discussed above.

Finally, while much of the discussion has focused on murine models of limb development, one cannot ignore the importance of the countless retinoid-dependent effects observed using the *in ovo* avian model. Proving to be an excellent amalgamation of *in vivo/in ovo* and *in vitro*, nature has provided researchers with an 'all-natural' system that can be manipulated just as easily as *in vitro* limb or whole embryo culture. Specifically, the introduction of plasmids housing exogenous genomic material (whether they be short-hairpin RNAs that knockdown gene function or complete gene sequences that promote protein production) into *in ovo* embryos by the well-documented RCAS method has allowed developmental biologists to manipulate avian embryos in countless ways (Hughes et al., 1987). In fact, an RCAS-MGP plasmid was transfected into chick embryo fibroblasts then injected into the developing chick limb to determine how the MGP overexpression could affect the chondrogenesis and osteogenesis of the developing long bones

(Yagami et al., 1999). Application of this robust technology *in ovo* will help determine the biological significance of the genes involved in RARγ-mediated limb dysmorphogenesis.

6.4 Perspectives

6.4.1 Other mechanisms of RARγ function

Although our studies have mainly focused on the relationship between RARγ activity and chondrogenesis, it is certain that RARγ can regulate many other pathways during embryo development. This section will address some of the other ways in which RARγ may mediate retinoid-induced limb dysmorphogenesis.

Firstly, the RARγ-dependent misregulation of *Meis1* and *Meis2* presented in Chapter 3 is a prime example of the RARγ-induced effects in pathways that are unrelated to chondrogenesis. Specifically, *Meis1/2* are homeodomain-containing genes that play distinct roles in patterning (Mercader et al., 1999). They exhibit proximalization activity and may mediate retinoid-induced limb truncation but have not been directly linked to chondrogenesis (Qin et al., 2002; Mercader et al., 2005). Examining the genetic and biochemical processes by which RARγ regulates *Meis* will be important in understanding the molecular bases underlying limb truncation. Additionally, the misregulation of other homeobox genes (albeit not in the limb) such as *Hoxa1* (Boylan et al., 1993), *Cdx1* (Allan et al., 2001) and *Cdx2* (Iulianella et al., 1999) has been linked to RARγ activity suggesting the possibility that RARγ may misregulate other patterning genes involved in limb development.

On the other hand, a plethora of work in the cancer field have uncovered a link between RARγ activity and apoptosis, a crucial process required from proper limb morphogenesis (Zakeri and Ahuja, 1994). Additionally, previous work from our lab has described the involvement of apoptosis in retinoid-induced limb dysmorphogenesis (Ali-Khan and Hales, 2003). Of particular interest, several RARγ-selective agonists have been demonstrated to induce apoptosis in various types of cells, some of which include carcinoma cell lines (Sun et al., 2000). Whereas the apoptotic cascade is complex, it is worth noting that RARγ has been shown to affect numerous targets pertaining to programmed cell death. For example, RARγ activity could induce Fas ligand expression in T cells (Toth et al., 2004), alter the Bcl-2/Bax status in pancreatic cancer cells (Pettersson et al., 2002), disrupt mitochondria function in malignant hematopoetic cells (Solary et al., 2003) and induce caspase activation in lung carcinoma cells (Kim and Lotan, 2005). These are all plausible mechanisms that may be misregulated in the limb during bouts of retinoid excess.

In contrast, the use of mouse F9 teratocarcinoma cells as a model for cellular differentation and some aspects of embryo development has also shed light on RARγ function (Strickland et al., 1980). F9 cells express all RAR subtypes and, in the presence of retinoids, undergo differentiation. Recent work examining RARγ-null F9 cells uncovered a novel target that requires the presence of RARγ to be expressed; tyrosine kinase 1 (*Tie1*; Su and Gudas, 2008). *Tie1* is expressed in all endothelial cells and is required for proper expansion (Puri et al., 1995) and serine/threonine kinase Akt-dependent survival of embryonic vasculature (Kontos et

al., 2002). Hence, these results provide evidence that RARγ may affect vascularization and cell survival in the developing limb.

Regardless of which signals are affected, a genome-wide gene array analysis would provide considerable insight into the potential mechanisms affected by RARγ. As in Chapter 3, this gene profile analysis following pharmacological activation of RARγ would identify the chondrogenesis-related and unrelated pathways downstream of its activity.

6.4.2 Beyond RARγ

In Chapter 3, our data uncovered another subset of non-RARγ-responsive genes that may potentially be involved in retinoid-mediated pathologies. Whereas this subset of chondrogenesis-related genes were unaffected by the pharmacological activation of RARγ, they were significantly misregulated (up- or down-regulation) by all-*trans* RA-induced pan-RAR activation.

Although these genes are all related to chondrogenesis, the signaling pathways in which they act vary considerably. For instance, the misregulation of *Bmp4* and *Smad2* is associated with transforming growth factor-beta (TGF-β) signaling (Whitman, 1998) whereas *Ctsk* (cathepsin K) is a protease involved in bone resorption and *Nfkb1* is an important player in redox regulation and inflammation (Iotsova et al., 1997; Kiviranta et al., 2005). Other extracellular matrix components such as *Dcn* (decorin) and *Col11a1* (type XI collagen) were also changed independently of RARγ activation (Mundlos, 1994).

These observations are an obvious indication that there is much more to retinoid-induced limb dysmorphogenesis than just RARγ. The functional roles of RARα and β have also been extensively studied. Selective pharmacological activation of each RAR has resulted in detrimental effects on limb development (Arafa et al., 2000). Additionally, aberrant RARα and β activation has been associated with observable effects on limb chondrogenesis *in vivo* and *in vitro* (Jiang et al., 1994; Jiang et al., 1995; Cash et al., 1997). Assessing how RARα and β misregulate the non-RA-responsive genes identified in Chapter 3 will be of particular interest. All in all, retinoid-induced teratogenicity does not solely rely on RARγ function, thus making the study of RARα and β involvement equally important in completely understanding the molecular basis underlying retinoid teratology.

Furthermore, as addressed in Section 1.1.5 of this thesis, in several experimental systems, retinoids can exhibit non-classical RAR-independent modes of action. Specifically, excess retinoid exposure has been associated with the RAR-independent activation of PPAR, AP-1 and ERK signaling (Shaulian and Karin, 2002; Zanotto-Filho et al., 2008). These various molecular players have been implicated in numerous aspects of embryonic development, therefore characterizing their involvement would provide insight as to the multi-dimensional mechanisms underlying retinoid-induced teratogenicity.

6.4.3 Conclusion

Ultimately, my work has contributed to the ever-growing field of retinoid teratology. Using an *in vitro* limb culture model, I have highlighting the particular

importance of RARγ in mediating aspects of retinoid-induced dysmorphogenesis and have proposed various mechanisms by which RARγ may disrupt chondrogenesis. Since the signaling processes controlling the limb development also play integral roles in the morphogenesis of many other embryonic structures (Capdevila and Izpisua Belmonte, 2001), further unraveling retinoid signaling in the limb will help uncover how retinoid teratogenicity may affect the developing embryo proper. Furthermore, with the increased mainstream use of newer generation RAR-specific retinoids for the treatment of various medical conditions and diseases affecting women of child-bearing age, understanding the mechanisms underlying their toxicity will help limit or remove their causing effects.

6.5 List of Original Contributions

1. Developing limbs deficient in RARγ (under an RARα1-null background) were less susceptible to malformations caused by retinoid excess than wildtype. RARγ activity was required to mediate the retinoid-induced misregulation of specific developmental genes required for cartilage formation (*Sox9* and *Col2a1)* and limb patterning (*Meis1* and *Meis2*).
2. Limb chondrogenesis was severely disrupted following the selective pharmacological activation of RARγ. A chondrogenesis-focused gene expression analysis identified two novel genes (*Mgp* and *Gdf10*) that were significantly upregulated following aberrant RARγ activation. These genes may be important players involved in mediating retinoid- and RARγ-induced limb insult. The expression and localization of these genes were characterized in the limb.
3. Pharmacological inhibition of MGP activity with warfarin resulted in the significant attenuation of dysmorphogenesis following excess retinoid exposure of the limb. MGP may represent a major mediator or effector of retinoid insult. Elucidating its mechanisms of action may provide insight towards the prevention of retinoid teratogenicity.
4. Ectopic overexpression of GDF10 negatively affected limb differentiation, providing the first evidence for GDF10 involvement in the development of the appendicular skeleton. Understanding the molecular mechanisms underlying GDF10 function will be crucial in understanding its role in mediating retinoid-induced malformations.

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Appendix

www.mcgill.ca/researchoffice/compliance/animal/forms/

McGill University Animal Care Committee
RENEWAL of Animal Use Protocol

For: Research ☐ Teaching ☐ project

For Office Use Only:
Protocol #:
Approval end date:
Facility Committee:
Renewal#: 1st 2nd

Principal Investigator: Hales, Barbara **Protocol #** 1825

Protocol Title: Teratogen-induced signaling pathways during limb development **Category:** B

Unit, Dept. & Address: Pharmacology & Therap., McIntyre Room 110, 3655 Prom. Sir-William-Osler

Email: barbara.hales@mcgill.ca **Phone:** 3610 **Fax:** 7120

Funding source: CIHR

Start of Funding: 01/01/2008 **End of Funding:** 31/03/2013

Emergency contact #1 + work AND home phone #s: Barbara Hales Work 3610; Home 514 935 3828

Emergency contact #2 + work AND home phone #s: Chunwei Huang Work 3634; Home 514 636 4415

1. Personnel and Qualifications

List the names of the Principal Investigator and of all individuals who will be in contact with animals in this study and their employment classification (investigator, technician, research assistant, undergraduate/ graduate student, fellow). Indicate if the Principal Investigator is not handling animals. If an undergraduate student is involved, the role of the student and the supervision received must be described. Training is mandatory for all personnel listed here. Refer to *www.animalcare.mcgill.ca* for details. Each person listed in this section must sign. *(Space will expand as needed)*

Name	Classification	Animal Related Training Information		Occupational Health Program *	Signature *"Has read the original full protocol"*
		UACC on-line Theory course	Workshops + others		
Barbara Hales, Principal Investigator		Yes	No, will not handle animals	No	
Chunwei Huang, Res. Technician		Yes	Yes, mouse and rat	No	
Eugene Galdones, Graduate student		Yes	Yes, mouse	No	
France-Helene Paradis, Undergrad student		Yes	Yes, mouse	No	
(JUNDER THE DIRECT SUPERVISION OF DR. C. HUANG, WILL EUTHANIZE MICE TO COLLECT EMBRYOS)					

*** Indicate for each person, if participating in the local Occupational Health Program, see http://www.mcgill.ca/researchoffice/compliance/animal/occupational/ for details.**

Approved by:

2. Approval Signatures

I will ensure that all collaborators and staff are aware of all changes to this protocol **Principal Investigator/ Course Director**		**Date:**
Chair, Facility Animal Care Committee		**Date:**
Animal Compliance Office		**Date:**
Chairperson, Ethics Subcommittee *(D level or Teaching Protocols Only)*		**Date:**
Approved Animal Use Period	**Start:**	**End:**

☐ **Renewal requires submission of full Animal Use Protocol form**

(Revised page)

3. Summary (in language that will be understood by members of the general public)
AIMS AND BENEFITS: Describe, in a short paragraph, the overall aim of the study and its potential benefit to human/animal health or to the advancement of scientific knowledge *(was section 5a in main protocol).*

The ultimate objective of this research program is to prevent birth defects. The overall goal is to decipher how teratogens disturb limb development to cause this birth defect. To do this we will determine the pathway(s) by which limbs respond to teratogenic insult.

4. Has there been any animal care issues? YES ☐ NO ☒ **if yes, supply details:**

5. If creating genetically modified animals or new combinations of genetic modifications, complete and attach a *Phenotype Disclosure form.*
If mice expressing new phenotype have been produced, submit a *Phenotype Disclosure form.*
Blank forms at *http://www.mcgill.ca/researchoffice/compliance/animal/forms/*

6. Procedures

a) For B and C level of invasiveness,

The procedures are the same as the original protocol: YES ☒ NO ☐

IF NO, complete the following:
Detail new procedures that are different from section 10a of the original protocol, including amendments *(include a copy of the entire revised procedure section 10a of the original protocol with the changes and/or new procedures in CAPS):*

b) For D level of invasiveness,

Include here ALL procedures described in the original protocol. New and changed procedures in CAPS *(was section 10a in main protocol); Please only attach SOPs related to new and changed procedures to this renewal form.*

7. Endpoints

a) For B and C level of invasiveness,

The procedures are the same as the original protocol: YES ☒ NO ☐

IF NO, supply new endpoints that are different from the original protocol:

Experimental endpoints:

Clinical endpoints:

b) For D level of invasiveness,

Include here ALL endpoints, including the ones described in the original protocol as well as new and changed endpoints in CAPS:

Experimental endpoints:

Clinical endpoints:

8. Hazards (check here if none are used: ☐)

a) Are the hazards different from original protocol? (infectious, radioactive, toxic, carcinogen, tumours)

YES ☐ NO ☒ if yes, supply details (material, risks, precautions):

b) Have the cell lines been tested for human and animal pathogens? YES:☐ NO:☐ None used:☒

9. Description of Animals to be used in the coming year (only):

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals. *If more than 6 columns are needed, please attach another page*

	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
Species	Mice - CD-1	CD-1				
Supplier/Source	Charles River	IRCM				
Strain	CD-1	RARE-hspLacZ				
Sex	F	M/F				
Age/Wt	Timed pregnant	2-6 mo./20 g				
# To be purchased	480					
# Produced by in-house breeding	-	25				
# Other (e.g.field studies)						
TOTAL# /YEAR	480	25				

10. Explanation of Animal Numbers:

BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT, describe the number of animals required for one year. Include information on experimental and control groups, # per group, and failure rates.

For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures. The arithmetic explaining how the total of animals for each column in the table above is calculated should be made clear.

(CD-1) We need an average of 4 -5 mice /experiment and project 2 experiments/week for 48 weeks. Most experiments are done with in vitro techniques; this does minimize the numbers of animals required. For each experiment we will euthanize gestation day 12.5 timed pregnant mice with CO2 overdose and excise the embryos. We need to test 30-40 limbs at the same stage of development, 3-5/bottle, with at least one control and several doses of each treatment. It is difficult to specify the exact number of adult mice required because

some are not pregnant while others may not have embryos at the right stage of development. The advantage of the in vitro techniques is that the "n" is the bottle, not the pregnant female. Limbs must be pooled to obtain sufficient tissue for analysis of many of the parameters to be assessed (eg. Gene expression arrays, inducible signalling pathways).

(RARE-lacZ) The RARE-lacZ reporter strain will be bred in house and timed pregnant females will be handled and euthanized as above, on gestation day 12.5, with CO2 overdose.

Submit to your local Facility Animal Care Committee. Please note that after two renewals, a full protocol needs to be submitted.

This approval does not imply that space will be made available. If a major increase of space needs is anticipated, please contact the appropriate animal facility manager.

Role of Retinoic Acid Receptors α1 and γ in the Response of Murine Limbs to Retinol In Vitro

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Received 19 July 2005; Revised August 16, 2005; Accepted 19 August 2005

BACKGROUND: Derivatives of retinol (vitamin A), commonly referred to as retinoids, signal through retinoic acid and retinoid X receptors (RARs/RXRs) and are essential for normal limb formation. Retinoid imbalances or perturbations in receptor function result in aberrant limb development. To examine the mechanisms underlying retinol-induced limb defects, we determined the responsiveness of limbs from RARα1$^{-/-}$γ mice to excess retinol in vitro. **METHODS:** RARα1$^{-/-}$γ$^{+/-}$ mice were bred and their embryos were recovered at gestational day (GD) 12.5. The forelimbs were excised and cultured in vitro in the presence of all-*trans* retinol acetate (0, 1.25, 12.5, or 62.5 μM) for 6 days. The expression profiles of genes known to affect chondrogenesis (*sox9* and *col2a1*) and limb outgrowth (*meis1*, *meis2*, and *pbx1a*) were examined by real-time qRT-PCR following retinol exposure for 3 hr. **RESULTS:** Whereas RARα1$^{-/-}$γ$^{+/+}$ and RARα1$^{-/-}$γ$^{+/-}$ limbs exhibited deleterious effects on limb outgrowth and chondrogenesis in the presence of exogenous retinol, this outcome was significantly attenuated in RARα1$^{-/-}$γ$^{-/-}$ limbs. The expressions of *sox9* and *col2a1* were significantly decreased in retinol-exposed RARα1$^{-/-}$γ$^{+/+}$ limbs. In contrast, expression was not altered in limbs from their RARα1$^{-/-}$γ$^{+/-}$ or RARα1$^{-/-}$γ$^{-/-}$ littermates. Retinol exposure upregulated the expression of *meis1* and *meis2* in RARα1$^{-/-}$γ$^{+/+}$ limbs; however, in RARα1$^{-/-}$γ$^{-/-}$ limbs the expression of both genes was unresponsive to retinol. *Pbx1a* remained unresponsive to retinol treatment in all genotypes. **CONCLUSION:** In the absence of RARα1, RARγ is a functionally important mediator of retinoid-induced limb dysmorphogenesis. *Birth Defects Research (Part A) 76:39–45, 2006.* © 2006 Wiley-Liss, Inc.

Key words: limb development; chondrogenesis; limb outgrowth; retinoid teratogenesis

INTRODUCTION

Vitamin A (retinol) and its derivatives, which are collectively referred to as retinoids, play key roles in vertebrate development. Many organs, including the developing limb, depend on tight control of retinoid concentrations for proper morphogenesis to occur (Ross et al., 2000). Either retinoid deficiency or excess during sensitive windows of development lead to limb dysmorphogenesis, which is commonly characterized by deleterious effects on chondrogenesis (Paulsen et al., 1994) and limb outgrowth (Kwasigroch and Kochhar, 1980).

Retinoid exposure has been implicated in the misregulation of genes required for proper chondrogenesis and outgrowth. Many signaling pathways have been identified that are key regulators in both of these aspects of limb development, and several of these key regulators have been shown to respond to retinoid signaling. With respect to chondrogenesis, various groups have shown a critical role for the SRY-box-containing protein 9 (Sox9) transcription factor in mesenchymal chondroprogenitor condensation (Lefebvre and de Crombrugghe, 1998). Sox9 enhances type II collagen (*Col2a1*) expression, the main building block of cartilage (Bell et al., 1997). In addition, limb-specific ablation of *Sox9* leads to limbless offspring (Akiyama et al., 2002). Moreover, *Sox9* expression is downregulated in micromass limb cell cultures or primary chondrocytes exposed to all-*trans* retinoic acid in vitro (Sekiya et al., 2001; Hoffman et al., 2003).

Limb truncation has been linked to the upregulation of genes that are important in proximal-distal limb pattern-

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ing. Although the apical ectodermal ridge initiates and promotes limb outgrowth, proximally expressed homeodomain transcription factors, such as the myeloid ecotropic viral insertion site (*Meis*) family members, dictate proximal limb identity and contribute to the rate of limb outgrowth (Capdevila et al., 1999; Mercader et al., 1999, 2000). Meis proteins can interact with and induce the nuclear localization of the homeodomain protein pre-B-cell leukemia transcription factor 1 (*Pbx1*), leading to altered gene expression through trimerization with diverse *Hox* family members and contributing to a proximal limb transcription program (Berthelsen et al., 1999). Under normal conditions, endogenous retinoids in the proximal limb bud are believed to control the expression of *Meis1* and *Meis2*. In the presence of excess retinoid, *Meis* members become expressed distally, resulting in limb proximalization and truncation (Mercader et al., 1999; Qin et al., 2002).

Retinoids signal through 2 members of the nuclear receptor superfamily: retinoic acid receptors (RARα, -β, and -γ) that bind both all-*trans* retinoic acid and *9-cis* retinoic acid, and retinoid X receptors (RXRα, -β, and -γ) that bind *9-cis* retinoic acid (Chambon, 1994). RAR-RXR heterodimers transduce the retinoid signal through binding to retinoic acid response elements and the ligand-dependent recruitment of coactivators involved in chromatin remodeling (Mangelsdorf et al., 1995; Kastner et al., 1997; Glass and Rosenfeld, 2000). Various N-terminal variant isoforms of both the RARs and the RXRs have been identified (Leid et al., 1992; Mangelsdorf et al., 1992; Lohnes et al., 1995); however, pharmacological manipulation has shown that although RAR activation causes a wide array of teratogenic effects, RXR-selective agonists are nonteratogenic, illustrating the importance of RARs in mediating limb teratogenesis (Kochhar et al., 1996; Elmazar et al., 1997; Arafa et al., 2000).

As the murine limb develops, the expression patterns of RARs change dynamically in a spatial and temporal manner (Dolle et al., 1989; Weston et al., 2003). This observation suggests that the different RARs play specific roles in limb development. However, RAR-ablation studies have provided evidence for functional receptor redundancy (Chambon, 1994). For instance, whereas ablation of a single RAR isoform (e.g., RARα1) or certain RAR subtypes (e.g., RARβ) does not affect normal development, complete ablation of other subtypes (RARγ) leads to specific malformations in the axial, but not the appendicular, skeleton (Lohnes et al., 1994; Luo et al., 1995; Iulianella and Lohnes, 1997). Of importance, only RARαγ double-null mice show severe limb dysmorphogenesis, whereas expression of RARα2 in $RAR\alpha1^{-/-}\gamma^{-/-}$ mice suffices for normal limb development in vivo (Lohnes et al., 1994).

The objective of the present study was to assess the role of specific RARs in retinoid-induced limb malformations. To do so, an in vitro limb bud culture system was used to elucidate the impact of compound ablation of RARα1 and γ on the response of limbs to teratogenic concentrations of retinol. We hypothesized that in the absence of RARα1, RARγ is a functionally important mediator of retinoid-induced limb teratogenicity and affects the transcription of genes involved in both chondrogenesis and limb outgrowth. Consistent with this hypothesis, we report that $RAR\alpha1^{-/-}\gamma^{-/-}$ limbs examined in this study were less susceptible to the teratogenic effects of all-*trans* retinol than their littermate controls ($RAR\alpha1^{-/-}\gamma^{+/+}$ or $RAR\alpha1^{-/-}\gamma^{+/-}$). Thus, RARγ, in the absence of RARα1, is an important factor in mediating the teratogenic retinoid signal in the limb.

MATERIALS AND METHODS

Animals and Limb Bud Culture

$RAR\alpha1^{-/-}\gamma^{+/-}$ mice in a C57BL/6-129Sv genetic background were crossed to generate $RAR\alpha1^{-/-}\gamma^{+/+}$, $RAR\alpha1^{-/-}\gamma^{+/-}$, and $RAR\alpha1^{-/-}\gamma^{-/-}$ embryos (Lohnes et al., 1994; Mendelsohn et al., 1994). The mice were mated overnight, and noon of the day of detection of a vaginal plug was considered gestational day (GD) 0.5. On GD 12.5 pregnant females were killed via cervical dislocation and their embryos were explanted. The embryos' forelimbs were excised just lateral to the somites and cultured in vitro in a chemically defined retinoid-free culture medium as previously described (Kwasigroch et al., 1984; Huang and Hales, 2002; Ali-Khan and Hales, 2003). Because all of the mice lacked RARα1, the embryonic head associated with each set of forelimbs was collected and genotyped for RARγ as previously described (Iulianella et al., 1999). All-*trans* retinol acetate (Sigma, St. Louis, MO) was dissolved in 100% ethanol and added to the culture medium at low (1.25 μM), medium (12.5 μM), and high (62.5 μM) concentrations at the onset of culture. The total volume of ethanol in vitro did not exceed 0.5% vol/vol. All of the animal studies complied with the guidelines set by the Canadian Council on Animal Care.

Limb Morphology and Morphometry

The limbs were cultured in vitro for 6 days, with 1 medium change on day 3 (at which time retinol was not added) to replenish the nutrient supply. They were then fixed in Bouin's solution (Harleco, Gibbstown, NJ) overnight, stained with 0.1% toluidine blue (wt/vol 70% ethanol; Fisher Scientific, Montreal, Canada) overnight, dehydrated with a graded ethanol series, cleared, and stored in cedarwood oil (Fisher Scientific, Montreal, Canada). The limbs were examined under a dissecting microscope (Wild Heerbrugg 99067; Heerbrugg, Rebstein, Switzerland), and digital images were acquired (GCQ-X3HD digital camera; JVC, Tokyo, Japan). With the use of computer-assisted image analysis software (MCID-7; Image Research, St. Catharine's, Canada), the limb surface and relative cartilage (cartilage area/total surface area) were measured as endpoints representing overall growth and chondrogenesis, respectively. The extent and quality of differentiation were assessed using a previously developed limb morphogenetic differentiation scoring system (Neubert and Barrach, 1977).

Gene Expression Analysis

After 3 hr, each pair of limbs was removed from culture, rinsed with PBS, and stored at −20°C in RNAlater RNA stabilization reagent (Qiagen, Mississauga, Canada) until further processing. Total RNA from each pair of limbs was extracted using the RNeasy Micro Kit (Qiagen) and quantified by spectrophotometric UV analysis. RNA was subsequently diluted to a working concentration of 10 ng/μl, and Quantitect One-Step SYBR Green RT-PCR (Qiagen) was done using the Roche LightCycler according to the manufacturer's instructions (Roche Diagnostics, Laval, Canada). The PCR thermal cycling parameters were as

Table 1
Genes, Accession Numbers, Primer IDs, Primer Sequences, and Amplicon Lengths

Gene	Accession number	Primer ID	5′ → 3′ Sequence	Amplicon length (bp)
β-actin	NM_007393	*β-actinL*	GCT CTT TTC CAG CCT TCC TT	101
		β-actinR	AGG TCT TTA CGG ATG TCA ACG	
Sox9	NM_011448	*Sox9L*	TAT GTG GAT CTG TGC GTG TG	137
		Sox9R	CCA GCC ACA GCA GTG AGT AA	
Col2a1	NM_031163	*Col2a1L*	GAA GGT GCT CAA GGT TCT CG	103
		Col2a1R	CTT TGG CTC CAG GAA TAC CA	
Meis1	NM_010789	*Meis1L*	CTC TCT CTC CTC TCT CTC CCT CTT	121
		Meis1R	GCA GTT TTT CCC ATC CTT CC	
Meis2	U57343	*Meis2L*	CTT CAA CGC ACA TAC ACA CA	101
		Meis2R	GCT CCT TTT TAT CCC CCA AG	
Pbx1a	NM_183355	*Pbx1aL*	CGG AAG AGA CGG AAT TTC AA	119
		Pbx1aR	CCG CAC TTC TTG GCT AAC TC	

follows: 95°C for 15 min (1 cycle) and then 94°C for 15 sec, 55°C for 30 sec, and 72°C for 20 sec (for 60 cycles). Each group consisted of RNA from 5 pairs of separately cultured limbs, and each sample was measured in duplicate. Embryonic head or hindlimb tissues from phenotypically normal RARα1$^{-/-}$γ$^{+/+}$ mice were also collected, and RNA was extracted to provide 1, 10, and 100 ng/μl RNA stocks used to generate standard curves for quantification. The primers used were generated with Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and produced at the Sheldon Biotechnology Centre (McGill University, Montreal, Canada; see Table 1 for sequence information). To normalize the output for each pair of forelimbs, the expression of each gene of interest was divided by β-actin gene expression, a commonly used housekeeping gene whose expression remains unchanged following retinoid exposure. Melting-curve analyses were done following each PCR to determine the output and detection quality (i.e., the formation of primer-dimers).

Statistical Analysis

All morphology and gene expression data sets were analyzed statistically using SigmaStat 3.0 (Systat Software, Point Richmond, CA). Two-way ANOVA followed by the post-hoc Holm-Sidak multiple-comparison test was used to compare all groups for all genotypes and exposures. The minimum level of significance was $P < .05$ for all tests.

RESULTS

Improved Morphology of RARα1γ$^{-/-}$ Limbs Following Retinol Exposure

To test the effects of exogenous retinoid exposure on RARα1$^{-/-}$γ$^{-/-}$ (RARα1γ$^{-/-}$) limbs, the forelimbs from RARα1γ$^{-/-}$ mice, as well as RARα1$^{-/-}$γ$^{+/+}$ (RARα1γ$^{+/+}$), and RARα1$^{-/-}$γ$^{+/-}$ (RARα1γ$^{+/-}$) littermate controls at E12.5 were cultured for 6 days in the absence or presence of various concentrations of retinol.

Limbs from RARα1γ$^{+/+}$ and RARα1γ$^{+/-}$ littermate controls developed normally in the absence of retinol, as shown by proper long-bone and digit formation (Fig. 1A and E). After escalating doses of retinol were administered, limb dysmorphogenesis was apparent in a dose-dependent manner, with marked effects on chondrogenesis, outgrowth, and patterning (Fig. 1A–D and E–H). This effect on limb development was similar in cultured CD-1 and C57BL/6 limbs (data not shown).

RARα1γ$^{-/-}$ limbs, by contrast, did not develop normally compared to their littermate controls, as evidenced by stunted long-bone outgrowth (Fig. 1A, E, and I). In addition, the abnormal development associated with exposure to excess retinol was attenuated, with treated limbs from RARα1γ$^{-/-}$ embryos appearing less susceptible to the teratogenic effects of retinol than the wild-type controls (Fig. 1J–L). Long bones were straighter following low-dose treatment (Fig. 1B, F, and J), and after medium-dose retinol exposure (Fig. 1C, G, and K) long bones and digits were more recognizable and more developed than those in dose-matched RARα1γ$^{+/+}$ or RARα1γ$^{+/-}$ littermates. Although RARα1γ$^{-/-}$ limbs exposed to the high dose of retinol appeared highly dysmorphogenic, cartilage structures were still visible, in contrast to either RARα1γ$^{+/+}$ or RARα1γ$^{+/-}$ controls (Fig. 1D, H, and L).

Computer-assisted analysis was used to measure limb surface area as a marker for overall limb growth. In RARα1γ$^{+/+}$ and RARα1γ$^{+/-}$ controls, exposure to increasing concentrations of retinol led to a decline in limb surface area, except for the RARα1γ$^{+/+}$ limbs that were exposed to low concentrations of retinol, which showed a slight increase in surface area (Fig. 2A). In the absence of retinol, the surface area of RARα1γ$^{-/-}$ limbs was significantly decreased compared to littermate controls. Of interest, a significant genotype-specific increase of RARα1γ$^{-/-}$ limb surface area was seen following exposure to the medium concentration of retinol.

We measured the effects on chondrogenesis by measuring the relative cartilage area (cartilage area/limb surface area). RARα1γ$^{+/+}$ and RARα1γ$^{+/-}$ limbs showed significant decreases in chondrogenesis following exposure to medium or high concentrations of retinol compared to genotype-matched control limbs. In RARα1γ$^{-/-}$ limbs a significant decrease was apparent only in cultures exposed to high concentrations of retinol. Compared to cartilage formation in RARα1γ$^{+/+}$ and RARα1γ$^{+/-}$ dose-matched controls, cartilage formation in the limbs of RARα1γ$^{-/-}$ compound null mice was less affected by retinol exposure (Fig. 2B).

The limbs were subsequently individually scored using the system developed by Neubert and Barrach (1977) to determine effects on limb pattern formation. The scores of control RARα1γ$^{-/-}$ limbs were significantly lower than those of their littermate counterparts. Retinol treatment led

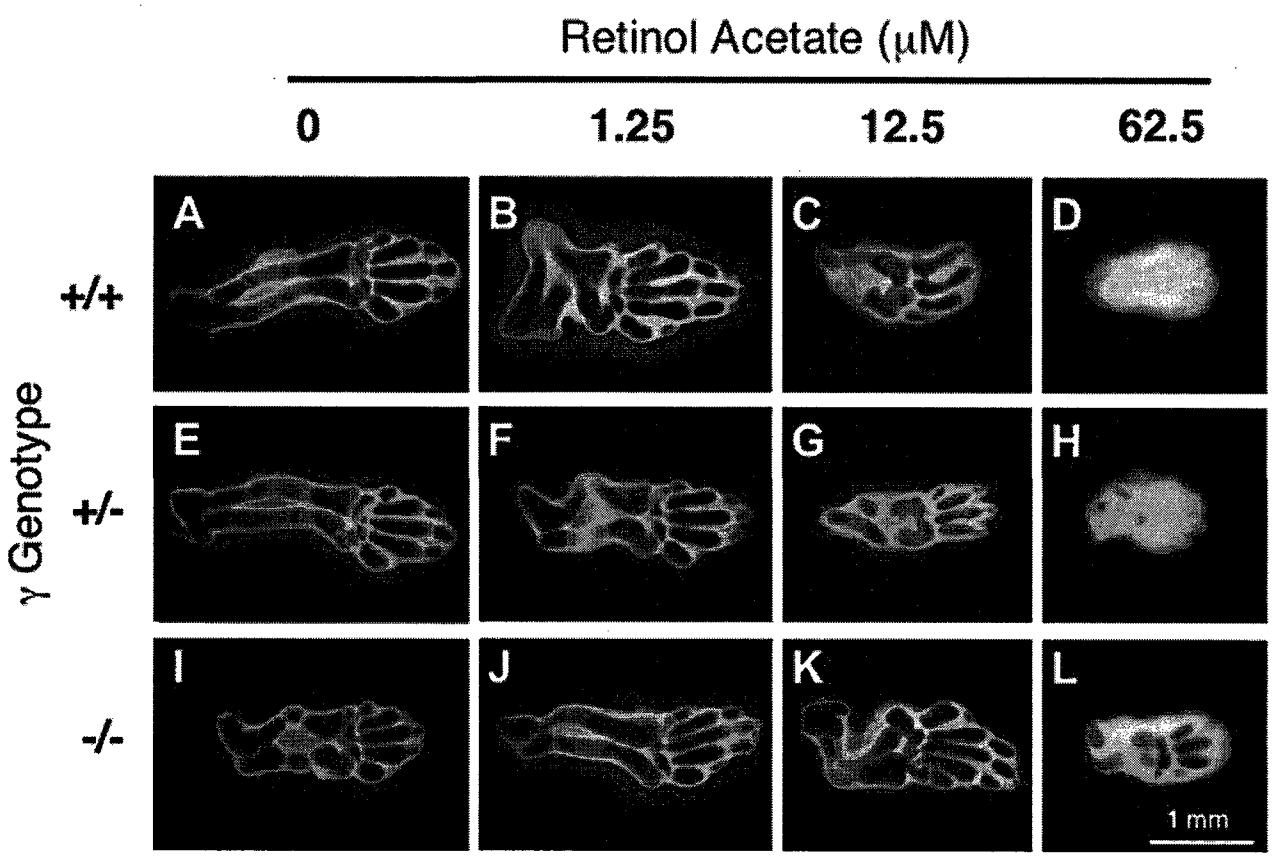

Figure 1. The effect of retinol treatment on RARα1γ$^{+/+}$, RARα1γ$^{+/-}$, and RARα1γ$^{-/-}$ limbs following in vitro limb bud culture. Limbs were excised from mice on GD 12.5 and cultured for 6 days in the presence of 1.25, 12.5, or 62.5 μM all-*trans* retinol acetate. Cartilage was stained with 0.1% toluidine blue. **A–H:** RARα1γ$^{+/+}$ and RARα1γ$^{+/-}$ limbs developed normally under control conditions and showed dose-dependent and detrimental effects on limb growth, chondrogenesis, and patterning following escalating doses of retinol. **I–L:** Although proper limb growth was not seen in RARα1γ$^{-/-}$ limbs under control conditions, these limbs were less susceptible to the teratogenic effects of retinol exposure than those of their littermates. Scale bar = 1 mm.

to a concentration-dependent decline in limb score in RARα1γ$^{+/+}$ and RARα1γ$^{+/-}$ limbs (Fig. 2C). Furthermore, RARα1γ$^{-/-}$ limbs exposed to retinol had higher limb scores than dose-matched RARα1γ$^{+/+}$ and RARα1γ$^{+/-}$ control limbs. Thus, RARα1γ$^{-/-}$ limbs were less susceptible to retinol-induced limb dysmorphogenesis.

Effects of Retinol on the Expression of *Sox9* and *Col2a1* in the Absence of RARα1 and γ

One of the most striking changes in limb development evoked by retinoid excess was a substantial decrease in cartilage formation. To examine the possible mechanisms underlying this decrease in chondrogenesis, we investigated the expression profiles of a key regulator of chondrogenesis, *Sox9*, and a downstream chondrocyte marker, *Col2a1*, following ablation of RARα1 and -γ.

A significant reduction in *Sox9* and *Col2a1* expression was observed in RARα1γ limbs exposed to 12.5 μM retinol (Fig. 3A and B). In contrast, retinol treatment of RARα1γ$^{+/-}$ and RARα1γ$^{-/-}$ limbs did not result in any significant changes in gene expression, suggesting that both copies of RARγ may be needed for retinoid-mediated downregulation of *Sox9* and *Col2a1*.

Effects of Retinol on the Expression of *Meis* and *Pbx* in the Absence of RARα1 and γ

Because retinol-induced limb truncation was noticeably reduced in the proximal long-bone regions in RARα1γ$^{-/-}$ limbs (Fig. 1J and K), we investigated the effects of retinol on *Meis1* and *Meis2* expression in RARα1γ$^{-/-}$ limbs compared to controls. In RARα1γ$^{+/+}$ limbs, the expression of *Meis1* and *Meis2* increased following exposure to 12.5 μM retinol for 3 hr (Fig. 4A and B). Of interest, in the absence of RARγ this retinol-dependent increase in *Meis1* and *Meis2* expression was not observed, which suggests that RARγ is necessary for the induction of *Meis1* and *Meis2* by retinol. In RARα1γ$^{+/-}$ limbs, retinol induced an increase in *Meis2*, but not *Meis1*, expression compared to untreated RARα1γ$^{+/-}$ controls (Fig. 4A and B). Expression of the *Meis* binding partner, *Pbx1a*, was not altered by retinol exposure or genotype (Fig. 4C).

DISCUSSION

Retinoid receptors not only play crucial roles in the proper development of the embryo, they also mediate retinoid-induced teratogenic events. In this study we in-

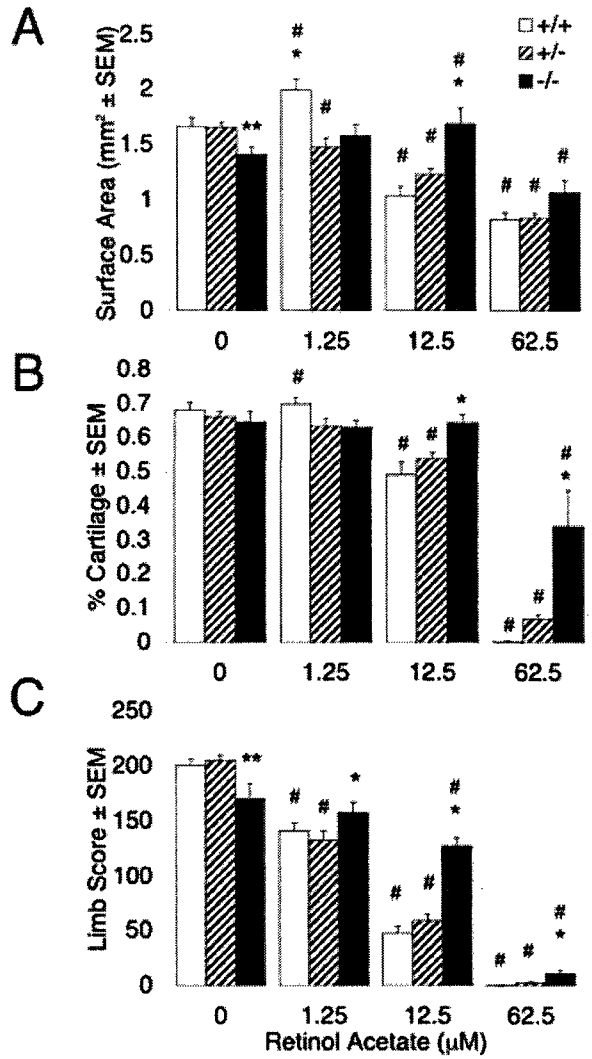

Figure 2. Effects of retinol on limb growth, chondrogenesis, and differentiation/patterning in vitro. **A:** Limb surface area, a surrogate marker for limb growth, was significantly decreased in untreated RARα1γ$^{-/-}$ limbs and significantly increased following exposure to 12.5 μM retinol compared to RARα1γ$^{+/+}$ and RARα1γ$^{+/-}$ controls. **B:** The percentage (%) of cartilage was quantified by calculating the ratio between the area of toluidine blue-stained cartilage and total surface area. Specific differences occurred in 12.5 μM and 62.5 μM retinol-treated limbs (i.e., RARα1γ$^{-/-}$ samples displayed greater cartilage formation compared to dose-matched genotypes). **C:** The limb morphogenetic differentiation score measured the amount and quality of the cartilage anlagen development. Limb scores were reduced in RARα1γ$^{-/-}$ limbs under control conditions compared to those of littermate controls, indicating impaired development in vitro. RARα1γ$^{-/-}$ limbs showed less disruption of development when treated with 1.25, 12.5, and 62.5 μM retinol, as indicated, compared to exposure-matched controls. Statistics were analyzed by 2-way ANOVA and the post-hoc Holm-Sidak multiple-comparison test (n = 10–35). *Significant increase, $P < .05$, vs. dose-matched genotypes; **significant decrease, $P < .05$, vs. dose-matched RARα1γ$^{+/+}$ genotype; #significantly different, $P < .01$, vs. genotype-matched controls.

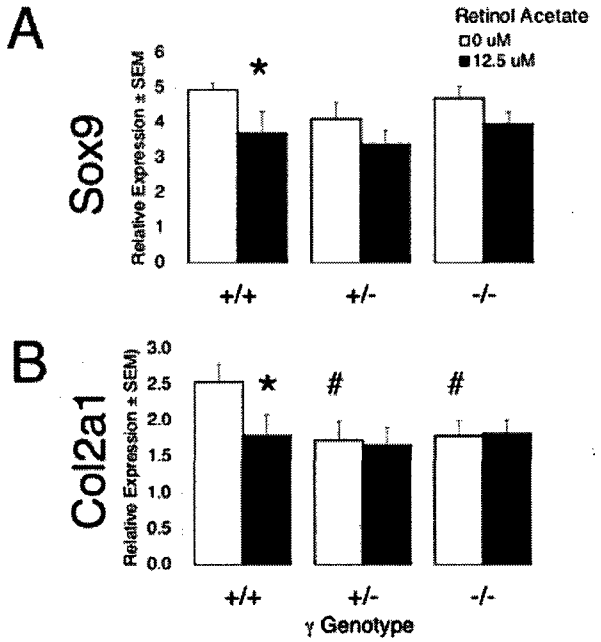

Figure 3. Effect of retinol treatment on the expression of chondrogenesis-related genes. Limbs were cultured in vitro for 3 hr in the absence or presence of 12.5 μM retinol. **A:** *Sox9* expression was reduced following retinol treatment in RARα1γ$^{+/+}$ limbs, but was not affected in RARα1γ$^{+/-}$ and RARα1γ$^{-/-}$ limbs. **B:** *Col2a1* gene expression paralleled *Sox9* expression, with marked effects elicited by retinol treatment in RARα1γ$^{+/+}$ but not RARα1γ$^{+/-}$ or RARα1γ$^{-/-}$ limbs. Each bar represents the mean gene expression of separately conducted limb cultures (n = 5) normalized against β-actin expression. Statistics were analyzed by 2-way ANOVA and the post-hoc Holm-Sidak multiple-comparison test. *Significant decrease, $P < .05$, vs. genotype-matched control limbs; #significant decrease, $P < .05$, vs. dose-matched RARα1γ$^{+/+}$ limbs.

vestigated the role of RARγ (in the absence of RARα1) in mediating retinol-induced limb teratogenesis. We demonstrated that in vitro cultured limbs from RARα1γ$^{+/+}$ and RARα1γ$^{+/-}$ mice develop normally and are highly susceptible to retinoid exposure. In contrast, there was a remarkable reduction in the sensitivity of RARα1γ$^{-/-}$ limbs to retinoid exposure. To determine an underlying molecular basis for this attenuated teratogenesis, we examined the expression profiles of genes required for proper limb development. In response to retinol exposure RARα1γ$^{-/-}$, RARα1γ$^{+/+}$, and RARα1γ$^{+/-}$ limbs exhibited different patterns of expression of genes involved in either chondrogenesis (*Sox9* and *Col2a1*) or limb outgrowth (*Meis1/2* and *Pbx1*).

Numerous studies have implicated retinoid signaling in the regulation of chondrogenesis. RAR activity has been closely linked to *Sox9* expression, and conditional null mutation studies have shown that Sox9 is essential for chondrogenesis within the limb (Foster et al., 1994; Weston et al., 2000, 2002; Akiyama et al., 2002). *Col2a1* is a gene known to be regulated by Sox9 (Bell et al., 1997). Following retinol exposure, similar decreases in both *Sox9* and *Col2a1* were observed in RARα1γ$^{+/+}$ limbs. The absence of changes in the expression of these genes in RARα1γ$^{+/-}$ and RARα1γ$^{-/-}$ limbs exposed to retinol suggests that

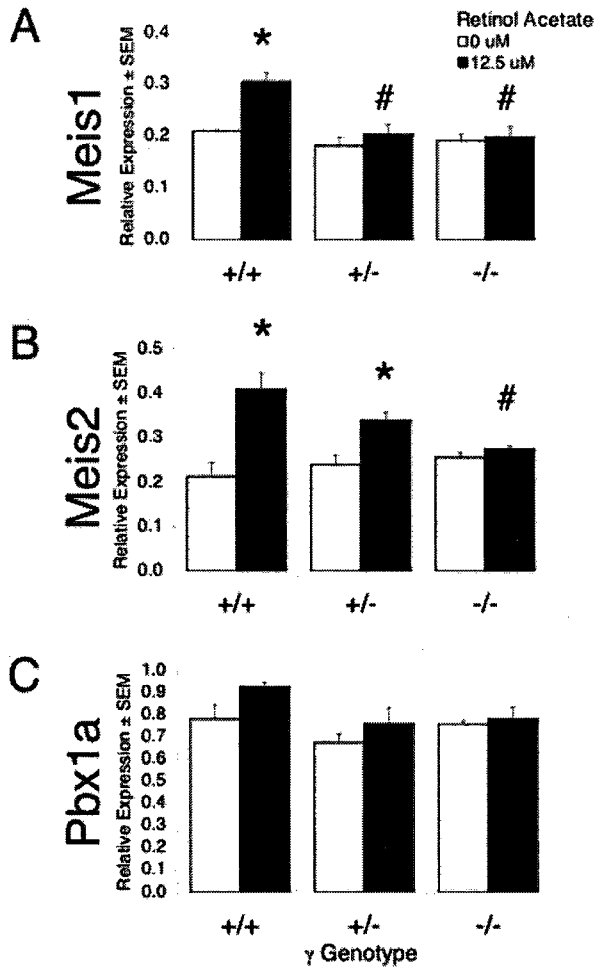

Figure 4. Changes in the expression of genes involved in proximodistal limb development following retinol treatment. Limbs were cultured in vitro for 3 hr in the absence or presence of 12.5 μM retinol and then RNA-extracted for qRT-PCR analysis. **A:** *Meis1* gene expression increased following retinol treatment in RARα1γ$^{+/+}$ limbs, but was unchanged in RARα1γ$^{+/-}$ and RARα1γ$^{-/-}$ limbs. **B:** *Meis2* gene expression increased following retinol treatment in both RARα1γ$^{+/+}$ and RARα1γ$^{+/-}$ limbs, but not in RARα1γ$^{-/-}$ limbs. **C:** *Pbx1a* gene expression was unaffected with respect to dose or genotype. Each bar represents the mean gene expression of separately conducted limb cultures (n = 5) normalized against β-actin expression. Statistics were analyzed by 2-way ANOVA and the post-hoc Holm-Sidak multiple-comparison test. *Significant increase, $P < .05$, vs. genotype-matched control limbs; #significant decrease, $P < .05$, vs. dose-matched RARα1γ$^{+/+}$ limbs.

RARγ has a role in regulating *Sox9* and *Col2a1* expression under the conditions used here.

The upregulation of *Meis* and *Pbx* members following in vivo treatment of GD11 pregnant female mice with 100 mg/kg of all-*trans* retinoic acid has been suggested to underlie retinoid-induced limb truncation (Qin et al., 2002). We observed similar increases in the expression of *Meis1* and *Meis2*, but not *Pbx1a*, in RARα1γ$^{+/+}$ limbs following retinol treatment. Our observation that retinol exposure did not alter *Meis1* or *Meis2* expression in RARα1γ$^{-/-}$ limbs, in which limb truncation was not severe, suggests that these genes may be downstream RARγ targets. Moreover, gene dosage is likely to be an important facet of this response, as both copies of RARγ were required for retinol regulation of *Meis1*. However, it is conceivable that the presence of only 1 copy of the RARγ gene may lead to a delay in, rather than an absence of, the regulation of downstream genes, since no noticeable resistance to retinoid teratogenicity was observed in RARα1γ$^{+/-}$ limbs in culture. Whereas the lack of a response of *Pbx1a* to retinol in RARα1γ$^{-/-}$ and control limbs conflicts with results of prior work by Qin et al. (2002), it is possible that significant change can be detected only at later time points because additional time may be needed for retinol acetate to be bioactivated (a step that the Qin group circumvented by treating pregnant mice with retinoic acid). RARα1 and γ are important factors in normal axial patterning. In 1 study the ablation of both RARα1 and γ synergistically increased the frequency and severity of axial defects inherent to RARγ null offspring (Lohnes et al., 1994). However, in in vivo studies the limbs of mice deficient in either RARα1 or RARγ developed normally and were sensitive to the teratogenic effects of excess retinoid exposure (Lohnes et al., 1994; Luo et al., 1996; Iulianella and Lohnes, 1997; Subbarayan et al., 1997), while in vitro the limbs of RARα1$^{-/-}$γ$^{-/-}$ mice appeared stunted in growth. RARα1γ$^{-/-}$ limbs may not be able to cope with the stress caused by limb excision or culture. In this regard, RARγ regulates at least 1 stress response gene, mitogen-activated protein kinase phosphatase 1 (*mkp-1*), which has a retinoid-dependent antiapoptotic effect following redox stress (Xu et al., 2002). Alternatively, the excised limbs of compound null mice may behave as retinoid-deficient tissues due to the lack retinoic acid generated by the lateral plate mesoderm and other tissues within the trunk. Previous work showed that targeted disruption of RARα and RARγ in F9 teratocarcinoma stem cells resulted in altered retinoic acid metabolism (Boylan et al., 1995), which suggests that the expression of enzymes required for the conversion of retinol into retinoic acid may depend on the presence of these RAR isoforms. This possibility is consistent with our observation of less-disrupted RARα1γ$^{-/-}$ limbs in the presence of low levels of retinol.

To date, only RXRα null mice have been associated with a resistance to retinoid-induced limb defects in vivo (Sucov et al., 1995). Here we demonstrate that RARγ, in the absence of RARα1, plays a role in transducing retinoid-induced limb dysmorphogenesis, and that these effects correlate with RARγ-dependent regulation of players in chondrogenesis (*Sox9* and *Col2a1*) and limb outgrowth (*Meis1* and *Meis2*).

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Retinoic Acid Receptor Gamma-Induced Misregulation of Chondrogenesis in the Murine Limb Bud *In Vitro*

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Vitamin A derivatives modulate gene expression through retinoic acid and rexinoid receptor (RAR/RXR) heterodimers and are indispensable for limb development. Of particular interest, RARγ is highly expressed in cartilage, a target affected following retinoid-induced limb insult. The goal of this study was to examine how selective activation of RARγ affects limb development. Forelimbs from E12.5 CD-1 mice were cultured for 6 days in the presence of all-*trans* RA (pan-RAR agonist; 0.1 or 1.0μM) or BMS-189961 (BMS961, RARγ-selective agonist; 0.01 or 0.1μM) and limb morphology assessed. Untreated limbs developed normal cartilage elements whereas pan-RAR or RARγ agonist-treated limbs exhibited reductive effects on chondrogenesis. Retinoid activity was assessed using RAREβ2 (retinoic acid response element β2)-lacZ reporter limbs; after 3 h of treatment, both drugs increased retinoid activity proximally. To elucidate the expression profiles of a subset of genes important for development, limbs were cultured for 3 h and cRNA hybridized to osteogenesis-focused microarrays. Two genes, matrix GLA protein (*Mgp*; chondrogenesis inhibitor) and growth differentiation factor-10 (*Gdf10*/*Bmp3b*) were induced by RA and BMS-189961. Real-time PCR was done to validate our results and whole mount *in situ* hybridizations against *Mgp* and *Gdf10* localized their upregulation to areas of cartilage and programmed cell death, respectively. Thus, our results illustrate the importance of RARγ in mediating the retinoid-induced upregulation of *Mgp* and *Gdf10*; determining their roles in chondrogenesis and cell death will help further unravel mechanisms underlying retinoid teratogenicity.

Key Words: **matrix GLA protein; growth differentiation factor 10; retinoic acid receptor gamma; retinoic acid; limb development; chondrogenesis.**

All-*trans* retinoic acid (RA), the most bioactive metabolite of vitamin A (retinol), is a potent morphogen that plays crucial roles during embryo development (Kochhar, 1967). Retinoid concentrations are tightly regulated during embryogenesis. Both deficiency (Hale, 1930) and excess can cause malformations of various embryonic structures in a time- and dose-dependent manner (Collins and Mao, 1999).

Limb development is an orchestrated process; a multitude of signals converge that specify and sculpt the heterogeneous (i.e., bone, muscle, skin) limb structure. Retinoids actively regulate various signaling pathways during normal limb morphogenesis, and it is no surprise that retinoid imbalances alter such developmentally important processes (Romand *et al.*, 2004). Additionally, the molecular mechanisms responsible for patterning the limb bear striking similarities to various other organ systems, making the limb an excellent model to help decipher how teratogens illicit their detrimental effects on organogenesis (Yamada, 2005).

As the limb develops, mesenchymal cells are recruited, migrate, and condense, initiating chondrogenesis, the earliest phase in skeletal development. These mesenchymal condensations differentiate and give rise to cartilaginous anlagen that eventually yield the ossified elements that comprise the skeleton (Olsen *et al.*, 2000). Previous studies have demonstrated that excess retinoid disrupts chondrogenesis (Kochhar, 1973; Kwasigroch and Kochhar, 1980). Although these effects on cartilage formation have been attributed to aberrant proliferation, differentiation and apoptosis (Ali-Khan and Hales, 2006; Zakeri and Ahuja, 1994), many of the genes and their underlying downstream mechanisms that regulate the retinoid-induced reduction in chondrogenesis are still unresolved.

Retinoids elicit their biological effects by regulating gene transcription. In the presence of ligand, retinoic acid:rexinoid nuclear receptor (RAR:RXR) heterodimers bind to retinoic acid response elements (RARE) and recruit and activate transcriptional machinery (Bastien and Rochette-Egly, 2004). Various RAR and RXR isotypes (and variant isoforms) have been identified (α, β, γ) yet only pharmacological activation of RARs, and not RXRs, causes teratogenic effects, illustrating the importance of RARs in mediating the teratogenic signal in the developing limb (Arafa *et al.*, 2000). Expression of RARs in the limb is temporally and spatially dynamic. During limb outgrowth, although RARα is expressed ubiquitously and RARβ is restricted to the webbing, the cartilage tissue which is highly sensitive to retinoid insult, expresses RARγ (Dolle *et al.*, 1989).

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Single RAR isoform ablation studies have uncovered the importance of RARγ in controlling axial development and patterning, but receptor redundancy has been linked to the lack of effect seen in the appendicular skeletons of RARγ-deficient mice (Chambon, 1994; Lohnes *et al.*, 1995). Notwithstanding, previous work in our lab has shown that limbs deficient in RARγ (on an α1-null background) were less susceptible to retinoid-induced limb dysmorphogenesis. Chondrogenesis and overall limb growth in these transgenic mice were vastly improved following retinoid insult, when compared with their retinoid-treated wild-type counterparts, indicating the importance of RARγ in mediating limb teratogenesis (Galdones *et al.*, 2006).

Contrary to RARγ ablation, the objective of the present study was to determine the outcomes of aberrant RARγ activation on limb morphogenesis. To do so, an *in vitro* limb bud culture system was employed (Kwasigroch and Skalko, 1983) and aberrant RARγ activation was induced pharmacologically using BMS-189961 (BMS961), a highly RARγ-selective agonist (Klaholz *et al.*, 2000). Given the predominantly high expression of RARγ in cartilage during limb development, we hypothesized that retinoid-induced limb dysmorphogenesis is mediated by a RARγ-dependent misregulation of genes important for proper chondrogenesis.

From a pharmacological standpoint, RARγ is an extensively pursued drug target for the treatment of several medical conditions including polycystic acne, psoriasis, diabetes, and various cancers (Johnson and Chandraratna, 1999). Unfortunately, such treatments are contraindicated for women of childbearing age. Understanding the modes of RARγ-mediated toxicity during development will effectively aid in the development of safer, less teratogenic retinoid analogs.

In this study, we demonstrate the teratogenic action of a RARγ-selective agonist on the developing limb. Retinoid-responsive reporter mice (RARβ2-lacZ) were employed to assess the location and extent of retinoid activity following activation of RARγ. Gene expression analysis using chondrogenesis-focused microarrays has identified two RARγ-responsive genes (*Mgp* [matrix GLA protein] and *Gdf10* [growth differentiation factor-10]) that may have novel uncharacterized roles in transducing the teratogenic signal in the limb. Furthermore, *in situ* hybridization (ISH) has localized *Mgp* to areas of developing cartilage and *Gdf10* to areas of programmed cell death in the limb, suggesting independent functions for each of these candidate genes.

MATERIALS AND METHODS

Limb bud culture and drug treatments. Pregnant gestational day (GD) 12 CD-1 mice were euthanized, embryos were explanted and forelimbs were excised just lateral to the somites and cultured *in vitro* in a chemically defined culture medium, as previously described (Ali-Khan and Hales, 2003). All-*trans* RA (Sigma, St Louis, MI) and BMS-189961, a RARγ-selective agonist (BMS961; a gift from Bristol-Myers Squibb, Wallingford, CT), were dissolved in 100% EtOH. Limbs were exposed to low and high concentrations of RA (0.1 and 1.0μM) or BMS961 (0.01 and 0.1μM) at the onset of culture. All animal studies complied with the guidelines established by the Canadian Council on Animal Care.

Limb morphology. Limbs were cultured for 6 days, with one change of the medium that was not supplemented with RA or BMS961, on day 3, as previously described (Galdones *et al.*, 2006). Briefly, limbs were fixed and stained with 0.1% toluidine blue (Fisher Scientific, Montreal, Canada) and examined under a dissecting microscope (Wild Heerbrugg 99067; Wild Leitz, Ottawa, Canada) and photographs acquired with a JVC digital camera (JVC GCQX3HD, Tokyo, Japan). The extent of proper limb development was quantified using a limb morphogenetic scoring system (Neubert and Barrach, 1977). Five separate replicates were completed and examined for morphological changes or markers.

RAREβ2-hsp68lacZ reporter mice and staining. To examine the extent and localization of retinoid activity, RAREβ2-hsp68*lacZ* double-transgenic males were mated with wild-type CD-1 females to produce single copy transgene offspring. These embryos were explanted at E12.5 and limbs cultured for 3 and 12 h as described above, in the absence or presence of RA or BMS961. Tissues were fixed immediately with 0.25% glutaraldehyde then stained with X-gal for 2–4 h, as previously described (Rossant *et al.*, 1991). Limbs were rinsed in phosphate-buffered saline (PBS) then visualized under a dissecting microscope. Results were acquired from three independent limb culture experiments.

RNA extraction. After 3 h *in vitro*, each group of six to eight forelimbs was removed from culture, rinsed with PBS and stored at −80°C in RNAlater RNA Stabilization Reagent (Qiagen, Mississauga, Canada) until further processing. Total RNA was extracted using the RNeasy Micro Kit (Qiagen) and quality and purity/quantity assessed by agarose gel electrophoresis and spectrophotometric ultraviolet analysis in 10mM Tris, pH 8.0 buffer, respectively. Five separate independent cultures of each treatment group were completed and used for microarray and quantitative RT-PCR analysis.

cRNA synthesis and hybridization. Starting with 2 μg of total mRNA, the TrueLabeling-AMP 2.0 Kit (Superarray Bioscience, Frederick, MD) was used to produce, amplify and biotinylate antisense cRNA for hybridization following the manufacturer's guidelines. The cRNA product was purified with ArrayGrade cRNA Cleanup (SuperArray Bioscience) and then hybridized overnight to Mouse Oligo GEArray Osteogenesis Microarrays (OMM-026, SuperArray Bioscience), using 3 μg of cRNA per array and following the manufacturer's suggested HybTube Standard Protocol. The following day, the membranes were stained with an alkaline-phosphatase-conjugated streptavidin antibody (1:8000, SuperArray Bioscience) and the chemiluminescence signal was developed using the CDP-Star Kit (SuperArray Bioscience). Five arrays, each from independent limb cultures, were completed for each treatment group.

Data acquisition and gene expression analysis. The array images were recorded using X-ray film, digitized using a desktop scanner, saved as 16-bit TIFF files and uploaded onto GEArray Expression Analysis Suite (GEASuite, SuperArray Bioscience). Array alignment and raw intensity quantification were done using GEASuite, then the data were imported into Agilent GeneSpring 7.3 GX (Agilent Technologies, Palo Alto, CA) where background correction and all further analysis was completed. For each individual array, the gene detection threshold was set at a raw signal intensity of 2× the background intensity; to minimize array-to-array variation, expression values were normalized to the median of all measurements on that individual array. Upon identification of the uniquely and commonly expressed genes in each group, statistical comparisons between control and RA or BMS961 treated groups were done using Student's *t*-tests ($p \leq 0.05$). Genes were only included in the significantly regulated group of genes when significance was detected in at least three out of five replicates, and the changes were all in the same direction (i.e., either all up- or all downregulated).

Quantitative real-time PCR. RNA was diluted to a final working solution of 10 ng/μl and Quantitect One-Step SYBR Green RT-PCR (Qiagen) was done

TABLE 1
Primer Sequences Used for Quantitative RT-PCR

Name	Symbol	Genbank no.	Left primer	Right primer
18S RNA	*Rn18s*	X00686	AAA CGG CTA CCA CAT CCA AG	CCT CCA ATG GAT CCT CGT TA
Matrix GLA protein	*Mgp*	NM_008597	GCG AAG AAA CAG TCA TTT GGT	TCA ACC CGC AGA AGG AAG
Growth differentiation factor 10	*Gdf10*	NM_145741	ATG CCC AGA ATT TCC ACA AG	AAG TCC AGC ACC TGA GAG GA
Cathepsin K	*Ctsk*	NM_007802	GAA CGA GAA AGC CCT GAA GA	CAC ACC TCT GCT GTA AAA CTG G
Decorin	*Dcn*	NM_007833	GTC TGG CCA ATG TTC CTC AT	AGG TCA TTT TGC CCA ACT GC

using the Roche LightCycler (Roche Diagnostics, Laval, Canada). The quantitative real-time PCR (qRT-PCR) cycling parameters were as follows: 95°C for 15 min (one cycle) and then 94°C for 15 s, 55°C for 30 s, and 72°C for 20 s (for 50 cycles) and each sample was measured in duplicate. Embryonic head tissue was also collected, and RNA extracted to provide 1, 10, and 50 ng/μl RNA stocks used to generate standard curves for quantification. The primers were generated with Primer3 software (http://frodo.wi.mit.edu) and produced by alpha DNA (Montreal, Canada; see Table 1 for sequence information). To normalize the output, the expression of each gene of interest was divided by 18S gene expression, a commonly used housekeeping gene whose expression remains unchanged in murine limbs following retinoid exposure (Ali-Khan and Hales, 2006). Melting-curve analyses were done following each PCR to determine the output and detection quality (i.e., the formation of primer-dimers). Each treatment consisted of RNA from four separate culture experiments and each sample was measured in duplicate.

Whole mount ISH. Limbs cultured for 3, 6, or 12 h were rinsed with PBS containing 0.1% Tween 20 (PBT) and fixed overnight at 4°C in 4% paraformaldehyde. The following day, the limbs were dehydrated through a methanol/PBT gradient and stored at −20°C until further use. For whole mount ISH, a DIG RNA labeling kit (Roche Diagnostics) was used to synthesize *Mgp* and *Gdf10* antisense probes from plasmids kindly provided by G. Karsenty (Columbia University, New York, NY) and S.-J. Lee (Johns Hopkins University, Baltimore, MD), respectively. Whole mount ISH was done as previously described (Decimo *et al.*, 1995). After hybridization, limbs were rinsed in PBT and photographed under a dissecting stereomicroscope (Wild Leitz). Three separate whole mount ISH experiments were done for each gene of interest.

RESULTS

Aberrant Activation of RARγ is Teratogenic to the Developing Limb

Because retinoid insult in the limb is attenuated in the absence of RARγ (Galdones *et al.*, 2006), our first goal was to determine if and how aberrant RARγ activation affects limb development. *In vitro* cultured murine limbs were treated with either RA (pan-RAR agonist) or BMS961 (RARγ-selective agonist) at E12.5. The treatment of limbs with either drug resulted in dose-dependent detrimental effects on gross morphology after culture for 6 days, with marked effects on chondrogenesis, as evidenced by decreases in toluidine blue stained cartilage (Fig. 1A). After exposure to low concentrations of RA or BMS961 (RA: 0.1μM, BMS961: 0.01μM), the zeugopod (long bones) appeared growth retarded, whereas the autopod (paw) remained intact. However, at high exposures (RA: 1.0μM, BMS961: 0.1μM) both limb regions were equally undifferentiated.

A limb scoring system was used to determine the quality and extent of morphogenetic differentiation of the cartilage elements that comprise the forming limb after exposure to the pan- or RARγ-selective agonist (Neubert and Barrach, 1977). In accordance with the gross morphology, limb scores were compromised following treatment with either the pan- or RARγ-selective agonist (Fig. 1B).

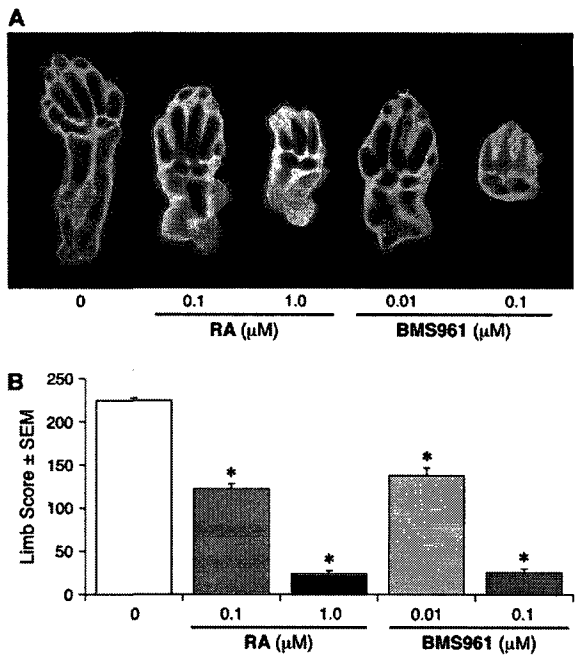

FIG. 1. The effects of pan-RAR and RARγ-selective activation on limbs following 6 days in culture. Limbs were collected from CD-1 mice at embryonic day 12.5 and cultured *in vitro* for 6 days in the presence or absence of pan-RAR agonist, all-*trans* RA (0.1, 1.0μM) or RARγ-selective agonist, BMS961 (0.01, 0.1μM). (A) Limbs were stained with 0.1% toluidine blue (wt/vol 70% ethanol). Both RA and BMS961 treatment result in marked effects on chondrogenesis, namely long bone outgrowth and digit formation. (B) The limb morphogenetic scores measured the extent of differentiation and chondrogenesis following treatment.

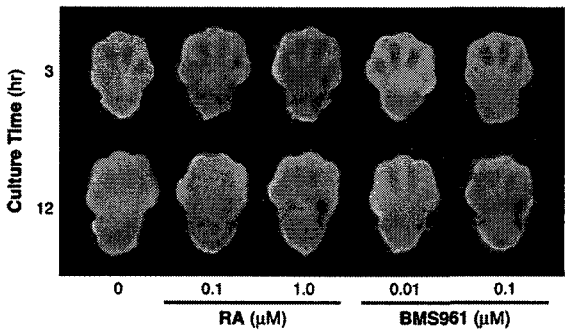

FIG. 2. Localization of RAR activity, as measured using the RAREβ2-hsp68*lacZ* retinoic-responsive reporter mice.

RARγ-Selective Agonism Induces Retinoid Activity in RAREβ2-hsp68lacZ Reporter Limbs

To determine the amount and distribution of retinoid activity in limbs following RA or BMS961 exposure, we treated transgenic limbs from a retinoid reporter strain containing a transgene under the control of the retinoid sensitive RARβ2 response element (Rossant *et al.*, 1991). In the absence of retinoid, the *lacZ* transgene was strongly expressed in the interdigital zones and observable along the axes of the forming long bones after 3 h *in vitro* (Fig. 2). With the addition of 0.1 or 1.0μM RA for 3 h, transgene expression was strongly upregulated in the proximal mesenchyme, necrotic zones, dorsal ectoderm, and interdigital webbing. At 3 h, BMS961 treatment (0.01 or 0.1μM) increased transgene expression in the proximal mesenchyme as well, but only in a modest fashion; transgene expression in the webbing was affected similarly in BMS961 and RA-treated limbs.

After 12 h *in vitro*, vehicle-treated limbs exhibited minimal transgene expression. In contrast, both pan-RAR and RARγ-selective activation induced restricted transgene expression in the zone of polarizing activity, the limb signaling center responsible for anterior-posterior patterning (Tickle, 2006). Additionally, although RA did not induce transgene expression in the interdigital zones at this later time point, BMS961 exposure resulted in a faint induction of staining in the webbing. Thus, because maximal effects on *lacZ* transgene expression were observed after 3 h *in vitro*, subsequent experiments were done at that early time point.

Changes in Gene Expression Induced by RA or BMS961 Treatment

It is clear that retinoids have detrimental effects on chondrogenesis (Jiang *et al.*, 1995) yet the various mechanisms underlying this process are still unresolved. We adopted a gene array approach to assess how the regulation of chondrogenesis-related genes in the limb was affected following pan-RAR or RARγ-selective insult. Limbs were treated for 3 h with either RA or BMS961 and RNA was hybridized to Mouse Oligo

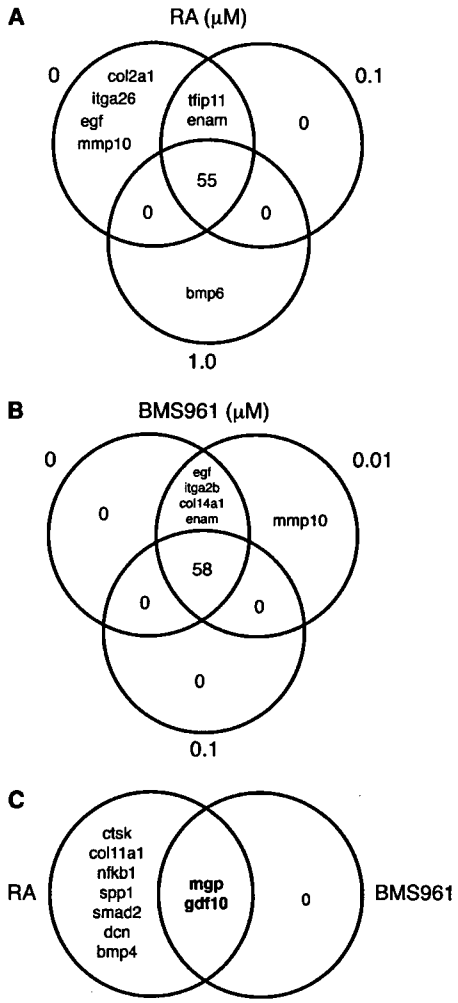

FIG. 3. Venn diagrams of gene expression. (A) The numbers of genes that were uniquely and commonly expressed following exposure to 0, 0.1, or 1.0μM RA for 3 h. (B) The numbers of genes that were uniquely and commonly expressed following exposure to 0, 0.01, or 0.1μM BMS961 for 3 h. (C) Genes that were significantly up- or downregulated when comparing control-treated limbs to those treated with either low and high RA and/or BMS961 treatment.

Osteogenesis Microarrays from GEArray containing approximately 100 genes important in various aspects of cartilage and bone development.

In comparing the vehicle-treated limbs to those treated with 0.1 or 1.0μM RA, 55 genes were commonly expressed in all groups (Supplementary Table 1). Four genes were expressed uniquely under control conditions, whereas one gene was expressed only after exposure to 1.0μM RA (Fig. 3A). Only two genes were expressed in two out of three treatment groups (0 and 0.1μM; genes listed in Fig. 3A). Limbs treated with BMS961 exhibited a similar outcome, with the majority of

TABLE 2
List of Genes Significantly Up- or Downregulated following RA or BMS961 Treatment

Gene	Symbol	Genbank	Relative intensity 0μM	Relative intensity 0.1μM	Direction	p
0 versus 0.1μM RA						
Matrix GLA protein	*Mgp*	NM_008597	1.84	2.52	↑	0.049
Growth differentiation factor 10	*Gdf10*	NM_145741	1.43	2.01	↑	0.009

Gene	Symbol	Genbank	Relative intensity 0μM	Relative intensity 0.1μM	Direction	p
0 versus 1.0μM RA						
Cathepsin K	*Ctsk*	NM_007802	1.18	0.90	↓	0.046
Type XI (1a) collagen	*Coll11a1*	NM_007729	1.41	1.01	↓	0.042
Nuclear factor of kappa light chain gene enhancer in b cells 1	*Nfkb1*	NM_008689	1.62	1.41	↓	0.039
Secreted phosphoprotein 1	*Spp1*	NM_009263	0.88	1.11	↑	0.022
Growth differentiation factor 10	*Gdf10*	NM_145741	1.43	2.08	↑	0.015
MAD homolog 2	*Smad2*	NM_010754	1.42	1.24	↓	0.007
Decorin	*Dcn*	NM_007833	1.68	1.13	↓	0.005

Gene	Symbol	Genbank	Relative intensity 0μM	Relative intensity 0.1μM	Direction	p
0.1 versus 1.0μM RA						
Bone morphogenetic protein 4	*Bmp4*	NM_007554	1.07	1.30	↑	0.043
Decorin	*Dcn*	NM_007833	1.69	1.13	↓	0.014

Gene	Symbol	Genbank	Relative intensity 0μM	Relative intensity 0.1μM	Direction	p
0 versus 0.01μM BMS961						
Matrix GLA protein	*Mgp*	NM_008597	1.84	2.33	↑	0.008

Gene	Symbol	Genbank	Relative intensity 0μM	Relative intensity 0.1μM	Direction	p
0 versus 0.1μM BMS961						
Growth differentiation factor 10	*Gdf10*	NM_145741	1.43	1.80	↑	0.023

genes commonly expressed between all groups (58; Supplementary Table 2) although one gene was expressed solely after exposure to low concentrations; four genes were expressed in both control and 0.01μM BMS961-treated limbs, but not in 0.1μM exposed limbs (genes listed in Fig. 3B). All uniquely expressed genes were only expressed at minimal baseline levels, therefore our analysis was limited to the groups of commonly expressed genes.

Activation of RARγ is Implicated in the Misregulation of Mgp *and Gdf10 Gene Expression*

To identify genes important in mediating retinoid-induced limb teratogenesis, the lists of commonly expressed genes in all RA or BMS961 treatment groups were analyzed for statistical significance ($p < 0.05$) using Agilent GeneSpring 7.3 GX software. Comparison between vehicle and the two RA-treated groups identified nine genes that were significantly up- or downregulated by treatment (Table 2). Our work identified a subset of genes that were significantly changed (either up- or downregulated) following RA exposure (Table 2). Several of the identified genes are related to bone morphogenetic protein (BMP)/transforming growth factor beta (TGF-β) signaling (*Smad2*, *Bmp4*, and *Gdf10*) and components of cartilage and extracellular matrix (*Coll1a1*, *Spp1*, and *Dcn*), whereas others are linked to matrix breakdown (*Ctsk*), oxidative stress (*Nfkb1*), and regulation of chondrogenesis (*Mgp*).

When comparing the control versus the two BMS961-treated groups, only two genes were identified as being upregulated by RARγ activation (Table 2). The lists of significantly altered genes were compared; both of the genes altered by RARγ-selective activation were also misregulated by RA exposure (Fig. 3C). These genes were *Mgp*, a potent inhibitor of chondrogenesis and osteogenesis (Price and Williamson, 1985)

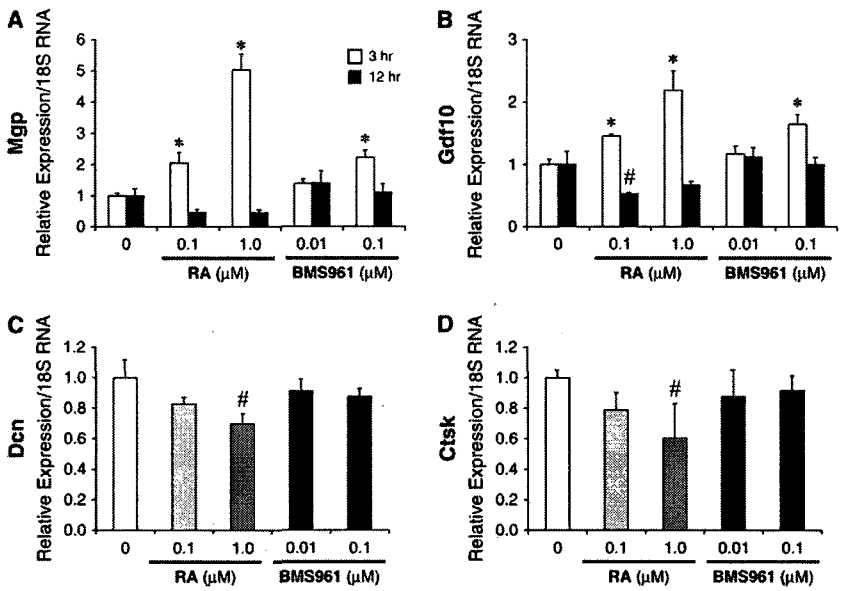

FIG. 4. qRT-PCR validation of the changes in expression of genes responsive to pan-RAR and RARγ-selective activation. Limbs were cultured *in vitro* for 3 (*open bars*) or 12 (*closed bars*) h in the absence or presence of RA (0.1, 1.0μM) or BMS961 (0.01, 0.1μM). (A) *Mgp* gene expression is upregulated by RA and BMS961 treatment after 3 h in culture but returns to baseline expression levels by 12 h. (B) *Gdf10* expression is upregulated in response to RA and BMS961 treatment following 3 h, but not 12 h *in vitro*. (C) *Dcn* and (D) *Ctsk* are both downregulated by RA but not BMS961 after 3 h in culture. Each bar represents the mean gene expression of four separately conducted limb cultures normalized against 18S mRNA expression. Statistical analysis was done using two-way ANOVA and the Holm-Sidak *post hoc* multiple-comparison test. *Significant increase, $p < 0.05$; #significant decrease, $p < 0.05$.

and *Gdf10*, a bone morphogenetic protein of uncharacterized function in the developing limb (Cunningham *et al.*, 1995).

Array Verification by qRT-PCR

We used qRT-PCR to assess the gene expression profiles of the two RARγ-responsive genes, *Mgp* and *Gdf10*. After culture for 3 h, the expression of both *Mgp* and *Gdf10* was significantly upregulated by exposure to low or high concentrations of RA as well as the high concentration of BMS961; the expression of these genes was not responsive to the low concentration of BMS961 (Figs. 4A and 4B). Interestingly, after 12 h in culture, the expression of both genes did not differ from control in the RA or BMS961 exposed limbs, illustrating that the effect of the retinoids on the expression of these genes in the cultured limbs is transient; indeed, the expression of *Gdf10* was significantly downregulated in 0.1μM RA-treated limbs at 12 h.

In addition, to further validate the results from the array experiments, we selected two genes that were pan-RAR, but not RARγ-responsive. *Dcn* (decorin), a proteoglycan associaed with collagen (Scott and Orford, 1981) and *ctsk* (cathepsin K), a cysteine protease expressed in bone (Garnero *et al.*, 1998) were both downregulated by exposure to 1.0μM RA for 3 h (Figs. 4C and 4D). As anticipated from the array data, exposure to either 0.1μM RA or BMS961 had no effect on the expression of these genes in limbs for 3 h *in vitro*.

Distribution of Mgp *and Gdf-10* Gene Transcripts Following pan-RAR and RARγ-*Selective Activation*

Whole mount ISHs were done to determine the distribution of *Mgp* and *Gdf10*, our gene transcripts misregulated by RARγ activation. Vehicle-treated limbs exhibited very little *Mgp* staining at 3 h; *Mgp* expression was induced by RA in a concentration-dependent manner. The induction of *Mgp* expression was restricted to the forming long bones at low RA concentrations and was extended distally into the rays at high RA levels. *Mgp* expression was upregulated strongly by BMS961 in the forming long bones, as well as the proximal mesenchyme, but was not induced in the digits by exposure to either concentration of BMS961 (Fig. 5A). At 6 h, *Mgp* transcripts were detected in the long bones in vehicle exposed limbs; exposure to either RA concentration further upregulated *Mgp* expression at this time point, whereas BMS961 did not. Interestingly, by 12 h *in vitro*, little *Mgp* expression was found in any treatment group, echoing the results seen by qRT-PCR where a transient decrease in expression was observed (Fig. 4A).

Localization of the expression of *Gdf10* was distinct from that of *Mgp*. G*df10* expression was faintly observable in the interdigital webbing under control conditions at 3 h (Fig. 5B). Ectopic expression was induced by exposure to either the low or high concentration of RA and shown to be highly localized to the interdigital webbing as well as the anterior and posterior necrotic

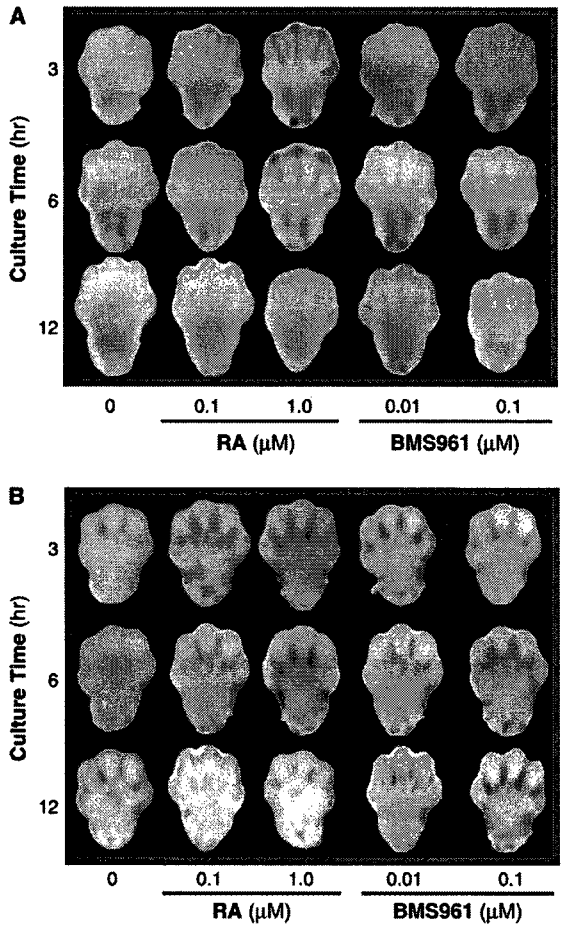

FIG. 5. Localization of RARγ-responsive gene transcripts following pan-RAR or RARγ-selective activation. Whole mount ISH of (A) *Mgp* and (B) *Gdf10* in E12.5 limbs following exposure to RA or BMS961 for 3, 6, or 12 h *in vitro*.

zones, all areas that are predestined to apoptose and help sculpt the developing limb. In addition, high concentrations of RA induced *Gdf10* transcripts in the proximal regions of the forming digits. BMS961 treatment resulted in similar expression patterns, albeit not as intense as their RA-treated counterparts. At 6 h, when control limbs expressed very little *Gdf10*, RA and BMS961 induced ectopic expression in the regions of programmed cell death and proximal rays. By 12 h *in vitro*, in RA-exposed limbs only faint staining was observable in the digits; however, BMS961-treated limbs still expressed low levels of *Gdf10* in the webbing, necrotic zones and proximal rays (Fig. 5B).

DISCUSSION

Although the effects of excess retinoids on development have been described, the mechanisms by which normal limb chondrogenesis and patterning are disrupted, and specifically the roles of distinct RARs, are not completely understood. Given that RARγ is highly expressed in the forming cartilage (Dolle *et al.*, 1989), we hypothesized that RARγ mediates retinoid-induced limb dysmorphogenesis. Specifically, the goal of this study was to examine how pharmacological activation of RARγ affects limb chondrogenesis.

Our results show that treatment of limbs with either RA (pan-RAR agonist) or BMS961 (RARγ-selective agonist) *in vitro* is detrimental to limb chondrogenesis and growth. Microarray-based expression analysis of chondrogenesis-related genes identified a group of RA-responsive genes and two RARγ-responsive genes (*Mgp* and *Gdf10*). The localization of upregulated *Mgp* transcripts to chondrocytes, and those of *Gdf10* to interdigital webbing/necrotic zones, suggests that they play novel roles during retinoid-induced limb dysmorphogenesis; namely the misregulation of chondrogenesis and programmed cell death, respectively.

RARγ-Induced Limb Defects

Both pan-RAR and RARγ-selective activation are detrimental to limb morphogenesis, significantly affecting the proper chondrogenesis of long bones, carpalia, and digits (Fig. 1). Specifically, although RA-treated limbs exhibited marked reductive effects on all cartilage elements at 0.1 and 1.0μM RA concentrations, BMS961 illicited phenotypically similar effects at concentrations that were 10-fold less (0.01 and 0.1μM). Although there is evidence that BMS961 can exhibit weak affinity for RARβ, the effective concentration (EC_{50}) at which BMS961 binds RARβ is threefold higher than our highest treatment (Klaholz *et al.*, 2000), suggesting that BMS961 is indeed acting as a RARγ-selective agonist in our limb bud cultures.

Retinoid Activity

The pattern of retinoid-induced lacZ upregulation in areas of endogenous RARβ expression has been observed previously (Rossant *et al.*, 1991). Given that transgene expression is driven by three copies of the highly retinoid-responsive RARE of RARβ2 (RAREβ2), the specific sequence of RARβ2 may not have a strong binding affinity for RARγ. Indeed, RARγ-associated retinoid activity is most likely higher than observed, because RARγ-selective activation induced gene expression changes in areas (i.e., chondrocytes) where no lacZ transgene expression was detected (Fig. 5A).

Pan-RAR Responsive Genes

Several groups, including our own, have studied global gene expression changes in the limb following retinoid excess (Ali-Khan and Hales, 2006; Qin *et al.*, 2002). To refine our search, and because chondrogenesis is severely affected by exogenous retinoid exposure, we assessed the expression changes of genes known to participate in aspects of cartilage and bone development.

Interestingly, the direction in which the genes were regulated was mixed; this is contrary to previous work illustrating the positive unidirectionally of the gene changes following exogenous retinol (vitamin A) exposure (Ali-Khan and Hales, 2006), a discrepancy that may be explained by the kinetics of the drug used. The immediate bioactivity of RA treatment leads to direct effects on their respective targets whereas retinol must be activated into RA, thus it would be of particular interest to assess retinol-induced gene changes following time points later than 3 h *in vitro*.

Classically, in the absence of ligand, RARs remain associated with RAREs and transcriptionally repress target genes; however, once a ligand is bound, the RARs change conformation, release their associated corepressors and in turn recruit coactivators and the transcriptional machinery that drive gene expression (Weston *et al.*, 2003). In our study, although several genes were upregulated by RA treatment (*Mgp*, *Gdf10*, *Spp1*, and *Bmp4*), others (*Ctsk*, *Col11a1*, *Nfkb1*, *Smad2*, and *Dcn*) were significantly downregulated. How this downregulation occurs is not well understood, yet at least two explanations exist: (1) RA may upregulate an intermediate gene upstream, which in turn inhibits the transcription of the observed target gene, or (2) the liganded RAR/RXR heterodimers may bind negative response elements (NRE) that repress rather than activate transcription. These NREs have been identified upstream of several retinoid-responsive genes, including *Mgp* (Kirfel *et al.*, 1997). However, because we have observed an upregulation of *Mgp* in our system, the activity of the NRE must be cell and tissue dependent.

We propose that these RA-responsive genes may play important novel roles in mediating retinoid-induced limb dysmorphogenesis. Because the scope of this study was focused on the RARγ-mediated regulation of gene expression, future studies will be required to determine how the misregulation of these newly identified RA-responsive genes affects limb morphogenesis.

RARγ Responsive Genes

In contrast to limbs treated with RA, BMS961-treated limbs exhibited a significant upregulation of only two genes: *Mgp* and *Gdf10* (Fig. 3C). Whole mount ISHs were performed to determine the extent and localization of gene upregulation; *Mgp* was expressed and significantly upregulated in chondrocytes (Fig. 5A). Of interest, *Gdf10* upregulation was limited to the interdigital webbing and the necrotic zones (Fig. 5B). These differential locations of expression suggest that each of these genes performs unique functions following retinoid-induced misregulation.

Inhibition of Chondrogenesis by Mgp

MGP, a γ-carboxyglutamic acid-rich, vitamin K–dependent protein (Price and Williamson, 1985), has been characterized as a potent inhibitor of chondrocyte maturation and mineralization (Yagami *et al.*, 1999). Mice lacking *Mgp* are viable, but exhibit spontaneous calcification of arteries and cartilage (Luo *et al.*, 1997). Our array experiments and follow-up qRT-PCR analysis show that *Mgp* expression is significantly upregulated by RA and BMS961. These results are consistent with those of Cancela and Price (1992) who identified a putative RARE upstream of *Mgp* bearing a striking resemblance to the RAREβ2 (de The *et al.*, 1990) and showed the RA-dependent upregulation of *Mgp* in various human tissues, including chondrocytes (Cancela and Price, 1992). However, although the RARγ-selective agonist did significantly upregulate *Mgp*, it did not do so to the same extent as RA (Fig. 3A), suggesting that other RARs (α and β) may participate in its complete transcriptional regulation.

Although *Mgp* transcripts were not detected by whole mount ISH in untreated limbs, RA, and BMS961-treated limbs aberrantly expressed *Mgp* in areas of chondrocyte development. Because chondrogenesis is a major target in retinoid-induced limb dysmorphogenesis, the distribution of *Mgp* transcripts may correlate with the reductive effects associated with retinoid insult. This is the first report implicating *Mgp* in a teratological context. MGP has been identified as playing a role as a functional inhibitor of BMP-2 and BMP-4 function (Yao *et al.*, 2007). Given that regulated BMPs are required for proper limb development, further work is needed to determine whether *Mgp* is indispensable for mediating specific retinoid-induced aberrations during development.

Gdf10 *and Programmed Cell Death*

GDF10 is a member of the TGFβ superfamily and is highly related to BMP-3 (Cunningham *et al.*, 1995), hence *Gdf10* is also commonly referred to as BMP-3b. Expression of *Gdf10* during organogenesis is highly localized in uterus, adipose and brain and less so in bone; ablation of *Gdf10* resulted in no apparent effects on developmental outcome (Zhao *et al.*, 1999). Many TGF-β superfamily members have been linked to various aspects of limb development: BMP-2, -4, and -7, the most highly characterized, play crucial roles in precartilaginous and chondrocyte differentiation, apical ectodermal ridge-dependent limb outgrowth, anterior-posterior patterning and interdigital cell death (Kawakami *et al.*, 1996), whereas TGF-β and GDF5 have been implicated in joint development (Storm and Kingsley, 1999). *Gdf10* function in the limb has not been previously characterized (Zhao *et al.*, 1999). Experiments targeting osteoblast function *in vitro* have recently revealed that GDF10 can inhibit BMP-2–dependent alkaline phosphatase activity, hence illustrating the negative regulation of Gdf10 on osteogenesis (Hino *et al.*, 1999). We are the first to show that *Gdf10* can be transcriptionally regulated by retinoids and that its upregulation is localized specifically to domains of programmed cell death in the limb, namely the webbing and necrotic zones (Fig. 5B). We postulate that *Gdf10* plays an integral role in regulating the apoptosis associated with retinoid teratogenicity (Ali-Khan and Hales, 2003).

SUMMARY

These results show the importance of RARγ agonists in mediating retinoid-induced limb dysmorphogenesis. Our chondrogenesis-focused gene expression analysis has identified a handful of retinoid-regulated candidate genes that may play novel roles in limb dysmorphogenesis. Notably, our work is the first indication that *Mgp* and *Gdf10* are significantly upregulated following pan-RAR or RARγ-selective activation. The identification of these candidate genes and their association with RARγ will help to unravel new mechanisms underlying retinoid-induced insult during development.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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