

Elucidation of the Chondrogenic Program Using a
Combination of Biology and Technology

by

Kamal Garcha

HBSc., The University of Western Ontario, 2000
MSc., The University of Western Ontario, 2003

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General Abstract

Skeletogenesis is associated with the aggregation of mesenchymal cells into condensations that prefigure the mature skeleton under the influence of numerous signaling molecules. In the developing murine limb, Fibroblast growth factors (FGFs) (principally 4 and 8) are expressed in the apical ectodermal ridge (AER) and signal to the underlying distal mesenchymal cells. In this manner, FGFs are thought to influence the proliferation of chondroprogenitors, thereby modulating the growth of pre-cartilage condensations. To study these effects the chondrogenic program can be effectively modeled *in vitro* using high-density cultures of primary cells isolated from the murine limb bud (E11.5). Treatment of distal mesenchymal cultures with FGF4 leads to a transient increase in proliferation and expansion of prechondrogenic condensations. Using transcriptional profiling with DNA microarrays we observed that consistent with the changes observed in cell proliferation, FGF4 treatment resulted in an ~ 18 fold increase in the expression of *Cdkn2b* – a cyclin dependent kinase inhibitor. SiRNA knockdown of *Cdkn2b* resulted in sustained mesenchymal proliferation. Further, we determined that FGF4 regulates *Cdkn2b* through a MEK1/ERK-dependent pathway. Additionally, we show that FGF4 promotes cell survival through the regulation of NF κ B via upregulation of its activator RIPK4.

To enhance our studies of chondrogenesis, a high throughput reporter gene-based assay was developed. Using this technology we performed a chemical genetic screen of ~1500 chemical compounds to assess their ability to regulate reporter gene activity. Of these compounds, 28 yielded a >2.5 fold increase in

SOX5/6/9 reporter gene activity. Secondary histological screens confirmed increased cartilage formation in response to treatment of primary limb cultures with several of these newly identified chemical enhancers of chondrogenesis. Through bioinformatic analysis of microarray data in comparison with the known targets of the screened chemical compounds, we have shown that inhibition of potassium channel KCND2 (Kv4.2) promotes chondrogenesis. Additionally, it was found that prochondrogenic bone morphogenetic protein 4 (BMP4) downregulated the expression of *Kcnd2*. These data have revealed an unanticipated role of potassium channels during chondrogenesis. Thus by using a combined approach of microarray analysis and chemical genetics we have further characterized the intricacies of the chondrogenic program.

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List of Abbreviations

| | |
|---------|---|
| AER | apical ectodermal ridge |
| Agc1 | aggrecan |
| BAB | butamben |
| BMP | bone morphogenetic protein |
| CDKN | cyclin dependant protein kinase inhibitor |
| cDNA | complementary deoxyribonucleic acid |
| col | collagen |
| cMEK | constitutively active MEK |
| Cspg4 | chondroitin sulfate proteoglycan 4 |
| DMSO | dimethyl sulfoxide |
| DM | distal mesenchyme |
| dn | dominant negative |
| DNA | deoxyribonucleic acid |
| E | embryonic day |
| EGFP | enhanced green fluorescent protein |
| Eomes | Eomesodermin |
| ERK | extracellular signal related kinase |
| ES cell | embryonic stem cell |
| FBS | fetal bovine serum |
| FGF | fibroblast growth factor |
| FGFR | FGF receptor |

| | |
|----------------|--|
| GDF | growth/differentiation factor |
| HMG | high mobility group |
| IDR | interdigital region |
| I κ B | inhibitory kappa B |
| IKK | I κ B kinase |
| KCND2 | potassium voltage -gated channel, Shal-related subfamily, member 2 |
| Sca1 | stem cell antigen 1 |
| MAPK | mitogen-activated protein kinase |
| MEK | MAPK kinase |
| mRNA | messenger ribonucleic acid |
| N-cad | N-cadherin |
| NCAM | neural cell adhesion molecule |
| NF- κ B | nuclear factor kappa B |
| Ogn | osteoglycin |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PD | proximo-distal |
| PKC | protein kinase C |
| PLM | primary limb mesenchyme |
| PM | proximal mesenchyme |
| PNA | peanut agglutinin |
| PZ | progress zone |
| qRT-PCR | quantitative real-time PCR |

| | |
|-------------|---|
| RA | retinoic acid |
| RARE | retinoic acid response element |
| Ripk4 | receptor-interacting serine- threonine kinase 4 |
| RNA | ribonucleic acid |
| rRNA | ribosomal RNA |
| siRNA | short interfering RNA |
| Shh | sonic hedgehog |
| Smad | homology to Sma and MAD (mothers against decapentaplegic) |
| Sox | SRY-like HMG box |
| SRY | sex-determining region of Y chromosome |
| TGF β | Transforming growth factor β |
| Wnt | wingless |
| Zic | zinc finger protein of the cerebellum |
| ZPA | zone of polarizing activity |

Co-Authorship Statement

Chapter 2: FGF Signals in the Embryonic Limb Regulate Cell Proliferation and Survival Through *Cdkn2b* and the NF- κ B Signaling Pathway

All of the following were carried out by Kamal Garcha and T. Michael Underhill:

identification and design of research program, performing the research, data analyses and manuscript preparation.

A former post-doctoral fellow, Lisa M. Hoffman, isolated RNA used in microarray experiments described in Chapter 2.

Chapter 3: Chemical Genetics Reveals a Novel Role for Potassium Channels in Chondrogenesis

All of the following were carried out by Kamal Garcha and T. Michael Underhill:

identification and design of research program, performing the research, data analyses and manuscript preparation.

Chapter 1

1.1 General Introduction

In the early mammalian embryo, coordinated signaling events lead to widespread changes in gene expression resulting in tissue morphogenesis. The temporal and spatial regulation of developmental programs is tightly regulated by various molecular mediators such that structures arise in a precise order. During limb development, an interplay of molecular signaling between the overlying ectoderm and the underlying mesenchyme gives rise to specialized tissues that direct the development of skeletal elements. Of particular interest is a specialized structure at the most distal edge of the developing limb comprised of a pseudostratified layer of epithelium –the apical ectodermal ridge (AER) that governs the outgrowth and patterning of the limb. This thesis examines the role of an AER expressed signaling molecule -fibroblast growth factor 4 (FGF4) in the developing limb, with a particular focus on FGF4 mediated mesenchymal cell proliferation and survival. Additionally, a chemical biology approach was employed to identify other pathways that regulate mesenchymal cell function and differentiation.

1.1.1 Significance

Cartilage functions as a template for endochondral bone formation. The differentiation and growth of cartilaginous tissues are processes vital to proper skeletal development and function, and in children cartilage within the growth plate also allows for longitudinal growth of bones. As such, developmental deformities arise from aberrant growth and differentiation of the chondrogenic anlagen during skeletogenesis. Cartilage has limited reparative ability, such that small chondrogenic

lesions in humans are a contributing factor to the development of osteoarthritis. In adults, osteoarthritis presents a major medical, social and economic burden on society, as the current incidence in North America is ~10% within the general population (www.arthritisnetwork.ca).

Little is known about cartilage repair; however, reparative processes in other tissues involve at least in part, the recapitulation of developmental programs utilized in their initial formation. Adult tissue regeneration resembles embryonic development, specifically, progenitor cells are recruited and induced to differentiate and give rise to new tissues that have the form and function of the original tissue. To better understand the molecular mechanisms involved in chondrogenesis, embryonic cartilage formation can be effectively modeled *in vitro* using mesenchymal cells isolated from the embryonic mouse limb. Our studies have involved signaling factors essential in limb induction with a particular focus on the transcriptional regulation of cartilage specific genes during the initial specification of the limb skeletal template. Understanding the molecular programs operating in embryonic chondrogenesis should provide therapeutic clues for enhancing adult cartilage repair.

Bioinformatic approaches are commonly used to examine changes in gene transcription in response to various stimuli. The aim of these approaches is to identify those genes and gene families that are downstream targets of the stimuli. Therefore, to further study the regulation of chondrogenesis, we performed microarray experiments on samples collected from primary limb mesenchymal (PLM) cultures treated with previously identified factors essential to limb formation. Validation of these data and the characterization of the role of several of these genes, is the focus

of chapter 2. Briefly, the role of FGF4 in the developing limb was examined at the level of transcription by examining previously generated microarray data. Previous observations indicated that treatment of PLM cultures with exogenous FGF4 induced transient PLM proliferation, and thereby yielded an increase in precartilaginous condensations. However, the mechanism responsible for the attenuation of FGF4 induced cellular proliferation had not been identified. We therefore hypothesized that a cell cycle inhibitor was likely responsible for attenuating FGF4 mediated proliferation. In Chapter 2 we provide evidence for the attenuation of FGF4 induced proliferation by one of its downstream targets - cyclin dependent kinase inhibitor 2b (*Cdkn2b*); a cell cycle inhibitor. Additionally Chapter 2 describes a new *in vitro* culture model using serum-free conditions to ascertain the role of developmental factors on chondrogenesis. Specifically we show that FGF4 promotes mesenchymal cell proliferation and survival.

The emergence of genome-wide approaches within the last few years has revolutionized our understanding of the molecular mechanisms underlying various biological processes. These new approaches enabled unbiased strategies to identify gene(s) that may have important roles in specific embryonic developmental programs. The importance of candidate genes can be evaluated empirically by overexpression or by gene silencing. To this end, Chapter 3 describes a novel transfection strategy that makes it possible to perform large scale gene screens by eliminating the constraints of time associated with the preparation of DNA transfection mixtures. As an extension of this methodology, a chemical-genetic

screen was also carried out to identify small molecules that modulate chondrogenesis. These screens yielded several novel stimulators of chondrogenesis. By comparing the known targets of the stimulatory small-molecules with data obtained by transcriptional profiling, we identify the importance of potassium channel KCND2 in the chondrogenic program. This technology has facilitated the implementation of large scale gene and chemical biology screens to delineate the molecular programs operating in chondrogenesis.

1.1.2 Background

The vertebrate limb is complex, being comprised of several tissues with an asymmetrical arrangement of components. During embryogenesis, the majority of the skeletal bones are formed via endochondral ossification, whereby the future bones are first formed as hyaline cartilage scaffolds. Specifically, mesenchymal progenitor cells differentiate into chondrocytes giving rise to a cartilage template that is subsequently replaced by bone in a precise arrangement. The limbs develop from paired primordial buds that appear on the lateral surface of the embryo at specific regions referred to as limb fields along the anterior-posterior body axis. Limb formation is initiated by the selective expansion of mesenchymal cells within the lateral plate mesoderm (Olsen et al., 2000). Subsequently, through ill-defined mechanisms, a subset of mesenchymal cells is fated to become chondrocytes. These cells first become evident within precartilaginous condensations, the earliest morphologically apparent event in skeletogenesis (Karsenty and Wagner, 2002; Mariani and Martin, 2003). During the condensation stage, numerous processes including mesenchymal cell recruitment, migration, and aggregation of progenitors

(Hall and Miyake, 2000; Tuan, 2004) coordinate to establish a high density of mesenchymal cells within the developing limb. Importantly, mutants defective in mesenchymal condensation as the result of a failure to meet the required high cell density, present with limb skeletal anomalies (Hall and Miyake, 1992; Mundlos and Olsen, 1997). Within the condensed mesenchyme, cells differentiate and establish a cartilaginous matrix that will support further skeletogenesis.

In an attempt to better define the molecular programs regulating the formation of the appendicular skeleton, our laboratory is characterizing the role of factors essential for chondrogenesis in the developing limb.

1.2 Overview of Vertebrate Limb Development

1.2.1 Limb Bud Initiation and Early Outgrowth

Limb development begins when mesenchyme cells proliferate from the somatic layer of the limb field lateral plate mesoderm (limb skeletal precursors), and from the somites (limb muscle precursors). These cells accumulate under the epidermal tissue to create a rounded bulge called a limb bud. Signals from the lateral plate mesoderm (LPM) are essential for limb bud formation and it is these LPM cells that will become the limb mesenchyme (Tickle and Munsterberg, 2001). The subsequent patterning of limb mesenchyme however, is due to interactions between the mesenchyme and the overlying ectoderm (Capdevila and Izpisua Belmonte, 2001) (Fig. 1.1A).

It is proposed that as mesenchymal cells enter the limb region they secrete factors that, through reciprocal signaling with the overlying ectoderm, induce the formation of a pseudostratified, columnar epithelium termed the apical ectodermal

ridge (AER). In the limb field, the presence of the T-box transcription factors TBX4 (hindlimb) and TBX5 (forelimb) precedes the signaling interaction between members of the FGF and Wingless (WNT) families (Agarwal et al., 2003). In *Tbx4*^{-/-} and *Tbx5*^{-/-} animals there is a failure of limb initiation, and FGF and WNT signaling. In the prevailing view of limb initiation, the role of the TBX transcription factors is to stimulate *Fgf10* expression, whereas the proposed role of WNT3 is to maintain high levels of FGF10 throughout limb initiation (Agarwal et al., 2003; Yang, 2003). Reciprocally, FGF10 is believed to maintain *Wnt3* expression during the formation of the AER (Barrow et al., 2003b). The AER runs along the distal margin of the limb and becomes a major signaling centre for the developing limb. The roles of the AER include maintaining the underlying mesenchyme in a plastic, proliferative phase that enables proximal-distal outgrowth of the limb.

The importance of the AER in limb outgrowth is emphasized in experiments involving its removal at successive stages of development. When the AER is removed at an early limb bud stage, the most proximal skeletal segment (stylopod: upper arm, thigh) forms, but middle (zeugopod: forearm, lower leg) and distal (autopod: wrist and hand, ankle and foot) segments are absent. When it is removed at a slightly later stage, only the autopod is missing (Rowe and Fallon, 1982; Saunders, 1998; Summerbell, 1974). Experiments showing that distal skeletal elements could be rescued by applying beads soaked in recombinant FGF protein to the tip of AER-denuded chick limb bud suggested that members of the FGF family of secreted proteins are the AER-derived signals required for limb development (Fallon et al., 1994; Niswander et al., 1993). Four of the 22 known *Fgf* genes, *Fgf4*, *Fgf8*,

Fgf9 and *Fgf17*, display AER-specific expression domains within the mouse limb bud (Itoh and Ornitz, 2004; Martin, 1998; Sun et al., 2000; Yu and Ornitz, 2008b) along with their respective receptors.

1.2.3 Models of Limb Patterning

The prevailing model of limb patterning has been the subject of some debate (Dudley et al., 2002; Mariani et al., 2008; Mariani and Martin, 2003; Saunders, 2002; Tabin and Wolpert, 2007). The reason for this controversy is related to the belief that cell fate is specified by the amount of time spent in proximity to the AER. Previously it has been shown that removal of the AER at successive stages results in the loss of increasingly distal limb elements (Summerbell et al., 1973). More recent data has suggested that this is merely a consequence of excessive cell death arising from the removal of the AER (Dudley et al., 2002; Niswander and Martin, 1993a; Saunders, 2002) (Fig. 1.2A). Confounding the understanding of limb patterning is more recent molecular data related to gene expression in the limb. To add to the possibilities, a new hybrid model is emerging based on knockout studies involving FGFs in the AER (Mariani et al., 2008). This new model, termed the “two-signal dynamic specification model”, proposes that the AER produces distal signals, whereas tissues near the body wall produce opposing proximal signals. Accordingly, a loss of a distal signal permits the proximal signal to extend more distally than normal, specifying more distal cells to give rise to structures normally found in the proximal limb (Fig. 1.2B). Regardless of the proposed model, the absolute necessity of the AER in outgrowth and patterning of the developing limb is undisputed.

1.2.4 Emergence of a Chondrogenic Anlagen

The aggregation of mesenchymal cells is an important transient event during chondrogenesis. Patterning signals are expressed and regulate skeletogenesis through the establishment of pre-cartilage condensations. The cells within the condensations have altered mitotic activity, and increased cell-cell signaling and interaction due to increased cell density. The condensed mesenchyme demarcates the regions in which a cartilage anlagen will form and eventually be replaced by bone. It is at this stage that condensed mesenchymal cells differentiate to chondrocytes, and begin to secrete an extracellular matrix (ECM) rich in aggrecan, collagen type II, IX, XI, fibronectin, hyaluronan as well as the link protein and tenascin (DeLise et al., 2000; Goldring et al., 2006; Kulyk et al., 1991; Stirpe et al., 1990; Swiderski and Solursh, 1992), whereas during differentiation the expression of collagen type I is downregulated. These condensed cells become encased by their secreted ECM and following further maturation and hypertrophy, they produce collagen type X and downregulate the expression of collagen type II, and support calcification. The calcified cartilage is subsequently invaded by blood vessels carrying osteoblasts, marking the initial stages of the template's mineralization to bone.

1.2.5 Signaling Networks Involved in Limb Development

Of the essential factors required for the coordination of signaling events culminating in the developed limb, ectodermal WNTs within the AER are among the earliest signals required to induce FGFs such as FGF10 and FGF8 (Ohuchi et al., 1997). These signaling pathways act in positive feedback loops (Niswander, 2003)

and promote proximal-distal growth. In this sequence of events as previously outlined, TBX transcription factors (TBX4/5) in the lateral plate mesoderm induce downstream factors FGF10 and WNT3. WNT3 acts via β -catenin to increase FGF8 in the ectoderm. FGF8 and WNT3 maintain FGF10 expression in the mesenchyme and vice versa (Agarwal et al., 2003; Barrow et al., 2003b; Tickle and Munsterberg, 2001). In this manner, reciprocal FGF signals regulate the outgrowth of the limb bud (Tickle, 2002; Yu and Ornitz, 2008a). Additionally, WNT7A produced by the dorsal ectoderm signals through its downstream target gene *Lmx1* in the underlying dorsal mesenchyme, and plays a critical role in dorsal-ventral patterning. *Wnt7a* is expressed early during limb bud development and maintain Sonic hedgehog (*Shh*) expression (Shum et al., 2003; Tickle, 2003) which is required for regulating anterior-posterior limb patterning, and is necessary to maintain *Fgf4* expression in the AER (Laufer et al., 1994; Niswander et al., 1994). Further adding to the complexity of this signaling network, the ventral ectoderm induces the expression of the transcription factor Engrailed (EN-1), which plays a role in dorsal-ventral patterning (Fig. 1.3). The homeobox (Hox) transcription factors encoded by the *HoxA* and *HoxD* gene clusters are also critical for the early events of limb patterning in the undifferentiated mesenchyme, and are required for the expression of *Fgf8* and *Shh* (Kmita et al., 2005; Tarchini and Duboule, 2006; Tarchini et al., 2006). Taken together, the limb is patterned through the coordinated action of multiple signals, including the FGFs, WNTs, Hedgehogs (HHs), across all three limb axes.

As limb patterning and outgrowth progress, multiple bone morphogenetic proteins (BMPs), as their name suggests, begin to influence bone morphogenesis by

initiating chondrogenesis. BMPs act at multiple stages to regulate the skeletogenic program. This process is highly dependent upon the temporal and spatial expression of BMP receptors and BMP antagonists, such as NOGGIN and CHORDIN (Niswander, 2002; Pizette and Niswander, 2000; Tickle, 2003). *In vitro* and *in vivo* studies have also shown that BMP signaling is required both for the formation of precartilaginous condensations and for the differentiation of precursors into chondrocytes (Yoon and Lyons, 2004; Yoon et al., 2005). During limb development, FGFs provide a proliferative signal, and delay cellular differentiation, whereas BMPs play a role in mesenchymal cell specification/determination and promote differentiation (Yoon et al., 2006). In general, it is thought that FGF signaling and BMP signaling function antagonistically to each other during the processes of limb outgrowth (Minina et al., 2002; Niswander and Martin, 1993a; Yoon et al., 2006).

1.2.6 FGFs and Their Associated Receptors

FGFs are involved in the earliest stages of limb development and in the formation of skeletal elements within the limb. Specifically, these factors signal from the AER to the underlying mesenchyme to promote cellular proliferation and survival. This mesenchymal region has been termed the progress zone (PZ), consisting of undifferentiated mesenchymal cells subjacent to the AER. FGFs within the AER signal to the underlying mesenchyme and maintain a population of progenitor cells required for the outward growth of the limb and the formation of skeletal elements (Li et al., 2005; Shum et al., 2003).

The expression patterns and function of FGFs in early limb development have been identified as key components of AER signaling to the underlying mesenchyme

and have been reviewed extensively (Mariani et al., 2008; Martin, 1998; Niswander et al., 1993; Sun et al., 2000; Sun et al., 2002b). Briefly, FGF ligands signal through binding and activation of one of four high-affinity FGF receptors (FGFRs) which represent a subclass of the tyrosine kinase receptor family (Itoh and Ornitz, 2004; Thisse and Thisse, 2005) (Fig. 1.4). FGF receptors contain, in their full-length form, a hydrophobic leader sequence, three immunoglobulin-like (Igl, II, and III) domains, an acidic box, a transmembrane domain, and a divided tyrosine kinase domain (Li et al., 2005). As a result of alternative splicing, numerous functional isoforms can also be generated. Individual FGFR proteins bind multiple FGFs but also display a unique pattern of affinities for the different ligands (De Moerlooze and Dickson, 1997). However, as ligand binding is also greatly influenced by the distribution of heparan sulfate proteoglycans (HSPGs) at the cell surface and in the extracellular matrix (ECM), it is unknown to what extent these *in vitro* assays reflect the ligand binding specificity of different FGFR proteins *in vivo*. Once released from cells, FGFs bind avidly to HSPGs such as the syndecans, glypican, and perlecan on the cell surface and in the extracellular matrix (ECM), which is thought to limit their diffusion from the source of production (Martin, 1998).

FGFs elicit their effects on cells by forming a complex that includes the ligand, a high affinity tyrosine kinase receptor, and a heparan-sulfate proteoglycan (Ornitz and Itoh, 2001; Thisse and Thisse, 2005; Yu and Ornitz, 2008a). Specifically, upon the binding of the ligand, FGF receptors dimerize and autophosphorylate several intracellular tyrosine residues that serve as docking sites for Src Homology 2 domain (SH2) containing polypeptides such as phospholipase C (Marie et al., 2005; Thisse

and Thisse, 2005). The phosphotyrosine binding domain (PTB) of the adaptor protein FGFR substrate 2 (FRS2) binds to the FGFR in a phosphotyrosine-independent manner and is tyrosine-phosphorylated upon activation of FGFRs (Kouhara et al., 1997). Once FRS2 is tyrosine-phosphorylated, it binds the SH2 domain containing adaptor protein GRB2 as well as the protein-tyrosine phosphatase SHP2 (Kouhara et al., 1997; Mohammadi et al., 1991; Xu et al., 1998). GRB2 then recruits the guanine nucleotide-releasing factor SOS to the plasma membrane where it subsequently leads to the activation of the RAS small guanosine triphosphatases (GTPases). RAS signaling activates the extracellular signal-regulated kinase (ERK) mitogen activated protein kinase (MAPK) pathway (Corson et al., 2003; Roberts and Der, 2007; Xu et al., 1998).

In the developing limb bud, the epithelial splice form of FGF receptor 2 (*Fgfr2b*) is expressed in the ectoderm, while the mesenchymal splice forms of FGF receptor 1 (*Fgfr1c*) and FGF receptor 2 (*Fgfr2c*) are expressed in the nascent limb mesenchyme (Niswander and Martin, 1993a; Orr-Urtreger et al., 1991). An early step in the initiation of limb bud formation involves, as previously mentioned, signaling from mesenchymally expressed FGF10 to FGFR2B which results in the formation of the apical ectodermal ridge. FGF8 is subsequently expressed in the apical ectodermal ridge and is thought to signal back to FGFR1C and FGFR2C in limb mesoderm (Naski et al., 1996; Ornitz and Marie, 2002a; Siliang Zhang, 2006). This pattern of reciprocal signaling is one of several essential events required for outgrowth and patterning of the limb.

Mesenchymal condensation is the first morphologic event leading to bone formation (Hall, 1987; Hall and Miyake, 1992). *Fgfr2* expression is first observed in the mesenchyme as mesenchymal cells begin to coalesce in the central core of the developing limb (Shum et al., 2003). At the condensation stage of limb development, *Fgfr1* expression persists in limb mesenchyme and in mesenchymal cells at the periphery of the condensation, whereas *Fgfr2* expression can be observed in the morphologically distinct mesenchymal condensations but not in the surrounding loose connective tissue (Ornitz and Marie, 2002a). These expression profiles appear to be evolutionarily conserved and have been observed in chicken, mouse, and human limb development (Delezoide et al., 1998; Orr-Urtreger et al., 1991; Peters et al., 1992; Szebenyi et al., 1995). At the initial onset of chondrogenesis *Fgfr3* expression is first observed in the chondrocytes within the center of the condensed mesenchyme (Peters et al., 1993). Importantly, although the FGFRs are dynamically expressed in the developing limb, the mechanisms regulating their expression are ill-defined.

FGF activity in the early limb is required for proliferation of the distal-tip cells and prevention of apoptosis (Dudley et al., 2002; Sun et al., 2002b). At later stages, FGF signaling plays an integral role in chondrocyte proliferation and differentiation (Ornitz and Marie, 2002a). Consistent with their expression, FGFs have been found to play multiple roles in limb and skeletal development while aberrant FGF signaling leads to a spectrum of limb malformations including syndactyly, truncations and dwarfism (Ornitz and Marie, 2002a). These studies have suggested multiple roles for

FGF signaling in accordance with the differential expression of *Fgfrs* in the chondrogenic program.

1.2.7 Mutations in FGFs and FGFRs

Targeted deletion of members of the FGF family and FGFRs in the mouse reveal that FGF signaling is essential for cell proliferation and survival in the preimplantation mouse embryo (Feldman et al., 1995), as well as for cell migration during gastrulation (Itoh and Ornitz, 2004; Zhang et al., 2004). Conditional knockouts enabled the analysis of later stages of embryogenesis and determined that FGF signaling plays a role in development of the limb buds, brain, and lung in addition to numerous other tissues and organs (Itoh and Ornitz, 2004).

The requirement for proper FGF signaling in skeletal development is evident in congenital anomalies that arise from mutations of the receptors affecting the extracellular, transmembrane, or intracellular domains. A severe condition known as Achondrodysplasia (ACH), characterized by reduced growth of the long bones with proximal segments more greatly affected than distal, arises from autosomal dominant mutations of FGFR3. Typically, the mutations result in the substitution of amino acids (glycine to arginine) in the FGFR3 transmembrane domain, making the receptor constitutively active (Naski et al., 1996).

Conditional knockout studies to determine the function of FGFs in the AER have revealed that only FGF8 removal significantly impacts limb development (Lewandoski et al., 2000; Moon and Capecchi, 2000) resulting in an absence of some skeletal elements. Although FGF4 mutants have normal limbs, compound mutations of both FGF4 and FGF8 result in mesenchymal cell death in the limb bud

and a failure of the limb structures to form (Boulet et al., 2004). In FGF8 mutants alone, FGF4 is believed to partially compensate for the lack of FGF8 and is upregulated in the AER (Lewandoski et al., 2000; Moon and Capecchi, 2000). The current view of the AER FGFs is that they exhibit overlapping functions, and it is the total amount of FGFs produced from the AER during limb development that is essential for skeletogenesis (Delgado et al., 2008). However some FGFs, such as FGF8, figure more prominently in this process.

1.2.8 Regulation of Cell Proliferation by the CDKN2/INK Family

In the developing limb, rapid cellular proliferation results in the outgrowth of the future appendicular structures. Proliferation is tightly regulated which is coordinated through the actions of protein effectors composed of cyclins and cyclin-dependent kinases (CDKs). Of the many types of kinases, the progression of cells through the G₁-S transition requires the activity of cyclin D-CDK4/6 and cyclin E/A-CDK2 complexes (Niswander et al., 1994; Reynisdottir et al., 1995a). The activity of these kinases is differentially regulated by the cyclin dependent kinase inhibitors (CDIs), also known as the CDKN2 family (or INK4 family), and the *CIP/KIP* families of cell cycle inhibitors. The *CIP/KIP* members p21^{cip21}, p27^{Kip1}, and p57^{Kip2} associate with and inactivate cyclin E-CDK2 and cyclin A-CDK2 complexes, whereas their association with cyclin D-CDK4 or cyclin D-CDK6 have been shown to be stimulatory (Niswander et al., 1994; Reynisdottir et al., 1995a; Wolfrim et al., 2004). By competitive interaction, binding of CIP/KIP proteins with cyclin D-CDK4/6 kinases prevents their interaction with cyclin E/A-CDK2. This facilitates the role of these kinases in completing the G₁ phase and their role in the DNA synthesis phase of the

cell cycle. The members of the CDKN2 family have a different mode of action. CDKN2A, CDKN2B, CDKN2C, and CDKN2D inhibit the catalytic binding between CDK4 and CDK6 kinases and the regulatory domains on the cyclin D subunits (Niswander et al., 1994; Quelle et al., 1995; Reynisdottir et al., 1995a; Takeuchi et al., 1995).

The role of the CDKN2 proteins in the regulation of cellular proliferation has been well studied in several cell types. Collectively the studies show an increase in CDKN2 proteins during the G₁ phase, resulting in a decrease in cell proliferation, followed by the differentiation of some cell lineages. Initially, interest in the CDKN2 family resulted from genetic linkage studies which showed that an inherited predisposition to melanoma in some families could be traced to a putative tumor suppressor on chromosome 9, specifically 9p21 (Quelle et al., 1995; Takeuchi et al., 1995). Through positional cloning, the *Cdkn2a* (*Ink4a*) gene was identified along with a closely related *Cdkn2b* (*Ink4b*) gene. CDKN2A and CDKN2B have similar modes of action and serve as tumor suppressors by acting as inhibitors of the CDKs that control cellular proliferation by regulating the progression of the cell cycle from the G₁ phase. Though the role of these genes is pivotal in determining the state of the cell, it has been difficult to detect *Cdkn2a* or *Cdkn2b* expression during embryogenesis (Gil and Peters, 2006). Given the nature of the developing limb, in which there is an acute phase of regulated growth, which is regulated at least in part by FGFs, we sought to characterize the function of FGFs in regulating mesenchymal cell proliferation.

1.2.9 Mitogen Activated Protein Kinase (MAPK) Signaling

MAPK cascades are essential signaling pathways involved in the regulation of cell proliferation, survival and differentiation (Corson et al., 2003; Roberts and Der, 2007), that function downstream of cell surface receptors such as the FGFRs. These signaling networks are comprised of three protein kinases that act as a signaling relay controlled in large part by protein phosphorylation. These kinases are: MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and a MAPK (Corson et al., 2003; Johnson and Lapadat, 2002). Through the action of RAS, its downstream effector RAF serine/threonine kinase is activated and plays a key role in regulating MAPK signaling. In an evolutionarily conserved pathway, RAF activates the MAPK/ERK kinase (MEK)1/2 dual specificity protein kinases, which then activate ERK1/2 (Roberts and Der, 2007). Activated ERKs then translocate to the nucleus, where they phosphorylate and regulate various transcription factors leading to changes in gene expression.

The MEK/ERK pathway is one of the major downstream pathways of FGF receptors (Corson et al., 2003; Murakami et al., 2000). Consistent with this, in the embryonic limb, the FGFRs and phospho-ERK show colocalized expression, and inhibition of FGFR results in the a loss of phospho-ERK signaling (Corson et al., 2003). Further, overexpression of constitutively active phospho-MEK1 in limb mesenchyme, inhibits chondrogenic differentiation, whereas pharmacological inhibition of phospho-MEK1 promotes chondrogenesis (Bobick and Kulyk, 2004; Oh et al., 2000). These data are consistent with the assertion that the role of FGFs is to

promote appendicular growth by negatively regulating chondrogenesis via MEK/ERK signaling.

1.2.10 Role of Nuclear Factor Kappa B in Limb Development

The AER FGFs stimulate mesenchymal proliferation to permit appositional growth of the limb, and also promote mesenchymal cell survival, requiring the activation of specific transcription factors. Originally identified as a nuclear factor that binds the kappa (κ) light chain enhancer in B-cells (Sen and Baltimore, 1986), NF- κ B is now recognized as an important transcription factor involved in many cellular processes including cell survival/death. The generalized name -NF- κ B can represent the NF- κ B and Rel protein superfamily, subfamily (p100, p105, and Relish), or the major heterodimer in most cells, p50-RelA (Gilmore, 2006). During limb morphogenesis, it has been established that NF- κ B is vital for the formation of the apical ectodermal ridge, and its transcription is regulated in part by AER derived signals such as the FGFs (Bushdid et al., 1998). As mentioned earlier, the AER is required to maintain the proliferative, undifferentiated state of the leading edge of the developing limb, whereas the underlying mesenchyme (the progress zone, PZ), is required to maintain the AER. Studies performed on chick embryos have shown that NF- κ B transcriptionally regulates the expression of factors necessary for the patterning of the limb, as well as limb mesenchymal cell survival (Bushdid et al., 1998; Kanegae et al., 1998). The establishment of a feedback loop between signals from the AER and the signals from the PZ is required for limb outgrowth. Disruption of the NF- κ B signaling pathway results in the loss of proper gene activation resulting in limb truncation (Bushdid et al., 1998; Kanegae et al., 1998; Perkins et al.,

1997), highlighting the importance of NF- κ B as a component of the network operating in epithelial-mesenchymal cell communication.

1.2.11 Regulation of NF- κ B

The complexity of NF- κ B regulation is highlighted by the observations that more than 200 physiological stimuli have been shown to activate these transcription factors (Tergaonkar, 2006). NF- κ B is initially located in the cytoplasm typically as a heterodimeric protein consisting of 50 and 65 kilodalton subunits (p50 and RelA) of the *Rel* family. NF- κ B is often found in an inactive complex associated with inhibitory I κ B proteins (Fig. 1.5). Under the appropriate stimulus or stimuli, I κ B is released from NF- κ B, subsequently NF- κ B translocates to the nucleus to initiate transcription of a variety of genes including those involved in the cell cycle/survival (Fig. 1.5). Spatiotemporal expression of two NF- κ B members - *c-Rel* and *RelA*, in the distal mesenchyme implies a role for NF- κ B signaling in the regulation of limb development. In support of this observation, *c-Rel* expression is diminished following AER ablation, and is subsequently rescued by the addition of FGF4 (Buschdid et al., 1998). Experiments involving I κ B mutants decreased the amount of nuclear translocated NF- κ B available for DNA binding, and have shown the decreased expression of critical genes in the PZ. Experiments with these mutants have also indirectly shown the decreased expression of genes in the AER and zone of polarizing activity (ZPA) (Kanegae et al., 1998). Overall, the intrinsic function of NF- κ B during limb development requires the proper functioning of all components of the pathway.

Due to the numerous pathways NF- κ B can influence and its implicated role in numerous biological processes and diseases, regulation of this diverse transcription factor has been studied quite extensively. The I κ B sequestering of NF- κ B in the cytoplasm represents a molecular mechanism of inhibiting NF- κ B signaling. Conversely, activators of NF- κ B facilitate its translocation to the nucleus to promote transcription of target genes. The receptor interacting proteins (RIPs) are important regulators of cell proliferation and differentiation (Adams et al., 2007). One such activator of NF- κ B is receptor-interacting serine-threonine kinase 4 - RIPK4 (also known as DIK, RIP4 and PKK). Overexpression studies have shown that RIPK4 increases NF- κ B activity, whereas kinase inactive versions of RIPK4 have a dominant negative effect on NF- κ B induction (Meylan et al., 2002b). Experiments involving NF- κ B loss-of-function have suggested that its role is to mediate mesenchymal maintenance of the AER as the mutant phenotype is a consequence of improper epithelium-mesenchyme communication resulting in severe limb truncation and defects (Bushdid et al., 1998). Interestingly, animals deficient in *Ripk4* have shortened limbs with syndactyly –a condition in which the fusion of two or more digits occurs involving the soft tissue, bones, or both (Holland et al., 2002).

1.3 Transcriptional Regulation of Chondrogenesis

1.3.1 Importance of SOX9

Appendicular skeletal development initiates shortly after the outgrowth of the limb bud with the formation of a histologically identifiable mesenchymal condensation, marked by the restricted expression of the nuclear transcription factor Sox9 (Fig. 1.1B). This transcription factor is required for the expression of the type II

collagen gene (*Col2a1*) (Kosher et al., 1986; Nah et al., 1988; Shum et al., 2003; Wright et al., 1995) and is essential for chondrogenesis. Sox9 belongs to the SRY (sex-determining region on the Y chromosome) family and contains an HMG (high mobility group) box DNA binding domain (Lefebvre et al., 1997; Ng et al., 1997; Wright et al., 1995). Mutations of Sox9 have shown it to be a master regulator of chondrogenesis (Healy et al., 1996; Wright et al., 1995). Sox9^{+/-} mouse embryos die perinatally and exhibit severe hypoplasia of the cartilage that is associated with lower levels of cartilage matrix genes (Bi et al., 2001). Similarly, Sox9 haploinsufficiency in humans causes campomelic dysplasia, characterized by severe skeletal dysmorphology (Wunderle et al., 1998). Further analyses with conditional inactivation of Sox9 at varying times during mouse limb development have revealed that it is required for mesenchymal condensation and subsequent chondroblast differentiation (Akiyama et al., 2002; Akiyama et al., 2004).

Two other members of the SOX family of transcription factors –SOX5 and SOX6 function cooperatively with SOX9 to regulate *Col2a1* expression (Lefebvre et al., 2001; Smits et al., 2001). It is proposed that the ability of SOX5 and SOX6 to form homo and hetero dimers facilitates their binding to 2 HMG domains simultaneously (Ikeda et al., 2005). SOX5/6/9 are commonly referred to as the SOX trio, as they are co-expressed in chondroprogenitors and chondrocytes (Akiyama et al., 2002; Lefebvre et al., 1998; Smits et al., 2001). SOX9 functions at multiple points during chondrogenesis and is involved in the commitment of undifferentiated mesenchymal cells into the chondrogenic lineage, and is required for mesenchymal condensation and the expression of Sox5 and Sox6. Embryonic limbs in which Sox9

was inactivated prior to mesenchymal condensation showed no expression of Sox5 and Sox6, whereas inactivation of Sox9 after the condensation stage resulted in embryos with skeletal defects characteristic of Sox5/6 double null mutants (Akiyama et al., 2002). Thus, SOX5/6 appear to be required to direct overt chondroblast differentiation, and the accompanying increase in expression of genes encoding extracellular matrix proteins (Ikeda et al., 2005). Proper SOX function during chondrogenesis is absolutely required for normal limb development.

1.4 Methodology and Strategies

1.4.1 Primary Cell Culture Model for Studying

Chondrogenesis

The basic parameters to study chondrogenesis *in vitro* were developed over 30 years ago by Ahrens and colleagues (Ahrens et al., 1977). The chondrogenic program can be effectively modeled *in vitro* using high-density micromass cultures of primary cells isolated from the vertebrate limb bud (murine embryonic age day 11.5). Under these conditions, prechondrogenic cells begin to condense within ~24 hrs and form cartilage nodules within ~72 hrs. Further, these cells exhibit robust phenotypic responses to signaling molecules such as BMPs, FGFs and WNTs. Although chondrogenesis can be studied using established chondrogenic cell lines, these cultures require ~10 days before the initial cartilage nodules become apparent, unless otherwise treated with chondrogenic factors (Denker et al., 1999). An obvious advantage of the primary cell micromass technique is the reduced time-frame required to elaborate the chondrogenic program, without the additional concerns of long-term cultures (eg. contamination). Additionally, primary cultures allow the ability

to study intrinsic genetic and cell cycle events in the developing mesenchyme that may be absent or misrepresented in established cells lines (ie. cell cycle inhibitors).

Although the traditional micromass technique has withstood the test of time, there are several shortcomings which include the absolute requirement of a high density of cells per micromass. This poses a particular problem in experiments in which cells are harvested from the distal mesenchyme yielding few cells. These cells provide a more homogeneous population, suitable for genetic analysis, and allow the study of the molecular events subjacent to the AER. Routinely, per dissected litter of murine E11.5 embryos, ~100 μL of distal limb mesenchymal cells are harvested (at a final density of 2.0×10^7 cells/ml), and are plated as 10 μL micromass cultures. This limited abundance of cells curtails large-scale experiments in the 24-well format, as the requirement of experimental controls/plate consumes one-sixth of the available cells since the majority of our experiments are performed in quadruplicate. The traditional 24-well based approach has severely hampered our throughput. To circumvent such limitations, and further delineate the chondrogenic program a novel 384-well based culture method has been developed. This more efficient method replaces the traditional 24-well based assays and allows the amount of primary cells and reagents consumed per experiment to be greatly reduced. The 384-well system provides a reliable means of assessing the role of factors on chondrogenesis while increasing the scale of throughput 16-fold.

At the onset of the research for this thesis, there was a necessity to develop technology that would enable high throughput screening of single genes and multiple gene crosses in PLM cultures. This need was brought about by the abundance of

transcriptional data obtained through bioinformatic data mining of previously generated microarrays. Logistically, it became apparent that transfections on a large scale would require the development of an enhanced transfection strategy to perform functional assays.

1.4.2 Assessing the Status of Chondroblast Differentiation

The effects of signaling molecules (BMPs, FGFs, etc.) on SOX5/6/9 activity, and consequently *Col2a1* expression, have been reported by our laboratory and others (Hoffman et al., 2006; Murakami et al., 2000; Semba et al., 2000; Weston et al., 2000; Zehentner et al., 1999; Zeng et al., 2002). To provide a read-out on the status of chondroblast differentiation, we have employed a SOX5/6/9-responsive reporter construct consisting of a 48 bp enhancer element derived from the first intron of *Col2a1* (Lefebvre et al., 1997; Ng et al., 1997; Zhao et al., 1997). This element has been reiterated 4 times, and placed upstream of a minimal *Col2a1* promoter and luciferase gene (Figure 1.1C). This 48 bp region is necessary for SOX5/6 and 9 binding, and thereby transcription of *Col2a1* (Lefebvre et al., 1997). Previously, using this reporter, we have assessed the action of numerous factors which regulate chondrogenesis such as BMPs and retinoids (Hoffman et al., 2006; Weston et al., 2002). These effects have been verified by alcian blue staining (staining glycosaminoglycans within cartilage nodules), which shows intense cartilage nodule staining in response to BMP4 treatment. Studies using this reporter in combination with other genes in co-transfection based assays have provided important information on the molecular networks operating in chondrogenesis.

1.4.3 Review of Transfection Technology

The ability to introduce nucleic acids into cells has enabled the advancement of our knowledge of genetic regulation and protein function within eukaryotic cells, tissues, and organisms. The transfer of recombinant genes into a variety of eukaryotic cultured cells, commonly known as transfection, is an extensively used approach in assessing gene function. Under specific conditions, eukaryotic cells can take up exogenous DNA, and a portion of this DNA becomes translocated to the nucleus. These events have been exploited to obtain both transient and stable expression of various genes. Factors that influence the entry of DNA into the cell include the barrier of the cell membrane to the charge of the DNA, as well as its size and conformation. Once inside the cell, the intact DNA must avoid enzymatic cleavage, in order to enter the nucleus where it can be transcribed. These factors hamper transfection efficiency. To circumvent these concerns, a wide range of methods have been developed to facilitate transfection, with the aim of efficiently delivering DNA into target cells and protecting it from nuclease degradation.

The use of viruses (retroviruses or adenoviruses) is the most efficient for cell transduction, but presents several disadvantages related to immunogenicity, DNA size restriction, and large-scale production constraints (Chen and Okayama, 1988; Felgner et al., 1987; Felgner and Ringold, 1989; Mannino and Gould-Fogerite, 1988). Synthetic means of delivering DNA to the nucleus have been developed to overcome these limitations and some of the primary advantages of this approach are that the composition of these synthetic reagents is known, they can form complexes with DNA that are either slightly toxic or completely non-toxic to cells, they have few

size limitations, and they are easy to prepare in large quantities (Gao et al., 2007). Synthetic transfection reagents induce cellular uptake of DNA by forming complexes with nucleic acids resulting in a positively charged complex. These complexes bind to the negatively charged cell membrane through ionic interaction and enter the cell through endocytosis, without significant cytotoxicity. Cationic lipids and polymers are prominent synthetic reagents. The combination of negatively charged DNA with positively charged lipids produces condensed particles known as lipoplexes (Felgner et al., 1987; Felgner and Ringold, 1989; Gao et al., 2007; Pedroso de Lima et al., 2001). Using positively charged polymers to compact DNA (called polyplexes), Felgner et al. (1987) showed an ability to transfect cells with nucleic acids. The original technology has since been modified and the number of synthetic reagents, along with the variety of commercially available forms, have greatly increased. As the demand for rapid, high efficiency transfections has increased, a number of other products (non-liposomal lipids, synthetic polymers, etc.) have been developed that mediate the transport of genes into cells.

A problem associated with the majority of non-viral gene-delivery agents is their relatively low transfection efficiency (Nikcevic et al., 2003) which can be influenced by a number of factors. Parameters that are taken into consideration when optimizing transfection efficiency include cell type or cell line to be used, culture conditions, and transfection reagent (Arnold et al., 2004). These concerns are based on observations that certain cell types are intrinsically easier to transfect than others. Although the exact reason for these differences is unknown, important factors influencing the success or failure of transfection include the quality of the

transferred DNA as well as its size, configuration, quantity, and the mitotic state of the cells to be transfected. Traditionally, primary cells have been difficult to transfect and various strategies have been devised to overcome these limitations.

1.4.4 Design and Application of New Technology

In order to study the regulation of genes involved in chondrogenic differentiation, it was essential for us to transiently introduce recombinant plasmid DNA into primary limb mesenchymal (PLM) cells isolated from the mouse at embryonic age 11.5 with high efficiency. Transient transfections are easy to prepare, and as a consequence facilitate high-throughput approaches. Earlier work from our lab involved transfections using Fugene6™ (Roche), a cationic lipid based reagent, which yielded sufficient transfection efficiency. However, these transfections required preparation in the absence of serum for maximal activity. It is presumed that a polyvalent negatively charged serum component inhibits the formation of transfection complexes. Ideally, transfection in the presence of serum yields better cell growth, function and viability, and reduced cytotoxic effects associated with transfection reagents (Arnold et al., 2004). These inherent issues of transfection, coupled with the value of the primary cells to be transfected prompted us to find a better solution and thereby circumvent some of the present limitations of transfection. To this end, we began experimenting with Effectene™ (Qiagen) which is also a cationic lipid based transfection reagent. Using Effectene™, transfections can be prepared in the presence of serum, with the additional benefit of being able to optimize several components of the transfection reagent, as they are not premixed by the manufacturer. Chapter 3 will describe the optimized transfection

parameters for Effectene™ with PLM cell cultures. We describe a means of increasing the transfection efficiency of this transfection reagent by using disaccharides (Fig. 1.6B).

1.4.5 Stabilization of Transfection Complexes

Preservation of biological materials such as proteins, enzymes, membranes and mammalian cells has been a source of great interest over the past 2 decades (Beattie et al., 1997; Crowe et al., 1998; Leslie et al., 1995; Powers et al., 1986; Sowemimo-Coker et al., 1993). In nature, stabilization in response to stresses such as desiccation or freezing is a common practice in many plants and animals. These organisms accumulate large amounts of sugars in response to physiological stress (Sun et al., 2002a). In particular, disaccharides such as sucrose and trehalose play a key role in the desiccation and preservation process.

Two different hypotheses have been postulated by which sugars protect biological materials in the desiccated state, namely the glass formation hypothesis, and the water-replacement hypothesis. In the glass formation hypothesis, the formation of stable glasses reduces molecular mobility and enables long-term storage (Leslie et al., 1994; Sun et al., 1996). The water-replacement hypothesis suggests that replacement of water molecules by sugar molecules and the direct interaction of sugars with polar residues (through hydrogen bonding during the desiccation process) allows biological structures to maintain their conformational structure in the dried state (Crowe et al., 1996; Sowemimo-Coker et al., 1993; Sun et al., 1996) (Fig. 1.6A). Given that disaccharides can impart stability to biological

components, such as lipids, we evaluated their ability to stabilize lipid-based DNA-transfections.

1.4.6 Enhancement of Experimental Throughput

The development of the 384-well based PLM transfection strategy has provided the means for enhancing our experimental throughput as the DNA-transfection mixtures can be prepared well in advance of their use, thereby eliminating the constraints of time imposed by conventional transfection strategies. Additionally, by adapting our 24-well based experiments to the new 384- well technology we have increased the scale of our experiments by 16-fold, in a highly reproducible fashion. We have demonstrated the merits of this technology by comparative studies of a small-scale gene-screen between conventional 24 well based experiments and 384 well based experiments using the aforementioned SOX5/6/9-luciferase reporter gene as a readout.

1.4.7 Small-Molecule Screening

A relatively new and powerful approach to defining the mechanisms underlying biological processes is the use of small-molecule chemical compound libraries. Small-molecules are defined as carbon-based compounds with a molecular weight under 500 (Kawasumi and Nghiem, 2007). Using a phenotype-based approach for screening small-molecules, compounds that produce a phenotype of interest can be identified and subsequently the target of the compound can provide information on the underlying biological process(es).

As an extension of the transfection technology, we performed a small molecule screen of a library of compounds with known biological activity. The goal of

this endeavor was to create a technology to enable identification of both pro- and anti-chondrogenic factors to aid in our understanding of the chondrogenic program. The data gathered from this study has proven beneficial in further characterizing the basic mechanisms regulating cartilage formation. We have also identified numerous new pro-chondrogenic compounds that will hopefully lead to the development of new therapeutics to treat the debilitating conditions associated with diseased and damaged cartilage.

1.5 Overview of Thesis

The progression of limb development involves an interplay of signaling networks that when investigated, provide as many answers as they do new questions. With an observed phenotype and working hypothesis, we show in Chapter 2 a systematic approach to identifying and characterizing genes of interest in the developing limb. Consequently, we also clarify the role of FGF4 in mesenchymal survival and provide new methodology to examine the role of developmental factors in isolation or limited combination.

Chapter 3 provides a technological approach aimed at increasing throughput to enable large-scale analysis of gene function in chondrogenesis. We provide a new technology to increase throughput, and to combine chemical biology with gene-based approaches. To this end, we used this technology to implement a chemical biology screen. The information from this screen combined with existing microarray data sets has provided new insights into the potential role of potassium channels in chondrogenesis. The overall goal has been to further define the chondrogenic

program using an integrated approach of hypothesis-based and discovery-driven research.

1.57 Topics Addressed

Chapter 2: FGF Signals in the Embryonic Limb Regulate Cell Proliferation and Survival Through *Cdkn2b* and the NF- κ B Signaling Pathway

Research outlined herein is directed towards defining the molecular networks regulated by fibroblast growth factor 4 in the limb mesenchyme.

Objectives:

- i To investigate the mechanisms underlying FGF4 action in cell proliferation and survival.
- ii To determine the role of FGF4 in chondrogenesis.

Chapter 3: Chemical Genetics Strategy Leads to the Identification of Novel Pathways Important in Chondrogenesis

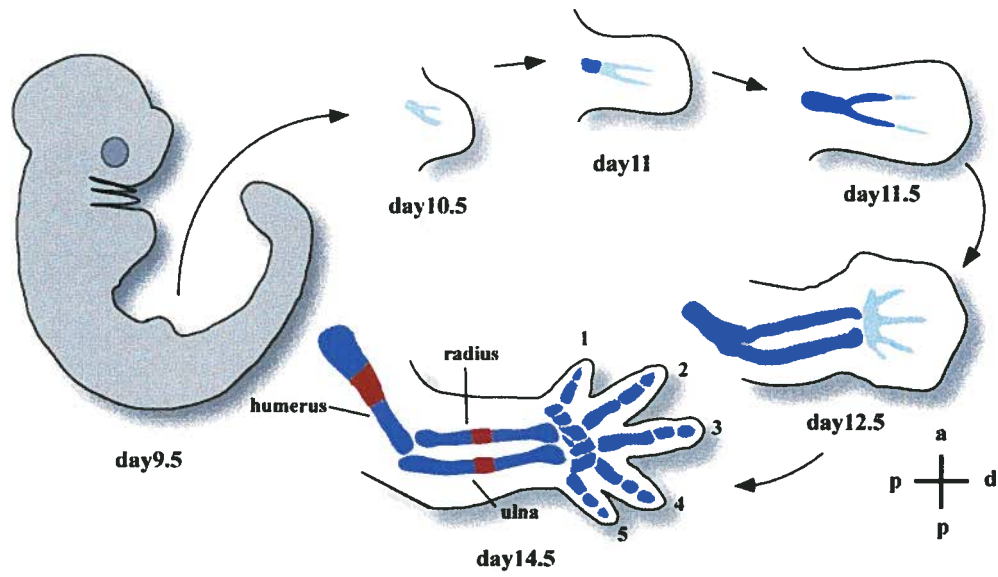
Objectives:

- i To develop and implement a chemical biology screen for identification of chondrogenic modulators.
- ii To gain new insights into molecular programs that regulate chondrogenesis.

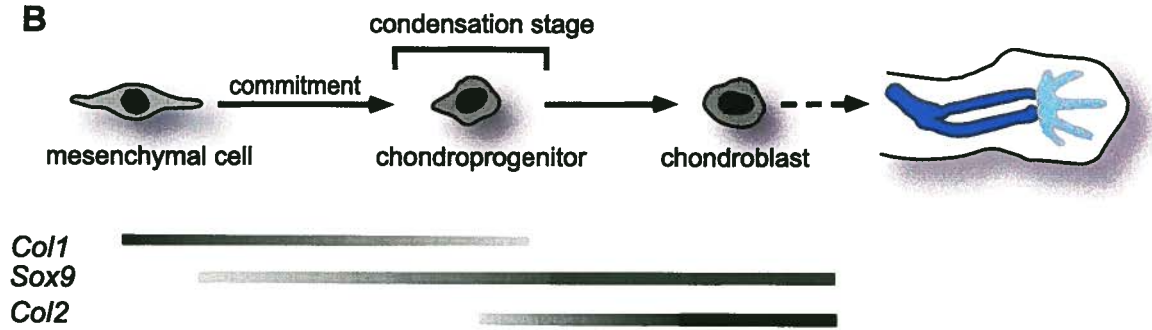
1.6 Figures

Figure 1.1. Overview of Murine Limb Skeletal Development. **A**, During endochondral ossification a cartilage template is established and subsequently remodeled to bone. This process is initiated through the action of multiple signals, culminating in the generation of cartilaginous anlagen that prefigure the skeleton. **B**, Type I collagen (*Col1*) expression is observed throughout the early limb and in precartilaginous condensations. SOX9 an HMG box-containing transcription factor, plays a central role in regulating chondrocyte commitment and differentiation. Type II collagen (*Col2a1*) is abundantly expressed in chondroblasts and its initial expression is dependent upon SOX5/6/9. Analysis of the *Col2a1* promoter has revealed a 48 bp sequence essential for SOX5/6/9 binding. **C**, Use of reporter genes (luciferase or fluorescent protein) containing multiple copies of the 48 bp sequence enable monitoring of SOX5/6/9 activity in PM cultures which provides a reliable readout on the status of chondroblast differentiation and cartilage formation.

A



B



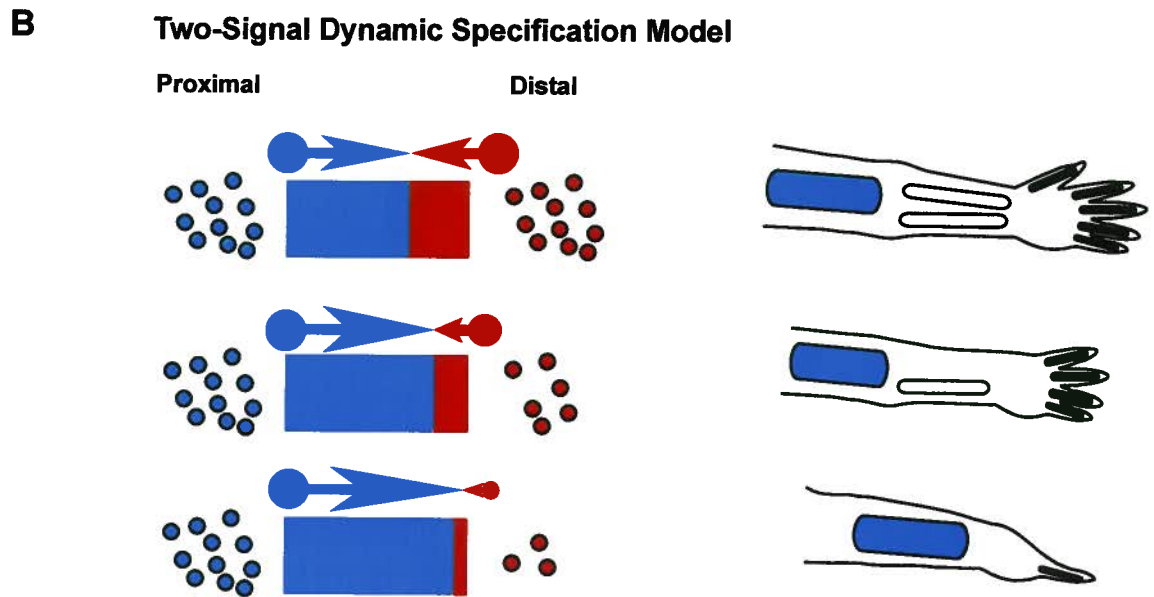
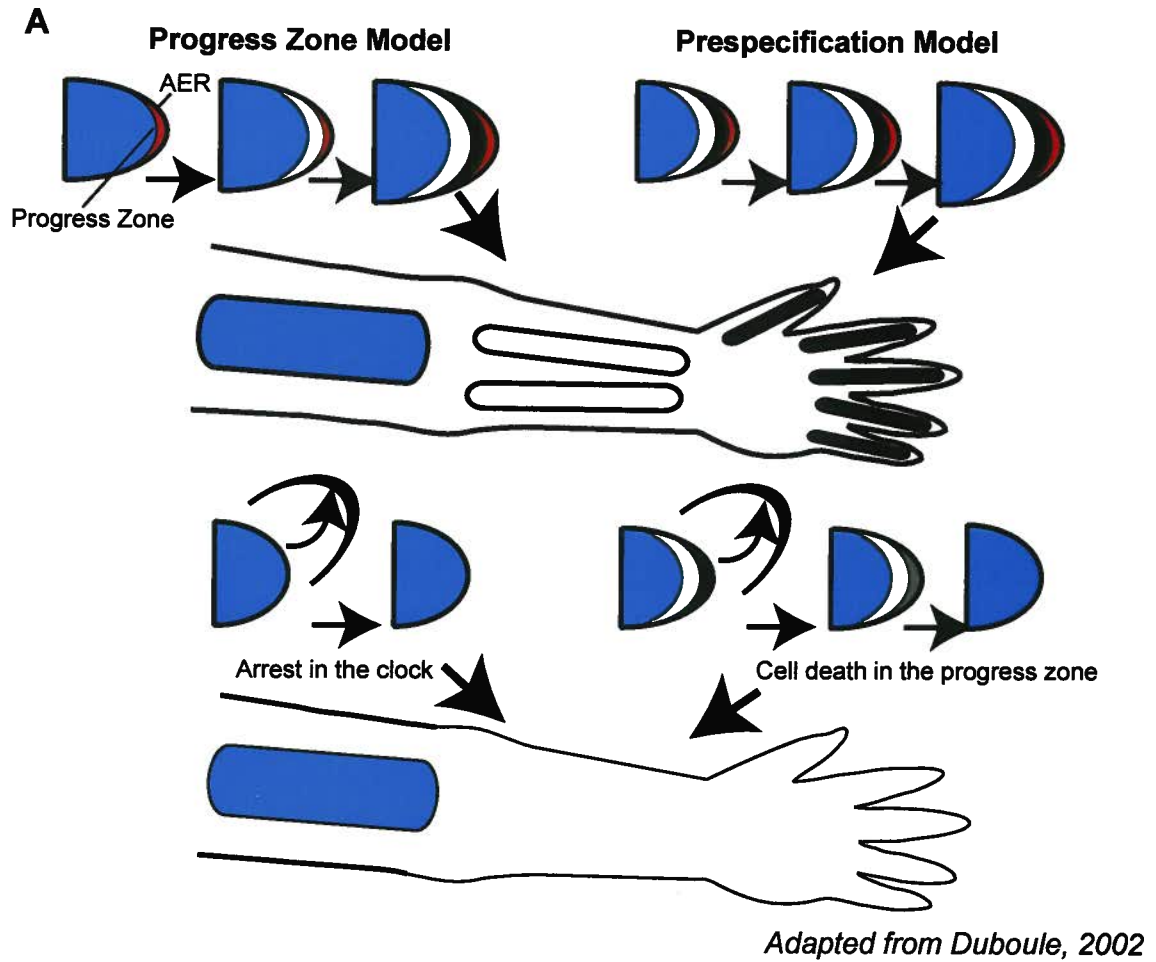
C



Figure 1.2. Three Perspectives on Proximal-to-Distal Patterning in Developing

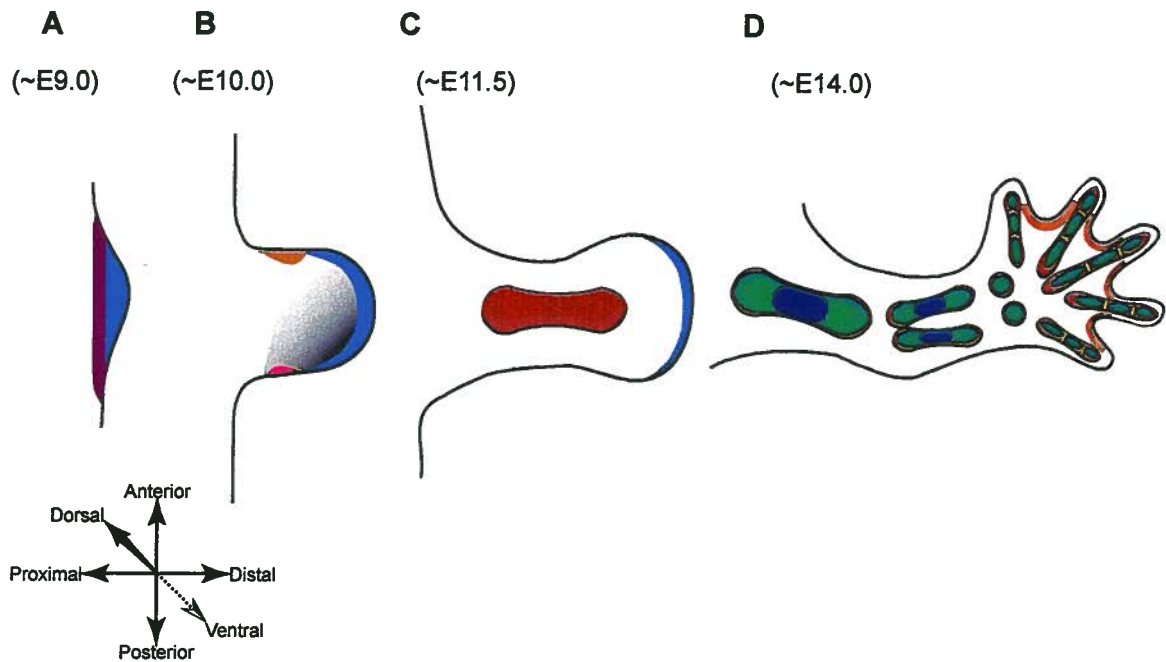
Limb Buds. A, The “progress zone” model, predicts that the fate of proximal (prospective upper arm; blue) elements is specified prior to the fate of more distal elements, as the limb grows. Changes in cell fate occur in the progress zone, adjacent to the AER. In the “prespecification” model, proximal-to-distal fates are believed to be prespecified early in development and the observed temporal events in skeletal development result from the selective expansion of these prespecified domains, along the acquisition of definitive cell fate. Following removal of the AER the “progress zone” model predicts that the specification clock is arrested and (in this case) distal specification never occurs. In the “prespecification” model removal of the AER prevents the expansion of prespecified distal domains because of cell death. In both cases, the same results are expected. (*adapted from Duboule, 2002*)

B, The “two-signal dynamic specification” model for limb proximal-distal patterning predicts that proximal domains of the embryonic limb containing proximal signals (blue) are specified by opposing distal signals (red) released from the AER. This model predicts that in AER mutants, or following surgical removal of the AER, the distal signal is reduced in proportion to the proximal signal. The proximal signal is not restricted and interacts with cells that in wild-type limbs would only receive distal signals. Due to decreased AER signaling, the limbs are smaller and distal structures are missing as the result of cell death in the distal region. (*adapted from Mariani et al., 2008*)



Adapted from Mariani et al., 2008

Figure 1.3. The Developmental Progression of Undifferentiated Limb Mesenchyme to Cartilaginous Skeletal Precursors in the Limb. **A**, Limb outgrowth begins as a protrusion of undifferentiated mesenchyme covered by ectoderm expressing FGF10 and WNT3 (purple) (embryonic age (E) ~9.0). The distal edge of the ectoderm thickens to form the apical ectodermal ridge (AER) comprised of pseudostratified columnar epithelium which initiates the expression of FGF8, BMP2, BMP4, and MSX2 (blue). **B**, During early appendicular growth, the mesenchymal core expresses numerous *Hox* genes (gray). The cells in the posterior domain of the proximal region of the ZPA secrete SHH and BMP4 (pink), while the anterior cells of the proximal region secrete only BMP4 (brown). The ectoderm secretes factors such as FGF4 in the posterior of the AER, WNT7A in the dorsal regions, and EN1 in the ventral segments (~E10.0). **C**, The mesenchyme condenses and the cells (red) secrete a variety of signaling factors such as GDF5, BMP2, BMP4, BMP7, and have elevated levels of SOX9 (~E11.5). **D**, Cellular differentiation occurs in a proximal to distal sequence such that the humerus differentiates prior to the radius and ulna, followed by the digits. Differentiating chondrocytes (green) secrete NOGGIN, and proliferating chondrocytes (blue) initiate IHH expression. The interdigital mesenchyme (orange) undergoes apoptosis, initiated by a combination of signals such as BMP2, BMP4, and MSX2. The cartilaginous templates are segmented into individual skeletal elements via joint formation (yellow) regulated by factors such as GDF5, WNT14, and CHORDIN (~E14.0). These elements are subsequently mineralized to form skeletal structures. (*adapted from Shum et al., 2003*)

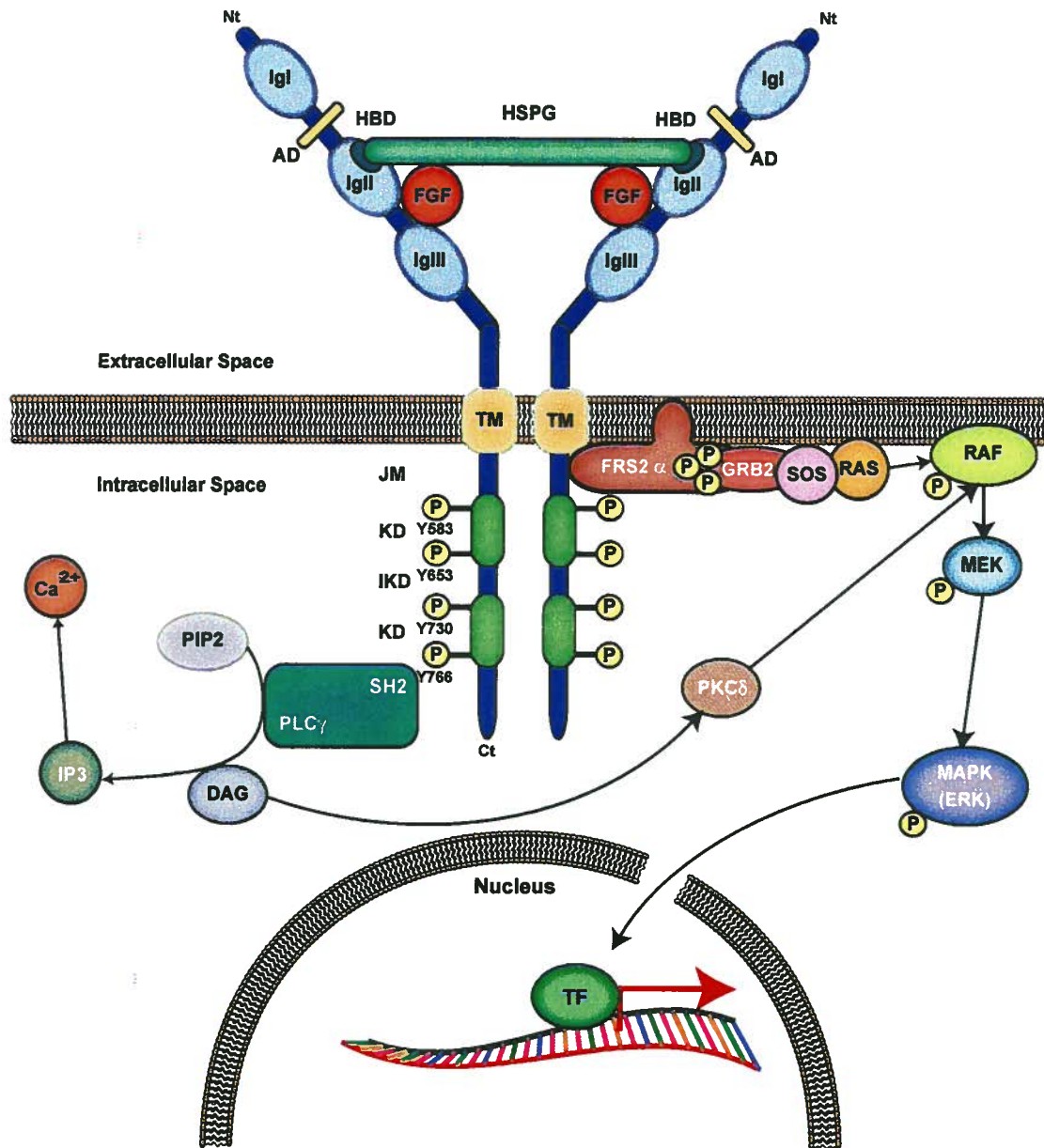


- **Intermediate Mesoderm:** FGF10, WNT3
- **Apical Ectodermal Ridge:** FGF8, BMP2, BMP4, MSX2, WNT3
- **Ventral Ectoderm:** EN-1
- **Dorsal Ectoderm:** WNT7A
- **Posterior Ridge Ectoderm:** FGF4
- **Entire Limb Bud:** *Hox* genes
- **Zone of Polarizing Activity:** SHH, BMP4

- **Anterior Mesoderm:** BMP4
- **Cell Condensation and Perichondrium:** GDF5, BMP2, BMP4, BMP7, SOX9
- **Developing Joints:** GDF5, WNT14, CHORDIN
- **Interdigital Mesenchymal Cells:** BMP2, BMP4, MSX2
- **Chondrocytes:** NOGGIN
- **Proliferating Chondrocytes:** IHH

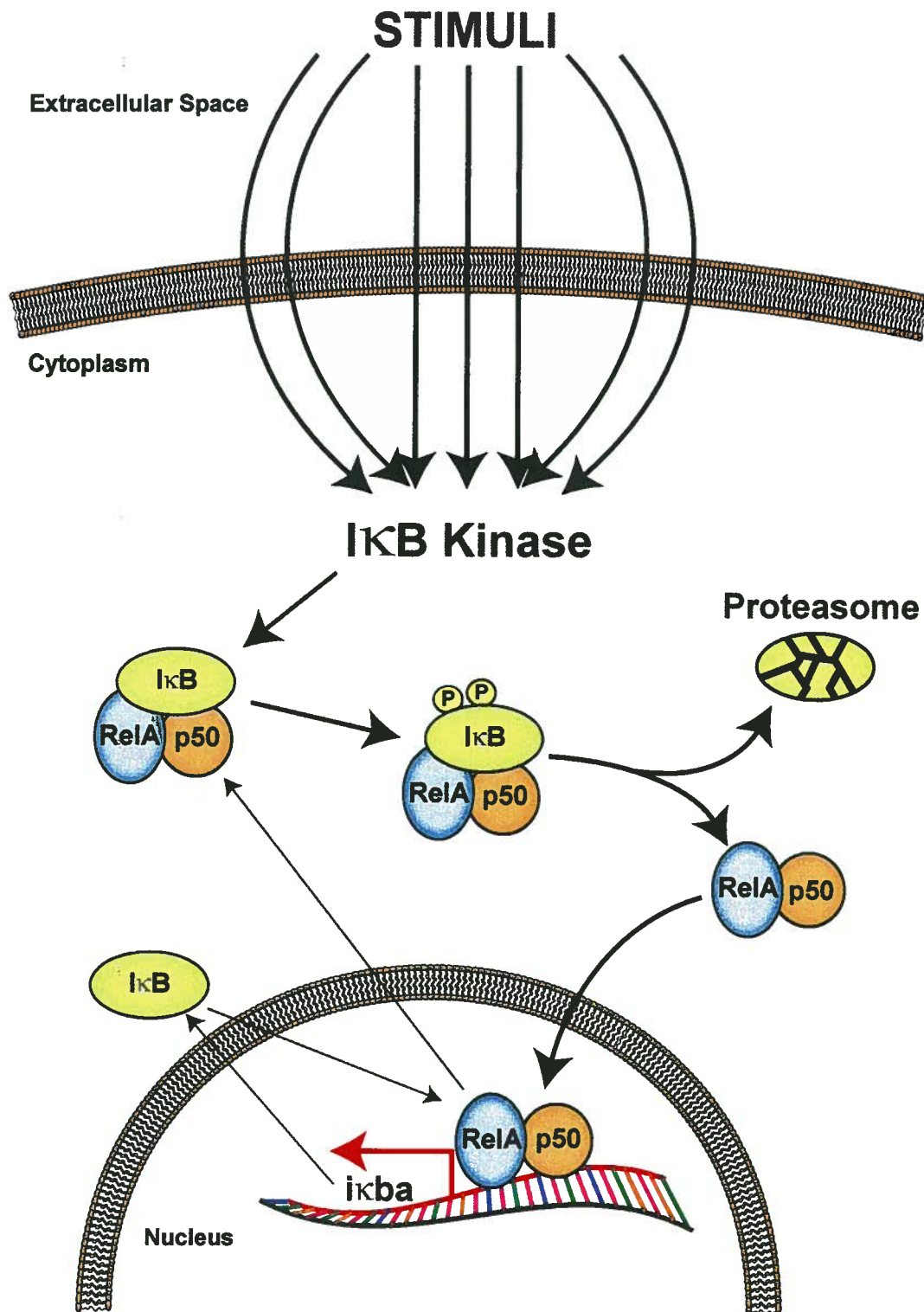
Adapted from Shum et al., 2003

Figure 1.4. FGF Receptors and FGF Signal Transduction. FGFRs are modular proteins comprising 3 immunoglobulin domains (IgI, IgII and IgIII). IgI and IgII are separated by an acidic box (AD). IgII contains a heparin binding domain (HBD). The IgIII domain is followed by a unique transmembrane (TM), a juxtamembrane (JM) and a kinase domain (KD) interrupted by an interkinase domain (IKD). FGF ligands linked to heparin sulfate proteoglycan (HSPG) bind to IgII and IgIII of FGFR. This causes the dimerization and subsequent transactivation by phosphorylation of specific tyrosine residues. The two main transduction pathways involve phospholipase C- γ (PLC γ) and RAS/MAP kinase. The SH2 domain of the PLC γ interacts with the phosphorylated Y766 of the activated receptor. The activated PLC γ hydrolyzes the phosphatidyl-inositol-4,5-diphosphate (PIP2) to inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 releases Ca^{2+} while DAG activates protein kinase C- δ (PKC δ). Activated PKC δ activates RAF by phosphorylating its S338 and stimulates the downstream pathway in a RAS independent manner. The main pathway involves the interaction of the docking protein FRS2 α with the amino-acid residues 407– 433. FRS2 α is activated by phosphorylation on multiple tyrosine residues and then interacts with and activates GRB2 linked to SOS, a nucleotide exchange factor involved in the activation of RAS. Once activated, RAS activates RAF which stimulates MEK which in turn phosphorylates MAP kinase ERK which translocates to the nucleus and phosphorylates specific transcription factors (TF). These TFs induce the expression of specific FGF target genes. (*Adapted from Thisse and Thisse, 2005*)



Adapted from Thisse and Thisse, 2005

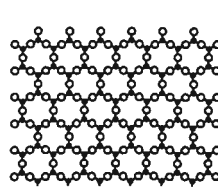
Figure 1.5. Rel/NF- κ B Signal Transduction. In the classical pathway, various signals converge on activation of the I κ B kinase (IKK) complex. IKK then phosphorylates I κ B at 2 N-terminal serines, which signals it for ubiquitination and proteolysis. Freed NF- κ B (p50-RelA, in this case) enters the nucleus and activates gene expression. One NF- κ B target gene encodes I κ B. The newly synthesized I κ B can enter the nucleus, remove NF- κ B from DNA, and export NF- κ B back to its resting state in the cytoplasm. Thick lines indicate the activating pathway; thin lines indicate the inactivating pathway.



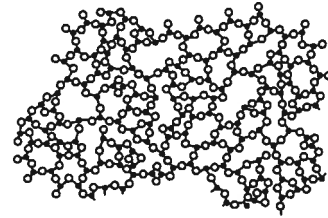
Adapted from Gilmore, 2006

Figure 1.6. Schematic Representation of the Modified Effectene™ Transfection Strategy for Enhanced Transfection and Storage of Transfection-Ready DNA Complexes Facilitating Large-Scale Transfections. **A**, One of the proposed biological roles of disaccharides such as trehalose, is to prevent the formation of crystal lattices which are known to fracture lipid micelles and lipid membranes under conditions of stress attributed to desiccation as well as freezing. Trehalose addition results in the formation of a glass like state and accommodates the fluidity of the lipid micelles and membranes. **B**, In preparation for transfection, DNA is condensed by interaction with the Enhancer and proprietary EC buffer containing 0.4M trehalose. Effectene reagent is then added to the condensed DNA to produce condensed Effectene-DNA complexes. Disaccharides within the proprietary buffer interact with the polar head-group of the Effectene™ reagent micelles which ensconce DNA-Effectene™ complexes.

A

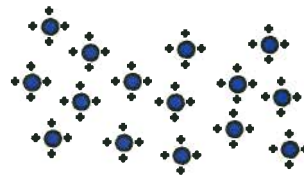
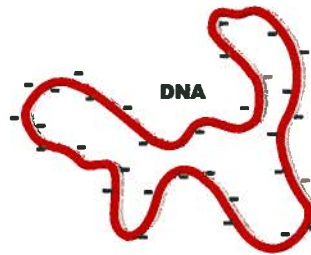


Crystal



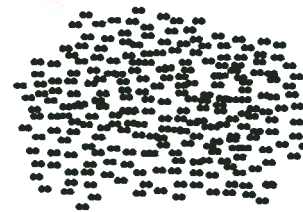
Glass

B

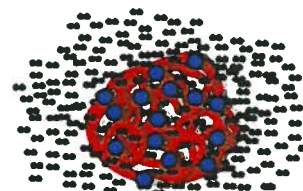


Enhancer Molecules

+

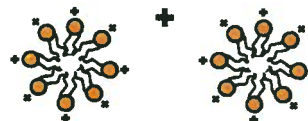


Trehalose Molecules

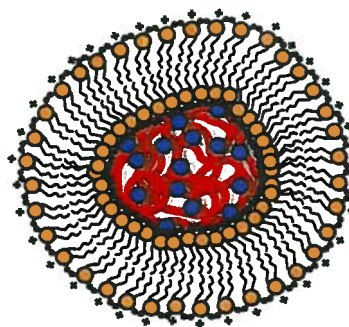


Condensed DNA

+



Effectene Reagent Micelles



Effectene-DNA Complex

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Chapter 2:

FGF Signals in the Embryonic Limb Regulate Cell Proliferation and Survival Through *Cdkn2b* and the NF- κ B Signaling Pathway

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2.1 Abstract

Genetic studies have defined critical roles for the fibroblast growth factor (FGF) family in multiple aspects of limb ontogenesis. However, the molecular basis of these activities is poorly defined. Here we demonstrate that FGF4 regulates cell proliferation in the limb mesenchyme through induction of *Cdkn2b* via a TGF β 1-independent MEK1/ERK-dependent pathway. FGF4 also expands the chondroprogenitor population and this is associated with increased expression of progenitor cell markers, *Ly6a* and *Zic2*, 3 and 5. In the limb bud FGFs are required for cell survival and we show that this involves FGF-mediated activation of NF- κ B signaling through MEK1-dependent localized upregulation of the NF- κ B activator, *Ripk4*. Further, in chondrogenic-deficient serum-free cultures of limb mesenchymal cells, FGF4 alone is sufficient to promote expression of *Sox6*, *Sox9* and a chondrocytic fate. Collectively, these findings define an important linkage between the FGF and NF- κ B signaling pathways, and establish a novel role for FGFs in chondrogenesis.

2.2 Introduction

Appendicular skeletogenesis involves the coordinated action of multiple signaling pathways that influence mesenchymal cell survival, proliferation, specification and differentiation. The fibroblast growth factor (FGF) family figures prominently in many of these processes. Several *Fgfs* are expressed in the developing limb, including *Fgf4* and *8* which are localized to the apical ectodermal ridge (AER), and whose encoded products signal to the underlying mesenchyme. Extirpation of the ridge leads to extensive mesenchymal cell death and limb truncations, which can be rescued by the addition of FGF2 or 4 (Fallon et al., 1994; Niswander et al., 1993).

The FGFs signal through their cognate receptors, the FGF receptors (FGFRs), which belong to the tyrosine receptor kinase superfamily. Four *Fgfrs* have been identified in addition to several splice variants. Within the skeletogenic program they are sequentially expressed (reviewed in Ornitz and Marie, 2002), with *Fgfr1* appearing in the limb mesenchyme, followed by *Fgfr2* in precartilaginous condensations and *Fgfr3* in chondrocytes. Deletion of *Fgfr1* in the mesenchyme of the early limb bud though leads to appreciable cell death in the mesenchyme and loss of distal skeletal elements (Li et al., 2005). Similarly, conditional knockout of *Fgfr2* in the AER leads to extensive cell death within the limb mesenchyme and loss of digits (Yu and Ornitz, 2008). Further, deletion of both *Fgfr1* and *2* in the limb mesenchyme leads to severe skeletal hypoplasia, reduced cell proliferation and increased cell death (Yu and Ornitz, 2008). Embryos conditionally deleted for *Fgf4* in the AER do not present with a limb phenotype, whereas *Fgf8* AER-specific

knockouts present with skeletal malformations that overlap with the defects observed in the *Fgfr* knockouts (Barrow et al., 2003; Boulet et al., 2004; Lewandoski et al., 2000; Sun et al., 2002; Trowbridge et al., 2006). *Fgf8* and *Fgf4/8* double knockouts exhibit increased mesenchymal cell death. Comparison of the phenotypes observed in the ligand versus receptor knockouts also suggests that FGFs function directly in chondrogenesis (Yu and Ornitz, 2008). These studies have defined critical roles for FGF signaling in regulating mesenchymal cell survival and proliferation, and establishment of the chondrogenic anlagen.

Secreted FGFs signal through the FGFRs to affect the activity of several potential downstream pathways that include the mitogen-activated protein kinase (MAPK), phosphatidylinositol-3' kinase, STAT and Src tyrosine kinase signaling pathways (reviewed in Eswarakumar et al., 2005). The MAPK pathway, in particular, the MEK1/ERK is an important downstream effector of FGF signaling in the developing limb, as activated ERK is observed in a proximal-distal gradient in the limb mesenchyme (Corson et al., 2003). Furthermore, evidence indicates that in chondrocytes, FGFs induce growth arrest via activation of the ERK1/2 pathway (Krejci et al., 2008; Raucci et al., 2004).

In the developing limb, signals from the apical ectodermal ridge (AER) play important roles in regulating cell proliferation, survival and chondrogenesis of underlying mesenchymal cells. Studies in chick and in mouse have shown that FGF signals from the ectoderm have critical roles in the regulation of these processes although the mechanisms and targets of FGF signaling in the mesenchyme are poorly defined. In this study, we provide mechanistic insights into

FGF action in cell survival and proliferation, and demonstrate that FGF alone stimulates chondrogenesis under conditions not conducive for cartilage formation.

2.3 Results

FGF signaling plays a fundamental role in regulating multiple aspects of mesenchymal cell behavior during limb outgrowth and associated skeletogenesis. To better understand FGF action(s), we have isolated distal mesenchymal (DM) cells subjacent to the AER and assessed their responsiveness to FGF4 and 8. When plated in high density or micromass culture conditions, these cells closely recapitulate the chondrogenic events observed *in vivo* (Fig. 2.1A). Indeed, analysis of *Col2a1* expression in these cultures reveals that the cells begin condensing within 24 h of plating (weak *Col2a1* expression), and form cartilage nodules shortly thereafter, as evidenced by the intense expression of *Col2a1*. In comparison, treatment of cultures with 20 ng/ml FGF4 delays expression of the chondroblastic phenotype, as observed with alcian blue staining, *Col2a1* expression and a SOX5/6/9 responsive reporter gene (Fig. 2.1A, C). SOX9 is both necessary and sufficient for chondroblast differentiation, and this reporter has been found to provide an accurate read-out on the status of chondroblast differentiation (Hoffman et al., 2006; Muramatsu et al., 2007; Weston et al., 2002). In organ culture of limb buds dissected from E11.5 Col2-EGFP transgenic mouse embryos (Grant et al., 2000), implantation of an FGF4-soaked bead similarly reduces transgene expression (Fig. 2.1B). Cartilage formation is also initially delayed in limb mesenchyme cultures established from Col2-EGFP mice, although analysis at later time points (Day 7) demonstrates an increase in the size and number of cartilage nodules. Consistent

with this observation, FGF4 treatment leads to an appreciable increase in the size of precartilaginous condensations in comparison to control cultures, as determined by rhodamine-PNA staining and *Col2a1* expression (Fig. 2.1A). Further, 12 h treatment with FGF4 significantly stimulates BrdU incorporation that is indicative of increased cell proliferation. This effect is transient, however, since cell proliferation in FGF-treated cultures returns to control levels by 24 h (Fig. 2.1D). Collectively, these findings indicate that FGF4, both *in vitro* and in organ culture, influences expression of the chondroblastic phenotype and regulates proliferation of primary limb mesenchyme (PLM) cells. To better understand the molecular basis of these actions, transcriptional profiling was subsequently employed.

Cultures derived from distal mesenchyme were established and treated with 20 ng/ml of FGF4, RNA collected and transcript abundance measured using Affymetrix U74 V2 arrays A and B. Two hundred and forty five genes were found to be induced at least 3-fold at 24 and 72 h post treatment; of particular interest, several of the *Zic* gene family members known to be associated with various progenitor cell populations were found to be markedly induced by FGF4 treatment (Fig. 2.2, Table I) (Aruga et al., 2002). Similarly, *Ly6a* (*Sca1*), a cell surface marker often associated with "stem" or progenitor populations was also significantly induced (Fig. 2.2, Table I) (Holmes and Stanford, 2007). Consistent with an FGF4-induced delay in differentiation, several cartilage-associated genes were down-regulated, as were several transcription factors including *Eomes*, *Foxp2* and *Sim2* (Fig. 2.2, Table I). Interestingly, the gene that exhibited the greatest increase (~ 18 fold) in expression within 24 h of treatment was *Cdkn2b*, a cyclin-dependent kinase inhibitor

that negatively regulates cell proliferation (Gil and Peters, 2006). While *Cdkn2a* and *Cdkn2b* are co-regulated, *Cdkn2a* expression only increased ~ 3 fold (Fig. 2.3C), and other detected *Cdkns* showed little change in expression. In a variety of cell types, including chondrocytes, FGF signaling has paradoxically been shown to reduce cell proliferation or induce growth arrest (Dailey et al., 2005). Thus, strong induction of *Cdkn2b* by FGF4 is consistent with our observation that FGF4 induces a transient increase in cell proliferation.

The ability of FGF4 to stimulate *Cdkn2b* at the transcript and protein level was validated using qRT-PCR and western blotting (Fig. 2.3B). Further analysis of *Cdkn2b* expression at 75' intervals over a 24 hour time period, reveals that FGF4 induces *Cdkn2b* expression almost immediately, with *Cdkn2b* expression being elevated ~ 10 fold, relative to untreated controls within 2.5 h of addition. Further, in limb organ culture, implantation of FGF4-soaked beads leads to a ~ 5-fold increase in *Cdkn2b* expression (Fig. 2.3D). Several previous reports have shown that *Cdkn2b* expression is stimulated by TGF β 1 (Reynisdottir et al., 1995); indeed, further analysis of our microarray data reveals that FGF4 also induces *Tgf β 1*, as further confirmed with qRT-PCR in treated PLM cultures and in organ culture (Fig. 2.4A). Thus, we hypothesized that FGFs regulate *Cdkn2b* expression through TGF β 1. To confirm the activity of the *Tgf β 1* used in this study, we evaluated it on the SOX9 reporter gene; consistent with previously described pro-chondrogenic activity of TGF β 1 (Chimal-Monroy et al., 2003), we demonstrate that treatment of PLM cultures with TGF β 1 increases reporter gene activity ~ 3-fold. Furthermore, this stimulatory effect can be effectively abrogated in the presence of the TGF β type

I receptor antagonist SB 431542 (Fig. 2.4B). Unexpectedly, treatment of DM cells with TGF β 1 has no significant effect on *Cdkn2b* transcript abundance or on the activity of a reporter gene that encompasses the TGF β 1-responsive region within the *Cdkn2b* promoter (Li et al., 1995) (Fig. 2.4C, D). In contrast, FGF4 stimulates *Cdkn2b* promoter activity. Together, these results indicate that FGF4 operates through a TGF β 1-independent pathway to regulate *Cdkn2b* expression.

FGFs function through several signaling pathways to affect cell behavior (Dailey et al., 2005; Eswarakumar et al., 2005). Several reports have illustrated an important role for the MEK/ERK signaling pathway in chondrogenesis and in FGF action, and as such, we sought to address the role of the MEK/ERK pathway in FGF regulation of *Cdkn2b*. As shown above, FGF4 treatment stimulates *Cdkn2b* expression ~ 8-fold, but not in the presence of an exogenous MAP2K1 (MEK1) inhibitor, U0126, to a final concentration of 10 μ M (Fig. 2.5A). FGF4-mediated stimulation of a *Cdkn2b* promoter-based reporter was similarly abrogated by the addition of U0126 (Fig. 2.5B). Cultures transfected with a minimal (-35) *Cdkn2b* promoter construct displayed at least 20 fold less activity (data not shown). Consistent with the MEK1/ERK pathway playing a central role in FGF-mediated regulation of *Cdkn2b*, overexpression of a constitutively active version of *Map2k1* in the presence or absence of FGF4 stimulates *Cdkn2b* promoter activity (Fig. 2.5C). Finally, the importance of *Cdkn2b* in FGF4-mediated cessation in DM cell proliferation was evaluated through knockdown of *Cdkn2b*. In control transfected cultures, the addition of FGF4 does not increase cell proliferation at 24 h, lending further support to our findings illustrated in Fig. 1D. In contrast, knockdown of

Cdkn2b leads to a similar increase in cell proliferation at both 12 h and 24 h. Cumulatively, these results show that FGFs regulate *Cdkn2b* expression and cell proliferation through the MEK1/ERK pathway.

Removal of the AER or deletion of *Fgf8* from the AER are both associated with severe limb truncations and increased cell death in the limb mesenchyme, effects shown to be exacerbated with deletion of *Fgf4*. Interestingly, inhibition of NF- κ B signaling in the mesenchyme phenocopies many of the limb defects in the *Fgf4/8* compound knockouts (Bushdid et al., 1998; Kanegae et al., 1998). NF- κ B is typically retained in the cytoplasm in a complex associated with IKK proteins. Upon activation, however, NF- κ B translocates to the nucleus where it activates target gene expression (Hayden and Ghosh, 2008). Spatiotemporal expression of two downstream targets of NF- κ B, *c-Rel* and *RelA*, in the distal mesenchyme further indicates a role for NF- κ B signaling in the regulation of limb development (Bushdid et al., 1998; Kanegae et al., 1998). In addition, *c-Rel* expression is diminished following AER ablation, and subsequently rescued by the addition of FGF4 (Bushdid et al., 1998). To determine if FGFs influence NF- κ B activity, we examined NF- κ B-responsive reporter gene activity in DM cells in the presence of FGF4. The addition of FGF4 to DM cells led to ~ 4 fold increase in reporter gene activity (Fig. 2.6A). Both basal NF- κ B activity and FGF4-induced activity were reduced upon co-transfection of *I κ B* or a dominant-negative version of *I κ B*, *I κ B-2N* (Fig. 2.6A). To identify potential mechanisms underlying FGF4 induction of NF- κ B activity, the microarray data set was queried for modulators of NF- κ B. These analyses led to the identification of receptor-interacting serine-threonine kinase 4 (*Ripk4*), a kinase that

has been shown to activate NF- κ B (Fig. 2.2, cluster 3) (Meylan et al., 2002). Induction of *Ripk4* expression in DM cultures was confirmed with qRT-PCR; interestingly, *Ripk4* expression in the DM quickly declines following establishment of cultures whereas its expression is maintained in the presence of FGF4 (Fig. 2.6B). This is consistent with the microarray profile in which *Ripk4* expression declines in control cultures after 24h, but is maintained in FGF4-treated cultures (Fig. 2.2, cluster 3). Furthermore, *Ripk4* transcripts are more abundant in the distal limb, consistent with the source of ectodermal FGFs (Fig. 2.6C). In accordance with previous reports, heterologous expression of *Ripk4* in PLM cells induces NF- κ B activity and this is further increased by FGF4 addition (Fig. 2.6D). Interestingly, expression of *Ripk4* also enhances SOX9 reporter gene expression, indicating that *Ripk4* activity may promote chondrogenesis (Fig. 2.6E). Similar to that observed for *Cdkn2b*, FGF4 also regulates *Ripk4* expression through the MEK1/ERK pathway, as the MEK1 inhibitor, U0126, completely inhibits FGF4-mediated induction of *Ripk4* (Fig. 2.6F), and overexpression of a constitutively active MEK1 increases *Ripk4* expression (Fig. 2.6G). Further, U0126 significantly decreases NF- κ B activity in DM cells, an effect that can be abrogated by expression of *Ripk4* (Fig. 2.6H). Together, these results demonstrate that FGFs activate NF- κ B signaling, at least in part, through up-regulation of *Ripk4*.

Numerous studies have suggested an important role for FGFs in regulating cell survival in the mesenchyme. To test this and the potential role of MEK1/ERK and NF- κ B signaling in the regulation of these activities, we developed a low-density serum free culture system for DM cells. When cells were seeded at sub-confluency

in 6-well dishes, and cultured in the absence of FGF4 or 8 (data not shown), no viable cells could be detected after 5 days of culture (Fig. 2.7A, B). In contrast, the addition of 50 ng/ml FGF4 maintained cell viability such that alcian blue-positive cartilage nodules were detectable at ~ 10 days (Fig. 2.7A, B). Hitherto, cartilage nodules failed to form in sub-confluent limb mesenchyme cultures either in the presence or absence of serum and/or other factors. Consistent with this observation, by day 6 of culture, precartilaginous condensations are observed, and staining with PNA-rhodamine reveals a “cobblestone” distribution pattern within precartilaginous condensations (Fig. 2.7B).

As shown previously, FGFs activate the MEK1 pathway, and as revealed herein, also activates the NF- κ B signaling pathways. To evaluate the importance of both MEK1 and NF- κ B signaling in cell survival, serum-free DM cultures were incubated in the presence of either U0126 or the NF- κ B inhibitor, Bay 11-7082, at concentrations shown to have no deleterious effect on DM cells maintained in high-density culture conditions with serum (data not shown) (Fig. 2.7A). Relative to control cultures, the addition of either inhibitor greatly decreases cell number, with the Bay inhibitor having the greatest negative impact. Further, both inhibitors also substantially reduce cell survival in FGF4-treated cultures (Fig. 2.7A). Bone morphogenetic proteins (BMPs) are abundantly expressed in the distal limb and have been shown to antagonize FGF function (Niswander and Martin, 1993b). In accordance with these earlier reports, we also note that the addition of BMP4 compromises cell survival either alone or in FGF4-treated cultures (Fig. 2.7A). In contrast, addition of the BMP antagonist NOGGIN leads to a small increase in cell

number in early cultures. Despite this, no viable cells were detected by day 6 of culture. Surprisingly, as seen with BMPs, albeit to a lesser extent, NOGGIN also compromises cell viability in FGF4-treated cultures, indicating that BMP signaling in combination with FGFs are needed for mesenchymal cell survival. Collectively, these results demonstrate a fundamental role for FGFs, in conjunction with both the MEK1 and NF- κ B signaling pathways, in regulating cell viability in limb mesenchymal cells.

To further evaluate the ability of FGF4 to stimulate chondrogenesis, the expression of Sox6 and Sox9 were followed over time following addition of FGF4 (Fig. 2.7C). Both genes are dynamically expressed, their expression peaking around day 4 of culture just prior to overt cell condensation, then subsequently decreasing before a second significant increase to almost peak levels by day 10, coincident with the appearance of alcian blue-positive, *Col2a1*-expressing cartilage nodules. Sox6 expression is up-regulated during chondroblast differentiation (Smits et al., 2001), thus the early decline in Sox6 expression likely reflects "dedifferentiation" of chondrogenic cells, with subsequent increases indicative of chondroblast differentiation; from day 6 to 10, Sox6 increases ~ 18-fold. These results clearly show that addition of FGF4 alone to serum-free cultures of DM is sufficient to promote expression of the chondroblastic phenotype.

2.4 Discussion

FGFs play critical roles at a number of steps within the skeletogenic program. Early in limb development, FGF signaling is required for outgrowth and patterning of the limb. Perturbation of FGF signaling is associated with severe limb truncations

and increased mesenchymal cell death. In this regard, AER-derived FGFs signal to the underlying mesenchyme to maintain a population of skeletogenic progenitors by maintaining viability, promoting expansion and preventing differentiation. To better understand the molecular basis of FGF action, we have used cultures derived from the DM of the developing limb, a region underlying the AER. These cells exhibit a spectrum of responses to FGFs that are consistent with its activities *in vivo*. Microarray analysis coupled with subsequent functional analysis has provided unprecedented insights into FGF action in the limb. More specifically, we have identified critical linkages between FGF signaling and the NF- κ B pathway, as well as FGF-regulated cell proliferation via *Cdkn2b* and the MEK1 signaling pathway.

2.4.1 FGFs and Cell Proliferation

FGFs can both stimulate and inhibit cell proliferation. FGF-mediated growth arrest has been well described in chondrocytes, and animals containing an activating mutation of FGFR3 exhibit reduced chondrocyte proliferation whereas inactivation mutations lead to increased chondrocyte proliferation. Interestingly, cell proliferation is increased in the absence of *Fgfr1* in the limb mesenchyme of the early developing limb (Li et al., 2005). Thus, FGFs appear to play paradoxical roles in the regulation of cell proliferation. PLM cells also exhibit varied growth responses to FGF4 and 8. For example, FGF4 or 8 induces a transient increase in cell proliferation that is quickly accompanied by a return to baseline proliferation rates. Herein *Cdkn2b* has been identified as mediating the growth suppressing activities of FGFs in mesenchymal cells. As has been demonstrated recently in chondrocytes, FGF-induced growth arrest requires the MEK1/ERK pathway, and herein this

pathway has also been shown to be necessary for FGF induction of *Cdkn2b* (Krejci et al., 2008; Raucci et al., 2004). Other studies have reported an important role for the Rb proteins p107 and p130 in FGF induced growth arrest in chondrocytes (Dailey et al., 2003; Raucci et al., 2004). FGF treatment was associated with dephosphorylation of p107 and p130 within 9-18h. Increased expression of CDKNs causes hypophosphorylation of Rb pocket proteins (Ashizawa, et al., 2001). Thus, these observations are also congruent with a role for FGF4-mediated growth arrest through *Cdkn2b*.

TGF β 1 has been previously reported to induce *Cdkn2b* expression through a mechanism involving SMAD-mediated downregulation of *Myc* and formation of a SMAD-activator complex on the *Cdkn2b* promoter (Seoane et al., 2001; Staller et al., 2001). Microarray analysis indicates that the various components (*Myc*, *Miz1*, *Smad2*, 3) of these complexes appear to be expressed (detected by DNA microarray) in DM cells, however, TGF β 1 addition did not influence *Cdkn2b* expression. Further, DM cells exhibit appropriate responses to TGF β 1, suggesting that TGF β 1 regulation of *Cdkn2b* expression is cell-context dependent.

Previous studies have shown that the cyclin-dependent kinase inhibitors *Cdkn2a* and *Cdkn2b* are regulated by the MEK1/ERK pathway (Gil and Peters, 2006; Malumbres et al., 2000), however, the linkage between FGF signaling and *Cdkn2b* expression has not been established. *Cdkn2b* and the closely linked *Cdkn2a* are both up-regulated by activation of MEK1/ERK; in accordance with these reports, microarray analysis conducted in our study reveals that *Cdkn2a* expression is also induced by FGF4 (~ 3-fold). Thus, it seems plausible to suggest that

upregulation of *Cdkn2a* may also contribute to FGF4-mediated cessation of cell proliferation, however, knockdown of *Cdkn2b* restores growth in FGF4 treated cultures at 24 h, indicating that if *Cdkn2a* is involved its role is likely minor. Moreover, our findings also explain how an absence of FGF signaling may lead to increased cell proliferation as observed in *Fgfr* knockout animals. Furthermore, activation of FGF signaling or constitutively active FGFRs are associated with reduced chondrocyte proliferation; this has been recently reported to involve the MEK1/ERK pathway, which is congruent with an involvement of *Cdkn2b*. In this manner, FGF-mediated induction of *Cdkn2b* provides a critical feedback loop ensuring that cell proliferation is tightly regulated even in the continued presence of a growth-promoting factor. Collectively, our findings demonstrate that FGFs also negatively impact cell proliferation through regulation of *Cdkn2b*, thereby providing a plausible mechanism for the contradictory mitogenic activities of FGFs.

2.4.2 FGFs, NF- κ B and Cell Survival

Inhibition NF- κ B activity in the limb mesenchyme leads to severe skeletal reductions or truncations, and increased cell death, perhaps as a direct consequence of perturbation of NF- κ B activity in the limb mesenchyme (Bushdid et al., 1998; Kanegae et al., 1998). REL is normally sequestered in a complex containing I κ B within the cytoplasm, however with the appropriate signal, I κ B is degraded and thereby allowing NF- κ B to enter the nucleus to regulate gene expression. The NF- κ B subunit, *Rel*, is abundantly expressed in the progress zone of the developing limb (Kanegae et al., 1998), but it is currently unclear as to how NF- κ B signaling is activated in this region. Herein, we have demonstrated that the

NF- κ B pathway is activated in distal mesenchymal cells in response to FGF4 (and 8, data not shown). Activation of NF- κ B by FGF4 requires the MEK1/ERK signaling pathway, and is associated with increased expression of the NF- κ B activating kinase *Ripk4*. RIPK4 is a member of a family of Ser/Thr kinases that influences signal transduction pathways and leads to the activation of NF- κ B. *Ripk4* is expressed in the distal limb, is activated in the limb mesenchyme by FGF4, and overexpression of *Ripk4* activates NF- κ B activity. In serum-free medium (SFM), limb mesenchymal cells exhibit limited survival, however this can be rescued by the addition of FGF4 (and 8, data not shown). Inhibition of either the MEK1/ERK signaling pathway or the NF- κ B signaling pathway in SFM conditions leads to increased cell death, indicating that these pathways are important in regulating mesenchymal cell survival. Interestingly, animals deficient in *Ripk4* present with severe skin anomalies associated with abnormal keratinocyte differentiation, and limb defects characterized by shortened limbs and syndactyly (Holland et al., 2002), the latter of which also appear in various FGFR mutants (Ornitz, 2005).

Previous studies have demonstrated an antagonistic action between FGFs and BMPs in limb outgrowth (Niswander and Martin, 1993a). While these actions are largely recapitulated in cultures of limb mesenchyme, cell survival does appear to require some level of BMP signaling. Indeed, NOGGIN reduces cell survival in FGF4-treated cultures, albeit not to the same extent as BMP4. In more proximal-derived mesenchymal cultures, BMP4 was found to support cell survival, but not chondrogenesis (data not shown).

2.4.3 FGF Signaling and Chondrogenesis

Deletion of ectodermally-expressed FGF4 and 8 in the developing limb leads to a spectrum of limb defects, characterized by a progressive loss of more distal elements. From these studies it has been proposed that the role of FGFs in limb development is to control the expansion of skeletal progenitor populations through the regulation of cell survival, proliferation and commitment (Sun et al., 2002; Yu and Ornitz, 2008). Together, these activities ensure that there are sufficient cells for the development of the various skeletal elements. Herein, we have clearly demonstrated that FGFs influence cell proliferation and cell survival, and also the expansion of chondroprogenitors mainly through the formation of precartilaginous condensations. Indeed, treatment of limb mesenchyme cultures with exogenous FGF4 (or 8, data not shown) leads to a marked increase in the size of precartilaginous condensations. More significantly, addition of FGF4 alone in sub-confluent SFM cultures is sufficient to promote cell survival, cell proliferation, and the formation of pre-cartilaginous condensations. Consistent with this, the chondroblastic marker *Sox6* is markedly up-regulated during the culture period and cartilage nodules appear by day 10. Furthermore, with respect to the expansion of chondroprogenitors, FGF4 up-regulates the expression of several markers associated with progenitor populations, including the *Zics* and *Ly6a* (*Sca1*). Microarray analysis reveals that expression of *Ly6a*, *Zic2*, 3 and 5 increases with FGF-4 treatment (~ 6, 7, 3 and 5-fold, respectively), as validated using qRT-PCR. Interestingly, these *Zics* in addition to *Ly6a* are all expressed in the distal limb (Ma et al., 2002), with the *Zic* transcripts being detected in the mesenchyme underlying the AER, and *Zic2* being expressed

also within pre-cartilaginous condensations, but not in chondroblasts (Nagai et al., 1997). All of these genes co-cluster with *Ripk4*, are enriched in the distal limb and similar to *Ripk4* exhibit diminishing expression following culture that can be maintained with FGF4. Further evidence consistent with a role in regulating expansion of chondroprogenitors, FGF4 delays expression of the chondroblastic phenotype, as shown by reduced *Col2a1* expression (Fig. 1A, and data not shown) and decreased SOX5/6/9 reporter gene activity. In aggregate, this study provides mechanistic insights into FGF regulation of cell proliferation and survival, and evidence supporting a direct role for FGFs in the early chondrogenic program.

2.5 Methods and Materials

2.5.1 Reagents

FGF4, BMP4, Noggin, and TGF β 1 (R&D Systems) were purchased from R&D Systems. FGF4 was resuspended in primary culture medium for all experiments with the exception of the SFM experiments in which stock solutions were prepared in SFM. FGF4 was added to media at a concentration of 20 ng/ml, with the exception of SFM experiments in which FGF4 was added at a concentration of 50 ng/ml. Noggin was resuspended in sterile PBS containing 0.1% BSA, and added to media at a concentration of 200 ng/ml. BMP4 and TGF- β 1 were resuspended in a reconstitution buffer which consisted of 4 mM hydrochloric acid containing 0.1% bovine serum albumin and were added to media at a concentration of 20 and 10 ng/ml respectively. BAY 11-7082 (Sigma), RBI (SB 431542) (Sigma), and U0126 (Promega) were dissolved in DMSO (BDH) and added to a final concentration of 5 μ M, 1 μ M, and 10 μ M, respectively.

2.5.2 Plasmid Constructs

The reporter gene plasmid containing SOX9 binding sites (pGL3) was previously described (Weston et al., 2002), whereas the NF- κ B responsive luciferase reporter was purchased from Stratagene. The *Cdkn2b*-35- and *Cdkn2b*-463-luciferase reporter plasmids were provided by X-F. Wang (Duke University, Durham, North Carolina). Constitutively active *Mek1* (cMEK1) was obtained from Clontech. Dr. J. Tschopp (University of Lausanne, Epilanges, Switzerland) provided us with the *Ripk4* plasmid. *I κ b α* and *I κ b-2N* (Algarde et al., 1999) plasmids were obtained from Dr. S. Bernier (The University of Western Ontario, London, Ontario). As a control vector, pcDNA3.1+ (Invitrogen) was used.

2.5.3 Establishment and Transfection of Primary Limb Mesenchymal Cultures

Primary limb mesenchymal (PLM) cultures were established from CD-1 murine embryonic limbs (E11.5) as previously described (Hoffman et al., 2006; Weston and Underhill, 2000). Briefly, the distal tips (subridge regions extending ~0.3mm from the distal apex of the limb to the proximal cut edge) of these limbs were dissected and separated from the proximal regions (described in Gay and Kosher, 1984). The ectoderm was enzymatically removed from these two limb regions by dispase treatment and separated by filtration through a 40 μ M cell strainer (BD Biosciences) giving rise to a single cell suspension. PLM cells were pelleted by centrifugation at 200 X g and resuspended to produce a stock cell suspension at a concentration of 2×10^7 cells/ml. For microarray analyses, 12-15 10 μ l aliquots of this suspension were plated into a 35 mm tissue culture dish (Nunc) and allowed to adhere for 1 h. After this period, 2 ml of culture medium consisting of

60% Ham's F12 nutrient mix/40 % Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10 % FBS (Qualified, Invitrogen) were added to each well with or without 20 ng/ml FGF4 (R&D Systems); this time was considered T=0. Cultures were maintained for a period of up to 3 d; to minimize handling, culture media was replaced on alternate days. Alcian blue staining of PLM cultures was carried out as previously described (Hoffman et al., 2006).

For transfection, stock plasmid DNAs were diluted to a concentration of 1 mg/ml. For co-transfections, a ratio of 3:1 – gene of interest to reporter gene was used. Luciferase reporter genes consist of a gene of interest promoter/enhancer driven firefly (*Photinus pyralis*) luciferase, and a constitutively active control *Renilla* (*Renilla reniformis*) luciferase to normalize for transfection efficiency. The ratio of firefly luciferase reporter to *Renilla* luciferase used was 20:1. A stock of this reporter gene mix was used at 1 mg/ml. PLM cultures were transfected using Effectene™ (Qiagen). Seven and a half microlitres of Effectene-complexed DNA was added to 35 µl of the PLM cell stock. Of this mixture, 10 µl spots of PLM cells were dispensed in the centre of each well. Plates were incubated under standard tissue culture conditions for 45 minutes at which time 0.5 ml of primary culture medium was added per well and the plates were incubated for an additional 30 minutes. Medium was replaced with 1 ml of fresh primary culture medium supplemented with factors or vehicle. At 16 h post-plating, culture medium was replaced with fresh medium. At 24 h post-treatment, cells were washed with PBS and lysed in 100 µl of passive lysis buffer (Promega) for 20 min., and dual luciferase measured as previously described.

Cdkn2b knockdown was performed using siRNAs purchased from Dharmacon. SiRNAs were transfected into PLM cells using LipofectamineTM RNAiMAX (Invitrogen). For BrdU incorporation assays, cells were transfected in suspension with *siCdkn2b* and 10 μ l micromass cultures were established as outlined above. For experiments involving the collection of RNA, 12-15 siRNA-transfected cultures were plated per well of a 6-well plate (Nunc), and 2 ml of media were added one hour post-plating. RNA was collected as described below.

2.5.4 Assessment of PLM Cell Proliferation

Cell proliferation in PLM cultures was determined using the 5-bromo-2'-deoxy-uridine Labeling and Detection Kit I (Roche) - an immunofluorescence assay for the detection of 5-bromo-2'-deoxy-uridine (BrdU) incorporated into cellular DNA. PLM cells were prepared as previously outlined, and 10 μ l micromass cultures were established on 4-well chambered glass slides (Nunc) and incubated under standard tissue culture conditions. Culture medium was added at 1 h post plating, at which time FGF4 (20ng/ml) and vehicle control were added to separate wells. This was considered to be time 0. After 12 h, FGF4 and vehicle control were added to the remaining wells. At 23.5 h, BrdU labeling medium (Roche) was added to each well, and the slides were incubated for 30 min. Medium was aspirated and the slides were washed with PBS and fixed in an ethanol fixative consisting of 30% 50 mM glycine solution : 70 % absolute ethanol for 30 min at -20°C. The subsequent steps to detect BrdU positive cells were performed according to the manufacturer's instructions. BrdU positive cells were visualized using a FITC filter set on an Axiovert S100 inverted fluorescence microscope.

2.5.5 Rhodamine - Peanut Agglutinin (PNA) Labeling

Primary cultures were washed twice with PBS, and fixed in 4% paraformaldehyde for 30 minutes at 4°C. Fixative was aspirated, and cultures were rinsed once with PBS. Under reduced light, rhodamine-labeled PNA (Vector Labs) was diluted in PBS to a final concentration of 10 mg/ml, and added to each well such that the cultures were completely covered. Plates were protected from light, and stored at 4°C overnight. Rhodamine-PNA solution was aspirated and cultures were washed twice using PBS. PNA bound cells were visualized using epi-fluorescence microscopy with a Texas red filter set.

2.5.6 Culture of PLM cells in the Absence of Serum Factors

Using the previously described method of preparing PLM cell stocks, a cell suspension was prepared at a concentration of 5×10^5 cells/ml in SFM. Of this stock, 1.5 ml were dispensed per well of a 6 well plate ($\sim 8 \times 10^5$ cells/well). One hour following seeding, culture medium was aspirated and replaced with fresh SFM containing factors or vehicle controls. Cells were incubated overnight under standard tissue culture conditions. Medium was changed daily. Cells were washed once with PBS, fixed on day 11 with 95% ethanol at -20°C overnight, and subsequently stained with Alcian blue.

2.5.7 Limb Bud Organ Culture and Bead Implantation

Limb buds from ~E11.5 Col2-EGFP mice (CD-1 background; derived from breeding of heterozygous transgenic males with CD-1 females) (Grant et al., 2000) were collected in cold PSA. Affi-Gel blue beads (Bio-Rad Laboratories) soaked in either vehicle or FGF4 (20ng/ μ l) for 2 h were transferred into the interdigital region

(IDR) of the limb buds. Limb buds were cultured on Nucleopore Track-Etch membranes (Whatman) at the air–liquid interface on top of stainless steel mesh in 12-well tissue culture plates (Corning). PSA was aspirated from each well and replaced with BGJb medium (Invitrogen) containing 10% FBS and antibiotics. The level of culture medium was maintained such that it did not exceed the height of the membranes. Limbs were incubated under standard tissue culture conditions. After a 24 h incubation, EGFP expression was visualized using a fluorescence dissection microscope (model MZ12; Leica).

For isolation of RNA from organ cultures, culture media was aspirated and wells were washed 3 times with PBS. Limbs were removed from the membranes by agitation and transferred to 15 ml polystyrene conical tubes. Nearly all PBS was removed and 5 ml of RNAlater (Ambion) were added to each tube. Limbs were stored at -20°C. Using Graefe knives, areas excluding the beads and those including the beads were dissected and transferred to individual microfuge tubes containing 700 µl of RLT lysis buffer (Qiagen RNeasy kit). Dissected limb regions were homogenized in the RLT lysis buffer by triturating, and RNA was isolated as per the manufacturer's protocol.

2.5.8 Transcriptional Profiling with Microarrays: Experimental Design and Analysis

RNA was harvested from primary cultures using RNeasy (QIAGEN) according to the manufacturer's instructions. For the zero time point, cells were allowed to attach for 1 h and were subsequently processed for RNA isolation. For other cultures, the media was gently aspirated, and any remaining media was

blotted from the well before the addition of the lysis reagent. After isolation, the RNA was precipitated, and resuspended at 2 µg/ml; RNA quality was examined on a Bioanalyzer 2100 (Agilent), and the expression of *Sox9* and *Col2a1* were measured using real-time PCR. For each time point, a minimum of two biological replicates were analyzed on U74V2 chips A and B. Ten µg of RNA was labeled and hybridized to the chips using the manufacturer's recommended protocol. Gene expression profiles were subsequently analyzed using MAS 5.0 (Affymetrix) and GeneTraffic UNO bioinformatics programs (Stratagene). All datasets were initially filtered to remove genes called absent by MAS 5.0, and were further filtered as indicated in the text using GeneSpring. Hierarchical clustering was performed in GeneSpring using the tree function and a Pearson correlation similarity metric.

2.5.9 Quantitative Real-Time PCR

To measure the abundance of various transcripts qRT-PCR was performed using the 7500 FAST Sequence Detection System (Applied Biosystems). The primer/probe sets used for detection of *Sox9* and *Col2a1* were as described in Weston et al. (2002). For detection of all other transcripts, TaqMan Gene Expression Assays (Applied Biosystems) were used. Total RNA was isolated from primary cultures and limb sections as described above, and an aliquot was reverse transcribed to cDNA using a High Capacity cDNA Archive kit (Applied Biosystems). Quantification was performed using ~10 ng of total RNA and the expression of all genes relative to endogenous *rRNA* was determined using TaqMan Ribosomal Control Reagents (Applied Biosystems).

2.5.10 Statistical analysis

All luciferase assays were performed in quadruplicate and repeated using three distinct preparations of primary cells. Real-time PCR analysis, with the exception of the 24 h time courses, was performed in duplicate and repeated a minimum of three times with independent RNA samples. Proliferation data were analyzed by one-way analysis of variance, followed by a Bonferroni post test for multiple comparisons using GraphPad Prism, Version 5.0 (Graph-Pad Software, Inc.). Significance is represented as follows: *, $P < 0.05$; **, $P < 0.01$; #, $P < 0.001$.

2.6 Acknowledgements

We would like to thank Dr. Wang (Duke University, Durham, NC) for the *Cdkn2b* promoter constructs and Dr. Tschopp (University of Lausanne, Switzerland) for the *Ripk4* cDNA and the London Regional Genomics Centre for carrying out the microarray experiments. K. Garcha was supported by pre-doctoral award from the Stem Cell Network, L.M. Hoffman was supported by a post-doctoral fellowship from the Canadian Arthritis Network, and this research was funded by a grant to T.M. Underhill from the Canadian Institutes of Health Research (CIHR). TMU holds an Investigator award from The Arthritis Society.

2.7 Figures

Figure 2.1: FGF4 Regulates Expression of the Chondroblastic Phenotype and Proliferation of Limb Mesenchymal Cells. **A**, FGF4 treatment (20 ng/ml) of PLM cultures delays chondroblastic differentiation and increases the size of pre-cartilaginous condensations. PNA-rhodamine staining at day 2 (D2) of pre-cartilaginous condensations is greatly increased in FGF4-treated cultures in comparison to untreated controls. Analysis of *Col2a1* expression by whole-mount in situ hybridization (WISH) shows that FGF4 treatment (20 ng/ml) leads to weak *Col2a1*-expressing foci of larger size as compared to control cultures. Similarly, FGF4-induced expansion of pre-cartilaginous condensations leads to larger chondrogenic nodules in PLM cultures derived from Col2-EGFP mice. Compare EGFP-expressing cells at D7 in untreated versus treated cultures, the former cultures are nodular in nature, whereas the FGF4 cultures form a sheet of transgene-expressing cells. Further, in FGF4-treated cultures alcian blue staining is more diffuse and widespread. **B**, in organ culture FGF4 delays chondroblast differentiation. Implantation of a bead (white arrowheads) soaked in FGF4 (50 ng/μl) (F4) decreases transgene expression versus vehicle controls (V) in organ culture of E11.5 limbs derived from Col2-EGFP mice; limbs were visualized 24 h post-implantation. Limbs are excised and cultured on a stainless steel mesh at the air/media interface, following 1 day of culture, all of the skeletal elements across the proximo-distal (PD) axis can be observed. **C**, treatment of DM and to a lesser extent PM-derived PLM cultures with FGF4 (10 ng/ml) reduces SOX5/6/9 activity, suggesting that FGF4 inhibits chondroblast differentiation. **D**, FGF4 (20 ng/ml)

transiently increases cell proliferation as revealed by a significant increase in BrdU incorporation at 12 h, but not at 24 h. Magnification bars in A from top to bottom, bar represents, 0.25 mm, 0.3 mm, 0.25 mm and 1 mm.

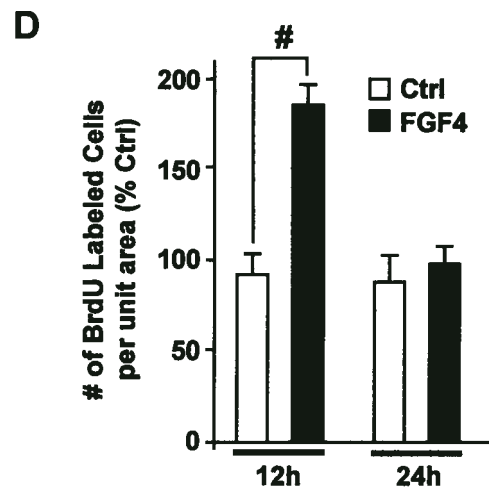
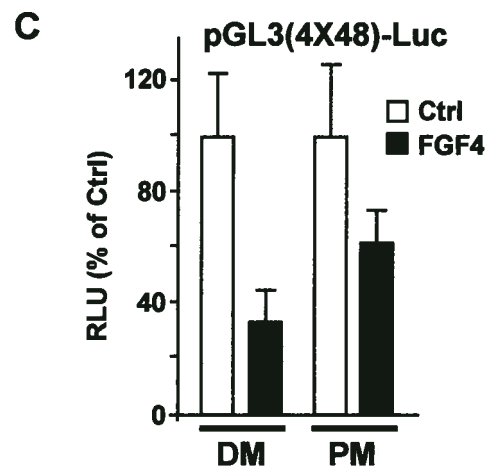
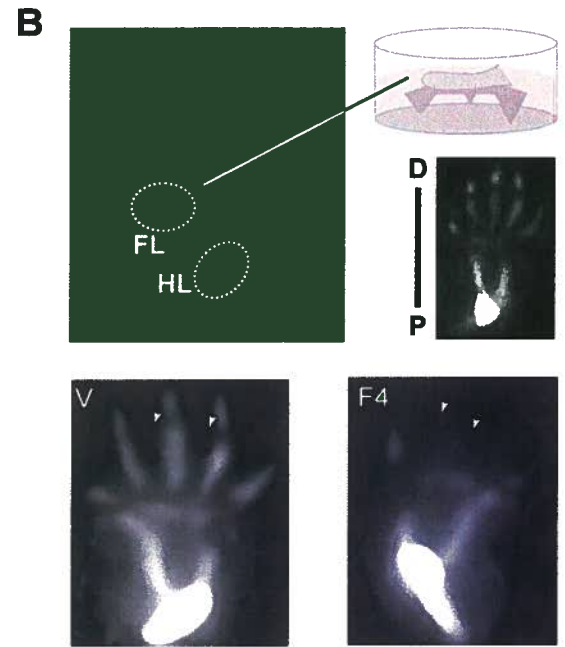
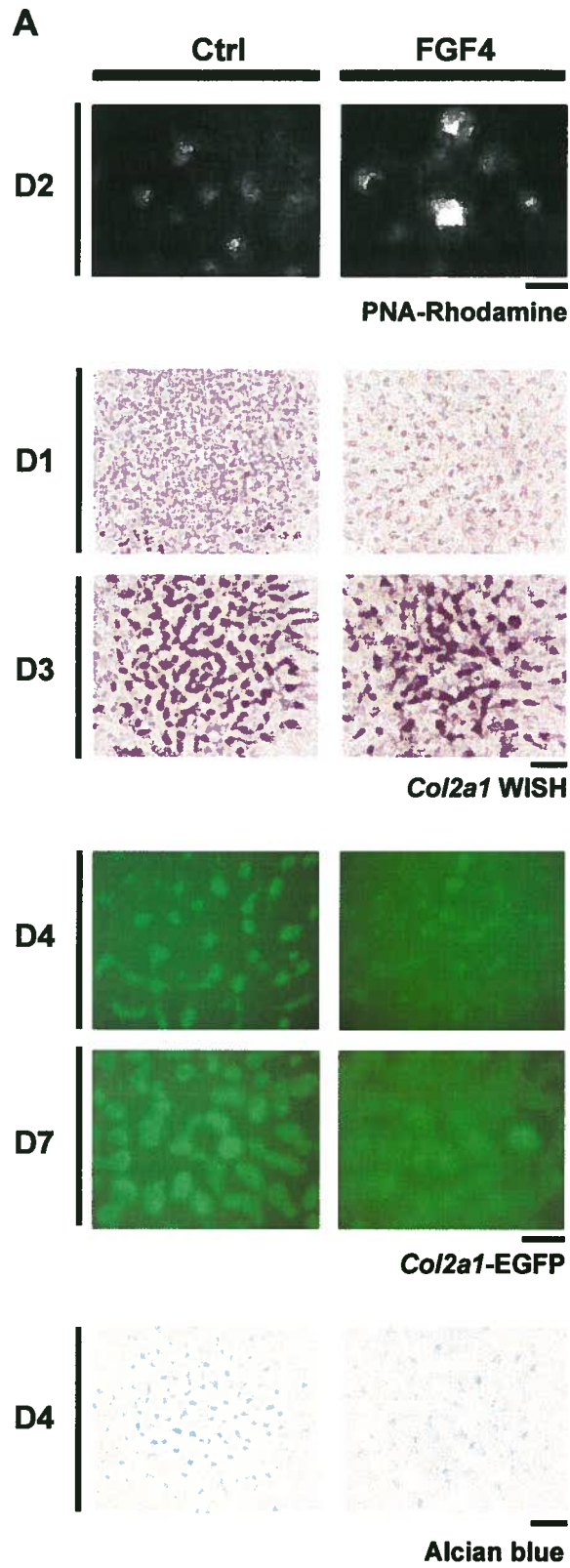


Figure 2.2: Identification of FGF4-Regulated Genes in Limb-Derived Distal Mesenchymal Cells. Hierarchical clustering in GeneSpring was performed using the Pearson correlation similarity measure to identify FGF-regulated genes exhibiting similar patterns of expression. Two-hundred and forty-five (3-fold cut-off) genes were clustered and several patterns emerged, four of which are shown. Many of the cartilage-expressed genes associated with chondroblast differentiation are down-regulated in FGF4-treated cultures (cluster 1), highlighted in red. Other clusters (2 and 4) contain genes that are up-regulated by FGF4 (highlighted in blue), whereas cluster 3 contains genes that are normally down-regulated upon culturing (highlighted in yellow), but whose expression is maintained in the presence of FGF4. Several genes connected with cell proliferation were also identified in FGF4-treated cultures including *Cdkn2b* (cluster 2) and *Tgf β 1* (cluster 3).

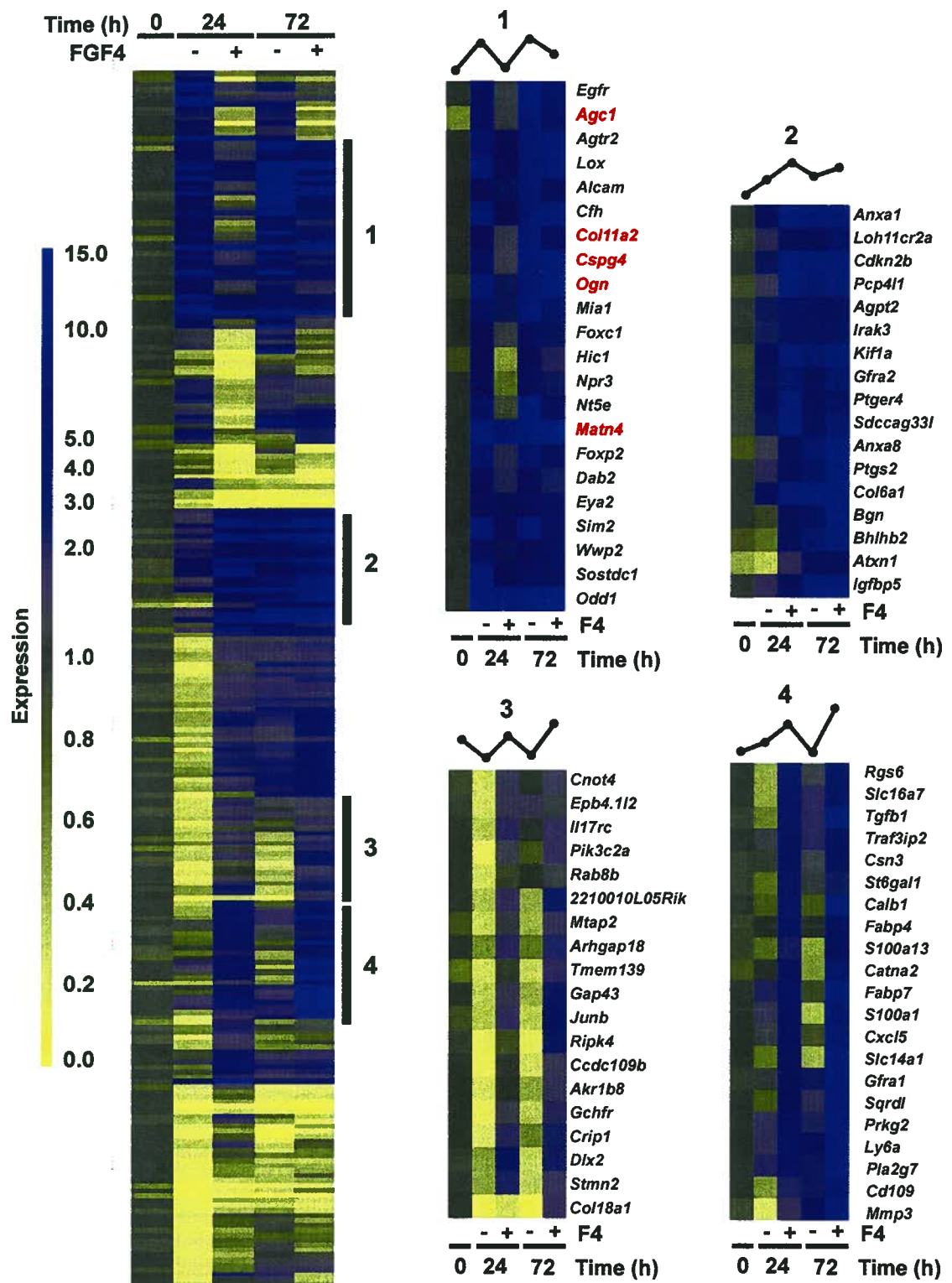


Figure 2.3: Cyclin Dependent Kinase Inhibitor 2b is Up-Regulated by FGF4 *in vitro* and in Organ Culture. **A**, FGF4 induced the expression of multiple members of the *Cdkn* family as determined by microarray analysis, and this is especially apparent for *Cdkn2b*, which is induced ~ 18 fold after 24 h of FGF4 treatment. All genes were called present in at least 4 samples. **B**, FGF4 induced the expression of *Cdkn2b* and CDKN2b as determined by qRT-PCR and western blotting, respectively. **C**, time course analysis of FGF4 induction of *Cdkn2b* by qRT-PCR revealed that within 2.5 h (arrowhead) *Cdkn2b* is induced ~ 10-fold in comparison to untreated cultures. **D**, in organ cultures, beads soaked in FGF4 lead to appreciable *Cdkn2b* expression in the regions surrounding the bead (BD), but less so in mesenchyme distal (DL) or proximal (PL) to the bead.

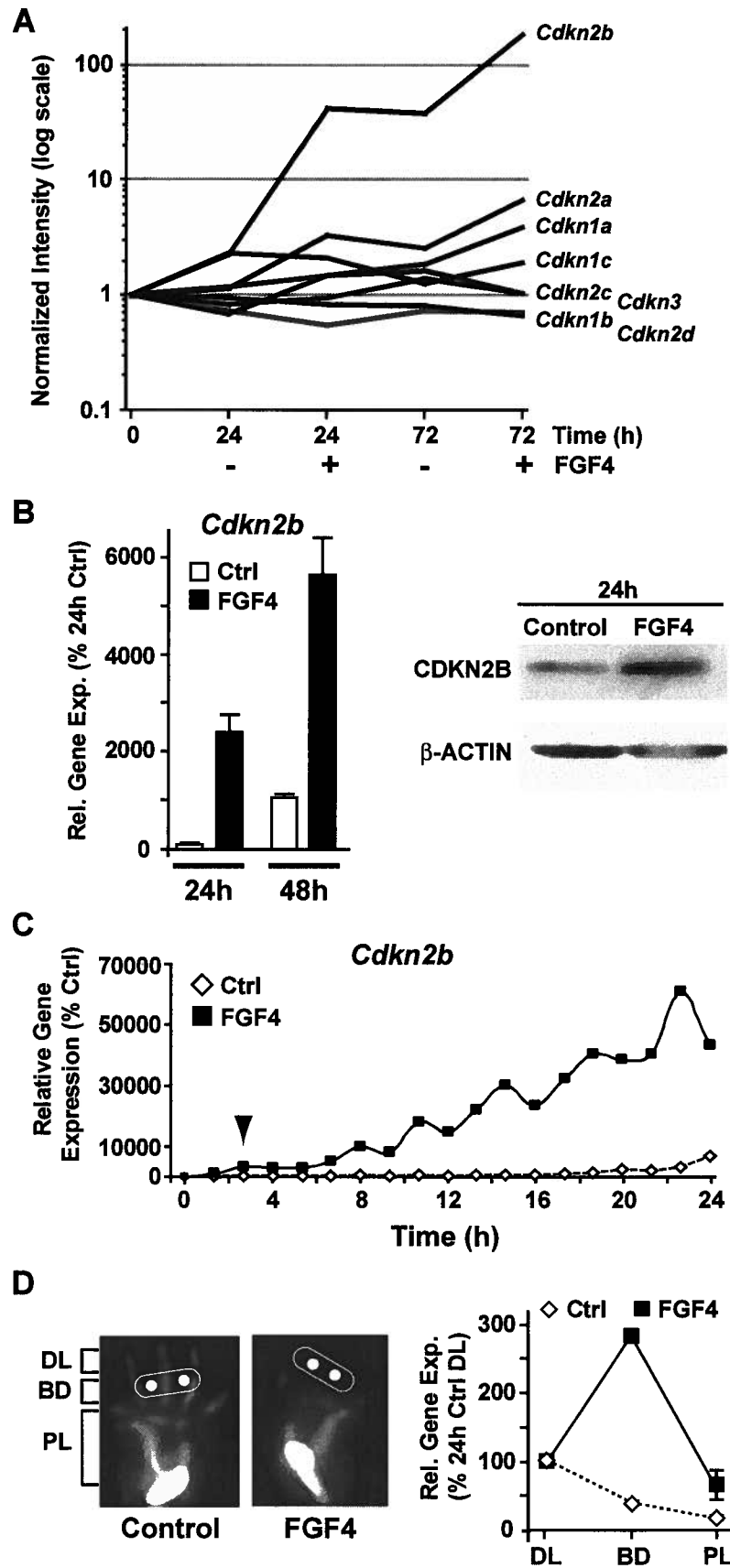


Figure 2.4: FGF4 Regulation of *Cdkn2b* Does Not Involve TGF β 1 Signaling. **A**, FGF4 (20 ng/ml) increases the expression of *Tgf β 1* both in PLM cultures and organ culture. **B**, TGF β 1 stimulates SOX5/6/9 activity and this can be inhibited by the TGF β 1R antagonist, SB 431542 (RBI). Further, TGF β 1 soaked beads stimulate ectopic cartilage formation in limb bud organ culture (inset). **C**, in comparison to FGF4, TGF β 1 does not stimulate the expression of *Cdkn2b* as determined by qRT-PCR. **D**, TGF β 1 does not stimulate the activity of a *Cdkn2b*-promoter derived reporter activity, whereas treatment with FGF4 does stimulate reporter gene activity. Note, this reporter contains the previously identified TGF β 1-responsive element.

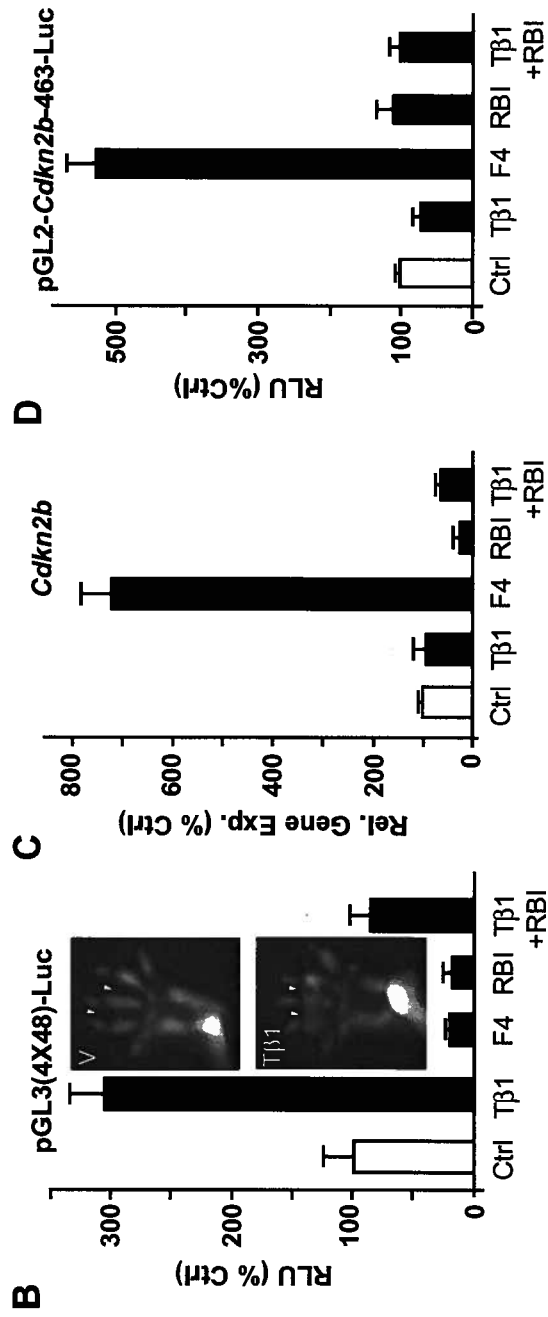
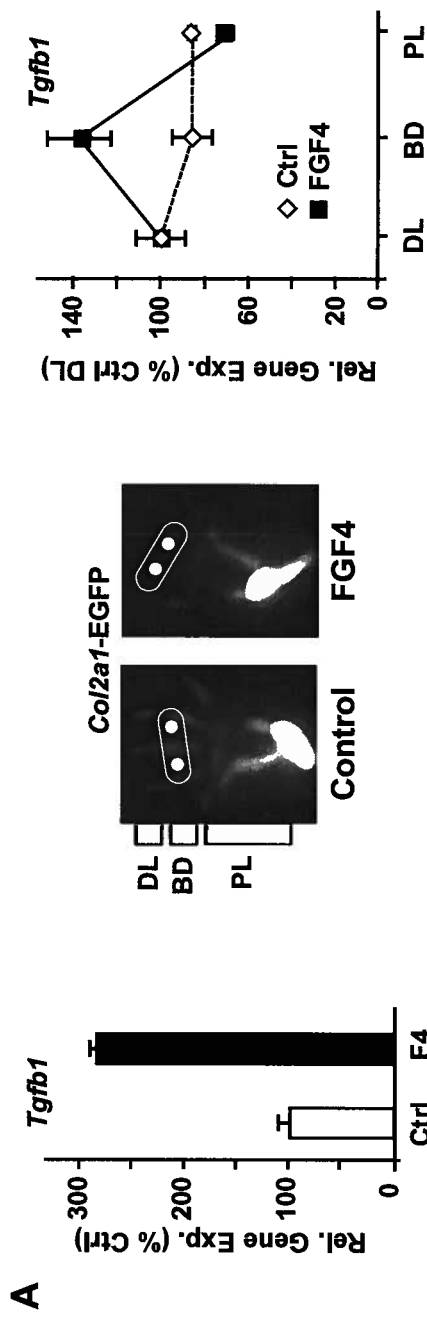


Figure 2.5: FGF4 Regulates Cell Proliferation Through *Cdkn2b* and This Involves the MEK1/ERK Signaling Pathway. **A**, induction of *Cdkn2b* by FGF4 (20 ng/ml) is abrogated in the presence of the MEK1 inhibitor U0126 (10 μ M). **B**, FGF4 (20 ng/ml) induces the reporter activity of a *Cdkn2b*-reporter encompassing the proximal region of the promoter (-463), and this reduced to less than control levels by U0126 (10 μ M). **C**, heterologous expression of a constitutively active version of MEK1 (cMEK1) induces activity of the -463 *Cdkn2b* reporter. **D**, siRNA-mediated knockdown of *Cdkn2b* in PLM cultures increases cell proliferation in comparison to siCtrl-transfected cultures. The extent of knockdown was determined using qRT-PCR and found to be ~ 65 %.

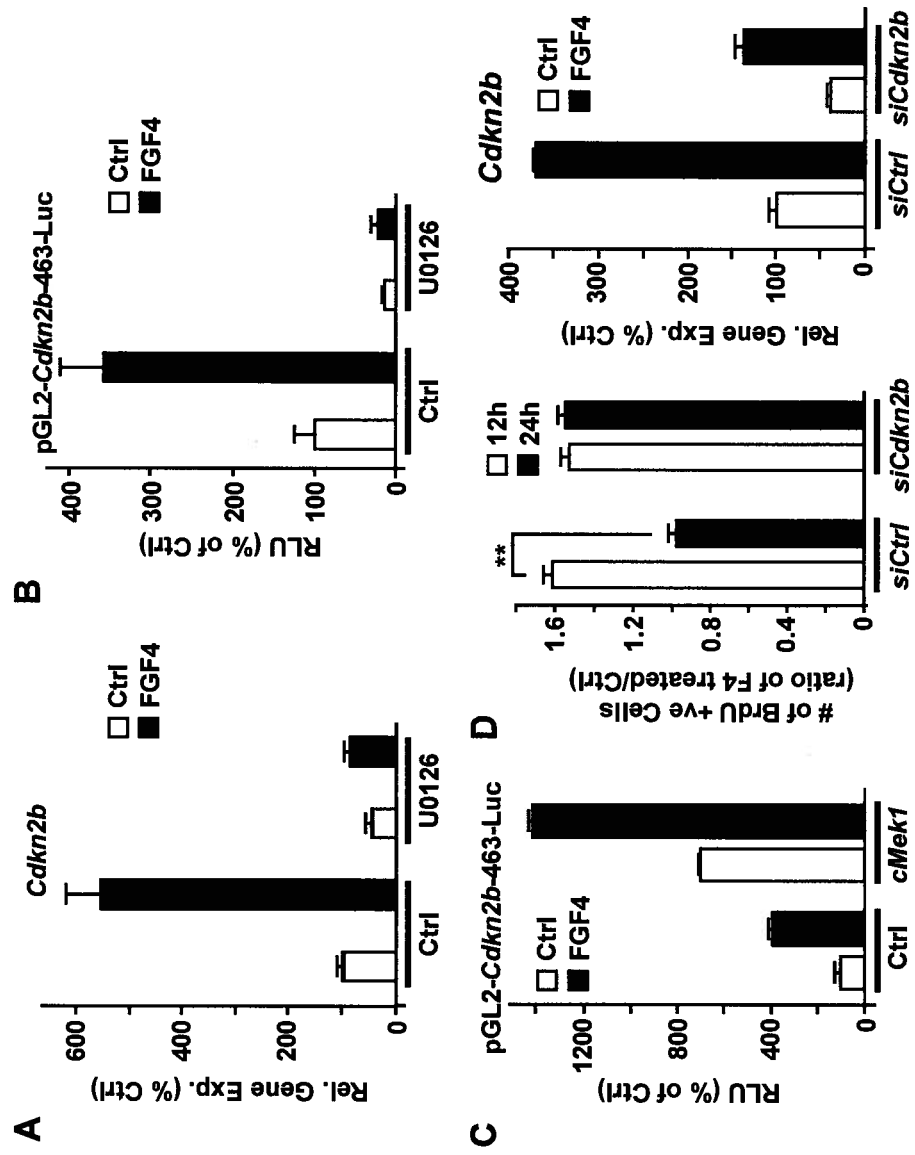


Figure 2.6: FGF4 Activates the NF- κ B Signaling Pathway in Part Through MEK1/ERK-Mediated Upregulation of *Ripk4*. **A**, FGF4 (20 ng/ml) treatment for 24h induces the activity of an NF- κ B reporter gene and this can be effectively inhibited by co-expression of either a construct encoding I κ B or a stabilized version of I κ B, I κ B-2N. **B**, the expression of *Ripk4*, an activator of the NF- κ B pathway, progressively declines following culture of PLM cells, however, *Ripk4* expression is maintained with FGF4 treatment (20 ng/ml). **C**, *Ripk4* transcripts are more abundant in distal limb sections (#1) as compared to proximal sections (#2-5). **D**, overexpression of *Ripk4* induces NF- κ B activity in PLM cultures and this is further increased in the presence of FGF (20 ng/ml). **E**, expression of *Ripk4* stimulates activity of the SOX5/6/9 reporter gene in PLM cells, however, this is attenuated for the most part by the addition of FGF4 (20 ng/ml). **F**, FGF4-mediated induction of *Ripk4* expression is inhibited by U0126. **G**, expression of a constitutively active MEK1 in PLM cells induces the expression of *Ripk4*. **H**, NF- κ B reporter activity is substantially decreased in U0126-treated PLM cultures and this can be partially rescued by overexpression of *Ripk4* at 24 h post transfection.

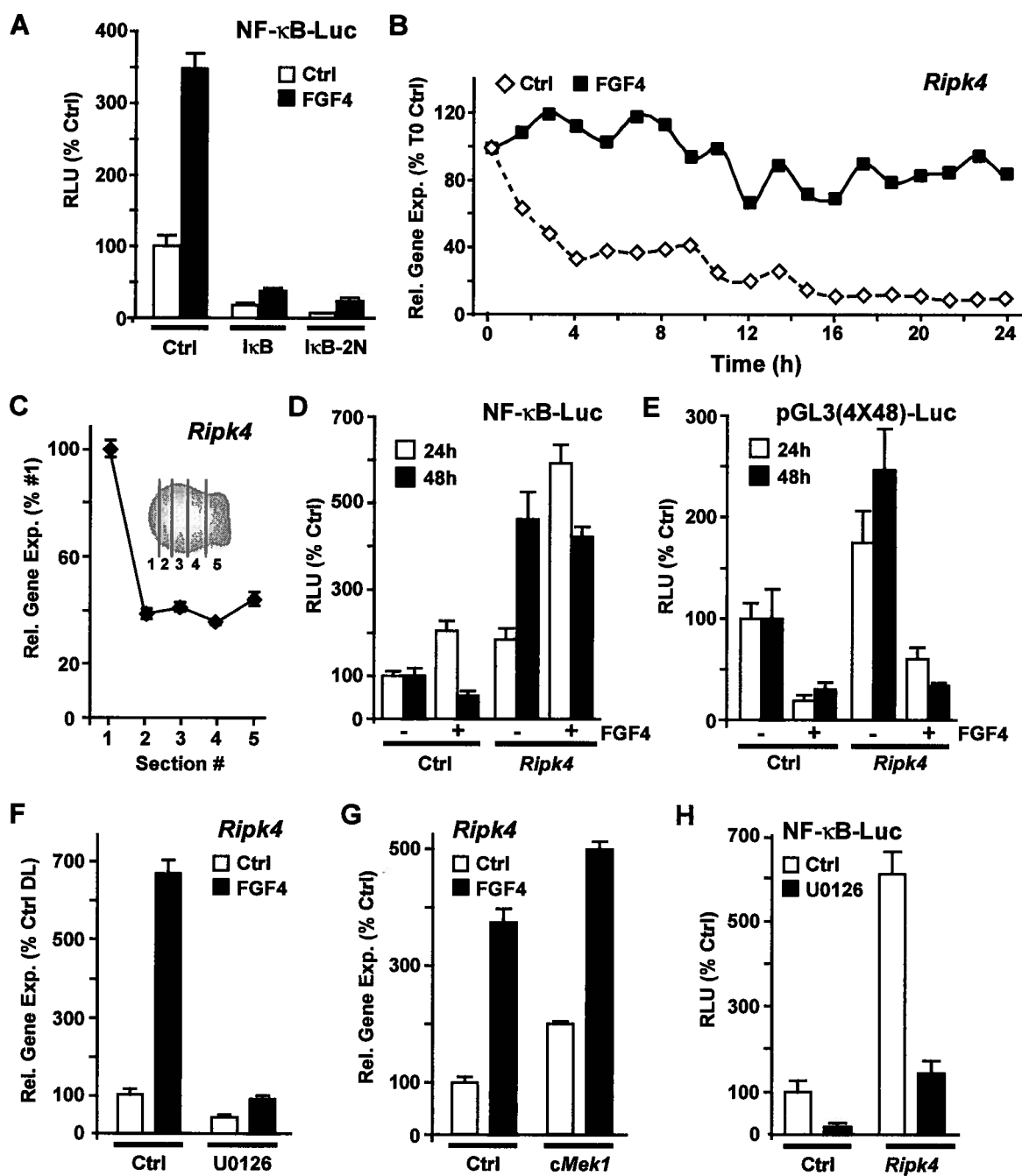


Figure 2.7: The MEK1/ERK and NF- κ B Signaling Pathways are Both Required for FGF4-Mediated Cell Survival and Cartilage Formation. **A**, a low-density serum free culture model was developed to assess the role of FGF4 and various signaling pathways in mesenchymal cell survival. In the absence of FGF4, no viable cells could be detected after 6 days of culture, and the addition of U0126 (10 μ M) or the NF- κ B inhibitor BAY 11-7082 (5 μ M) reduced cell viability to 4 and 1 days, respectively. The addition of FGF4 (50 ng/ml) markedly stimulated cell number, however, this was inhibited by the addition of either U0126 or BAY 11-7082. The addition of BMP4 (10 ng/ml) also negatively influenced cell viability in the presence or absence of FGF4, whereas, NOGGIN (100 ng/ml) reduced cell viability in older cultures (> 4 days). **B**, FGF4 is sufficient to stimulate chondrogenesis in sub-confluent monolayer PLM cultures. After 1 day (D1), there are visibly more cells in the FGF4-treated cultures in comparison to controls. By day 10 (D10), numerous chondrogenic nodules can be visualized in the FGF4-treated cultures and these can be stained with alcian blue. Further, several regions of PNA-rhodamine staining (arrowheads) can be visualized in the FGF4-treated cultures and at higher magnification, intensely PNA-positive cells (arrows) can be visualized overlying weaker PNA-positive cells (bright field). These latter cells exhibit a honeycomb-like pattern of PNA binding (arrowhead), consistent with condensation formation. **C**, the Sox genes 6 and 9 are dynamically expressed under these conditions. Sox6 and 9 exhibit 2 waves of expression, the first wave appearing just before overt condensation (D4) followed by a large increase in Sox6 from D7-10 that accompanies chondroblast differentiation. Magnification bars, in B, top panel left to

right, the bars represent, 0.25 mm, 0.25 mm, 0.5 mm and 0.5 mm; bottom panel, left to right, the bars represent 0.25 mm, 0.06 mm and 0.06 mm.

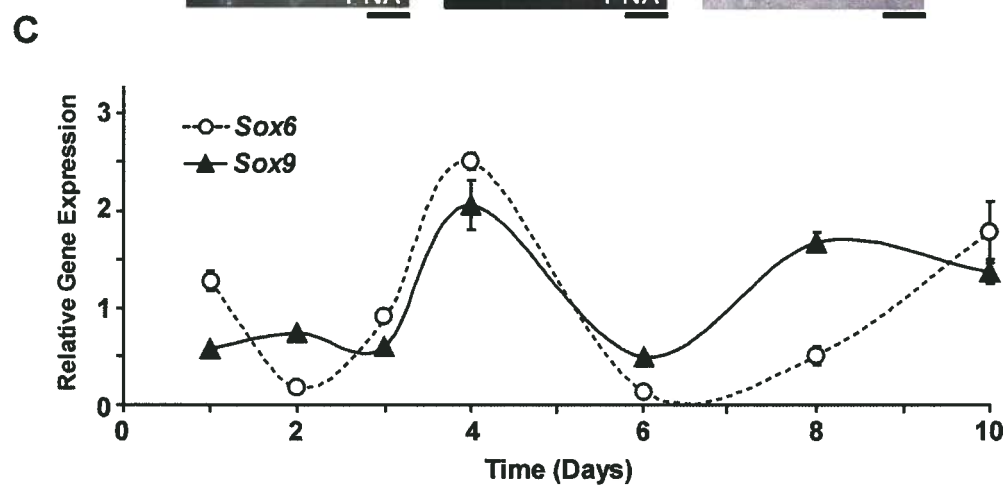
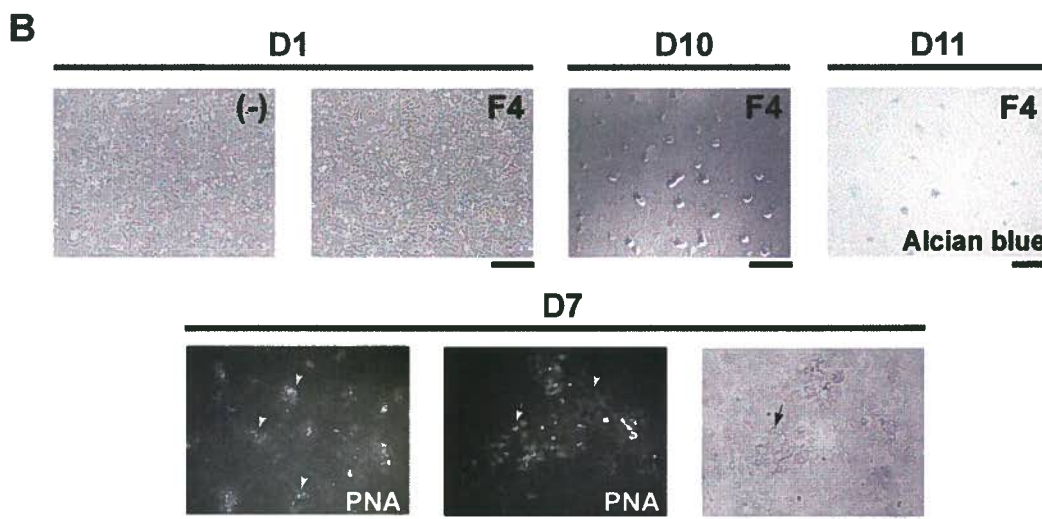
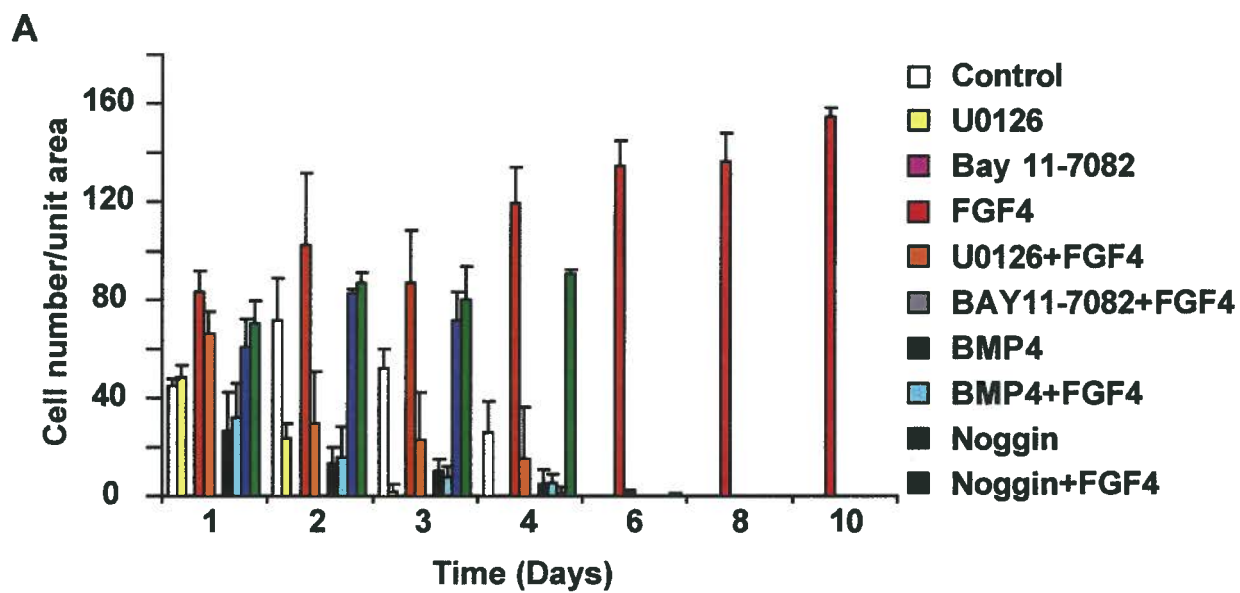


Table 2.1: qRT-PCR Analysis of Selected Genes in E11.5 DM Cultures

| Gene | 24h | | 48h | |
|----------------------|-------------|-------------|-------------|-------------|
| | Ctrl | FGF4 | Ctrl | FGF4 |
| <i>Bhlhb2</i> | 1.0 | 4.6 | 5.2 | 4.5 |
| <i>Dach1</i> | 1.0 | 1.5 | 1.4 | 2.1 |
| <i>Eomes</i> | 1.0 | 0.1 | 0.5 | 0.3 |
| <i>Foxp2</i> | 1.0 | 0.2 | 1.6 | 0.1 |
| <i>Ly6a</i> | 1.0 | 7.9 | 1.0 | 6.7 |
| <i>Runx1</i> | 1.0 | 4.6 | 2.0 | 3.8 |
| <i>Sim2</i> | 1.0 | 0.1 | 2.0 | 0.5 |
| <i>Zic2</i> | 1.0 | 6.6 | 0.2 | 3.9 |

*** Fold change is determined by normalization to untreated 24h control culture.**

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Chapter 3:

Chemical Genetics Reveals a Novel Role for Potassium Channels in Chondrogenesis

A version of this chapter will be submitted for publication. Garcha, K., and T. M. Underhill. Chemical Genetics Reveals a Novel Role for Potassium Channels in Chondrogenesis.

3.1 Abstract

To gain insights into the molecular programs regulating chondrogenesis a chemical genetics approach was employed. Implementation of these strategies involved the development and validation of screens in primary cultures of murine limb mesenchymal (PLM) cells. PLM cultures closely recapitulate *in vivo* chondrogenesis and within 2-3 days in culture, chondrogenic progenitors differentiate into matrix-secreting chondroblasts. Chondroblast differentiation is associated with increased SOX5/6/9 activity and a SOX5/6/9-responsive reporter gene was used to follow expression of the chondroblastic phenotype. Compound libraries representing ~ 1,500 compounds were screened in 384-well format, and 28 compounds were found to increase reporter gene activity > 2.5 fold. In secondary screens, 7 of 28 active compounds stimulated cartilage formation as assessed by alcian blue staining. Interestingly, one of these compounds, butamben (butyl 4-aminobenzoate), a member of the benzocaine family of analgesics, exhibited pro-chondrogenic activity. The high affinity target of this compound is the potassium channel, KCND2, which through transcriptional profiling was found to be downregulated by bone morphogenetic protein (BMP) 4. Further, butamben could rescue cartilage formation in the presence of the BMP antagonist, NOGGIN. Together, these results reveal an unanticipated and novel role for potassium channels in chondrogenesis.

3.2 Introduction

Cartilage plays a fundamental role in the development of much of the skeleton, as long bones are formed from a cartilage precursor, which is derived from condensed mesenchyme. Numerous factors and pathways have been shown to play an important role in regulating the chondrogenic program. These studies have been greatly aided by the development of *in vitro* primary cultures that faithfully recapitulate the *in vivo* sequence of events. High density or micromass cultures of primary limb mesenchymal (PLM) cells can be established from the embryonic limb mesenchyme and within a short period these cultures give rise to *bona fide* cartilage nodules. Two main stages can be readily identified, precartilaginous condensations and cartilage nodules. Initially prechondrogenic cells aggregate to form precartilaginous condensations and subsequently cells within the centre differentiate into chondroblasts. Critical to these steps is SOX9, a transcription factor belonging to the Sry-related high mobility group (HMG) box gene family, whose expression presages and regulates the expression of type II collagen (*Col2a1*) (Lefebvre et al., 1997; Ng et al., 1997). Sox9 is required for both commitment and differentiation of chondroblasts. The onset of chondroblast differentiation is associated with increased expression of *L-Sox5* and *Sox6*, and together with Sox9 they increase the expression of *Col2a1* and other chondrogenic genes. By transfecting PLM cultures with a firefly luciferase based reporter gene consisting of four, 48bp- fragments of the *Col2a1* enhancer, to which SOX5/6/9 have been shown to bind, our group among others have demonstrated a reliable means of assessing the status of chondroblast differentiation (Hoffman et al., 2006; Lefebvre et al., 1997; Ng et al.,

1997; Weston et al., 2002; Weston et al., 2000). Using this assay, in previous studies we showed an inverse correlation between the activity of the retinoid signaling pathway and chondroblast differentiation (Weston et al., 2000). Molecules that enhance retinoid signaling interfere with chondroblast differentiation and this is associated with decreased SOX5/6/9 activity. In addition to following SOX5/6/9 activity in PLM cultures, monitoring activity of the retinoid pathway with a RA responsive reporter gene has been found to provide a useful measure of cartilage formation (Weston et al., 2002). Recent studies have also shown that the pro-chondrogenic BMPs regulate cartilage formation in part by decreasing activity of the retinoid signaling pathway (Hoffman et al., 2006).

Chemical genetics has proven a powerful approach to identifying new pathways and targets operating within a biological program (Yeh and Crews, 2003). Further, this strategy has enabled identification of useful molecular tools in which to manipulate molecular and cellular processes. Herein, a chemical genetics strategy was implemented to identify novel modulators of the chondrogenic program. High throughput screens utilizing PLM cultures were developed and validated in 384-well format and small molecule libraries were screened to identify compounds that influenced SOX5/6/9 activity; these compounds were also tested on a retinoic acid response element (RARE) reporter. Several small molecules with pro-chondrogenic activity were identified and one of these, a potassium channel modulator, appears to function downstream of BMP signaling in chondrogenesis.

3.3 Results and Discussion

To better understand the molecular mechanisms underlying chondrogenesis we previously developed low throughput reporter gene-based assays to interrogate gene function in this program (Hoffman et al., 2006; Weston et al., 2002). The premise for the assay was based on the observations that at the onset of chondroblast differentiation *Col2a1* expression is greatly elevated and this is preceded by increased expression of L-Sox5 and 6. To efficiently follow this event, a reporter gene derived from the *Col2a1* gene containing binding sites for SOX5/6/9 was used and subsequently shown to provide an accurate measure of the status of chondroblast differentiation (Hoffman et al., 2006; Weston et al., 2002). Briefly the assay involves co-transfection of PLM cultures at the time of seeding and analysis of reporter gene typically at 48 h post-transfection. Importantly, it is during this time frame that chondroprogenitors in the culture differentiate into chondroblasts. In its present incarnation, the chondrogenic assay was implemented in a 24-well format, which limited throughput as PLM cultures had to be transfected and seeded manually. Further, PLM cells have to be cultured at high-density and isolated on the day of transfection as low-density cultures or prior culturing limits their chondrogenic potential. To enable implementation of robust large-scale screens for chondrogenic modulators, methods were developed to increase transfection efficiency while permitting storage of a prepared DNA/transfection mixture.

To improve PLM transfection efficiency several transfection reagents were evaluated and Effectene was found to exhibit greater transfection activity in PLM cells than other reagents such as Fugene6 (Roche) (data not shown). To further

streamline and increase transfection efficiency and throughput, methods were developed whereby DNA/transfections could be prepared en masse and stored. For this purpose, various disaccharide stabilizers were tested for their potential to enhance the integrity of the lipid-based EffecteneTM transfection reagent and thereby allow longer-term storage. Disaccharides have been used in both cryopreservation and desiccation to enhance preservation of biologics or cells (Beattie et al., 1997; Chen et al., 2000; Crowe et al., 1998; Crowe et al., 1994; Leslie et al., 1995). The EffecteneTM transfection methodology condenses DNA in a buffered solution (EC buffer) and to this buffer the disaccharide sucrose or trehalose was added to a final concentration of 0.4M. Inclusion of either 0.4M sucrose (S) or trehalose (T) to the EffecteneTM EC buffer led to an increase in transfection efficiency of PLM cultures (Fig. 3.1A). Transfection efficiency with 0.4M sucrose supplementation was increased ~1.7 fold, and that of 0.4M trehalose containing mixtures increased 2.7 fold as determined by measuring the activity of phRL-SV40. This plasmid expresses *Renilla* luciferase under the control of a constitutive SV40 promoter/enhancer (Fig. 3.1A). Importantly, the addition of either sucrose or trehalose to the transfection mixtures does not affect culture biology, as the addition of BMP4 (20 ng/ml) led to similar increases in the activity of a SOX5/6/9-responsive reporter gene [pGL3(4X48)] (Fig. 3.1B). To test the ability of the disaccharides to enable short and long-term storage of DNA-EffecteneTM transfection mixtures, aliquots of these transfection formulations were stored at -20⁰C for a period of 1 month or 3 years. Following which time they were transfected into freshly isolated PLM cells, and cultured in the presence or absence of BMP4. Disaccharide containing preparations

retained a minimum of ~80% transfection activity following 1 month of storage, however standard formulations showed a ~ 40 fold reduction in transfection efficiency (Fig. 3.1A). Unexpectedly, after 3 years of storage the disaccharide-containing transfection mixtures still retained robust activity that was > 15,000 fold higher than control transfections (Fig. 3.1A). It is difficult to make a direct comparison between the 2 groups (1 month versus 3 year storage), as serum lot and transfection lots are different. Nonetheless, in comparison to control transfections, the addition of either disaccharide (trehalose afforded a 4-fold greater protection than sucrose) enabled short or long-term storage of the DNA/transfection mixture with minimal loss of activity (Fig. 3.1A). Further, as observed in the fresh and short-term transfections, BMP4 exhibited similar trends in biological activity in the disaccharide-containing 3-year stored DNA/transfection mixtures. Together, these results demonstrate that supplementation of transfection mixtures with trehalose improves transfection efficiency in addition to permitting long-term storage with minimal loss of transfection activity.

The increased transfection efficiency afforded by the inclusion of trehalose into the transfection reagent was key in enabling implementation of screens in 384-well plates. Previous attempts in adopting this higher-density format were met with problems related to reduced transfection efficiency, and consequently higher variability and lower signal/noise ratios (Garcha and Underhill, unpublished). Transfection methodology and cell density was optimized for the 384-well format (Fig. 3.2A). Optimization was carried out with a series of co-transfections with known chondrogenic-modulatory genes at various cell densities and compared to

activity in assays conducted in standard 24-well format (Fig. 3.2B). Consistent with previous studies, modulation of the retinoid and/or TGF β /BMP signaling pathways affected activity of the SOX5/6/9-responsive reporter (Fig. 3.2B) (Hoffman et al., 2006; Weston et al., 2002). Inhibition of retinoid signaling (*Cyp26a1*) or activation of BMP signaling (BMP4, *Bmpr1b*) was associated with increased reporter gene activity and *vice versa*, and this was in accordance with the previously reported effects of these pathways on chondroblast differentiation and cartilage formation (Hoffman et al., 2006). Further, overexpression of Sox5, Sox6 or a gene encoding an activated form of *Mkk6* (MKK6E) also increased reporter gene activity as reported previously (Lefebvre et al., 1998; Weston et al., 2002). In general, the 90,000 cells/well in 384-well plates correlated well with the results from the 24-well format and provided a reliable and robust read-out of SOX5/6/9 activity.

The aforementioned chondrogenic assay was used to execute a forward chemical genetics screen to identify chondrogenic-modulatory molecules, with the intention that the identity of these molecules would provide insights into the molecular programs regulating chondrogenesis. For these reasons, screens were initially carried out with compound libraries where some target information was available, such as natural product and drug libraries. Two collections were screened including, the Prestwick Chemical Library[®] and the Biomol Natural Products Library. The Prestwick Chemical Library[®] contains 1120 small molecules, 90% being marketed drugs and 10% bioactive alkaloids or related substances, whereas compounds included in the Biomol Natural Products Library (361 compounds) consist of highly purified natural products of known structure and pharmacological

activity. Compounds ($\sim 15 \mu\text{M}$) were transferred by manual pinning into 384-well plates ~ 16 h post-transfection and luciferase activity was measured following 24 h of subsequent incubation. Compounds that reduced Renilla luciferase activity 50% or more (with the exception of 1 compound, Cytochalasin B, see below) in both reporter gene assays (4X48 and RARE -a retinoic acid response element reporter gene—as an indicator of anti-chondrogenic activity) in comparison to controls were eliminated from further analysis as these were deemed to have cytotoxic activities, this yielded a list of 1418 compounds out of 1481. Based on previous reporter gene analyses with various factors, a 2.5 fold RLU cut-off was initially used for selection of molecules with putative anti-chondrogenic or pro-chondrogenic activity, and 65 and 28 compounds were found to meet this criterion, respectively (Fig. 3.3A, B). Of the compounds that reduced 4X48 activity, several CYP inhibitors were identified including the azole-containing compounds (i.e. ketoconazole, enilconazole, and butoconazole nitrate) that also stimulate RARE activity in addition to several retinoids (i.e. retinoic acid, isotretinoin and 9-cis retinoic acid). Ketoconazole has previously been shown to be both a potent inhibitor of chondrogenesis and to reduce 4X48 activity in PLM cultures (Hoffman et al., 2006). The 28 "pro-chondrogenic" compounds were further tested for their ability to stimulate chondrogenesis in histological assays (Fig. 3.3B). Of the 28, 7 compounds were found to stimulate alcian blue staining, while the other compounds were all found to inhibit alcian blue staining (Fig. 3. 3C). This latter observation may be a consequence of the fact that cultures stained for alcian blue were treated for 3 days with compound, as opposed to transfected cultures that were exposed for 1 day. These comments

notwithstanding, this screening strategy successfully identified several novel chondrogenic modulators with a hit frequency of 0.5% (7 of 1418 compounds). The emphasis for subsequent analyses was placed on alcian blue positive hits, as negative hits while informative could result from modulation of more generic pathways/targets.

The seven validated pro-chondrogenic compounds represent chemically distinct structures. Of these compounds cytochalasin B exhibited the greatest pro-chondrogenic activity as measured by reporter gene activity (Fig. 3.3B). Cytochalasin B functions primarily as a cytoskeleton disruptor by interfering with actin polymerization (Cooper, 1987). Interestingly, cytochalasin D shares this activity, but in these screens cytochalasin D or E had no effect (data not shown), whereas other reports have shown cytochalasin D stimulates Sox9 expression (Woods et al., 2005). In addition to its actions on actin polymerization, cytochalasin B has been shown to have additional activities distinct from cytochalasin D, which include inhibition of glucose transport (Cooper, 1987). It may be these additional properties which are responsible for and/or augment cytochalasin B's prochondrogenic activities.

Butamben (butyl 4-aminobenzoate) another identified pro-chondrogenic small molecule has at least 2 targets. Butamben belongs to the benzocaine class of analgesics, which are known modulators of sodium and calcium channels (Beekwilder et al., 2006). Therapeutically these compounds are used in the > 100 mM range. Interestingly, of this broad class of compounds, only butamben was found to significantly increase 4X48 reporter gene activity (Fig. 3.4A). In addition, to

interfering with calcium channels, butamben also inhibits potassium channels in particular, Kv4.2 (*Kcnd2*) (Winkelman et al., 2005). This latter property does not appear to be shared by the other structurally-related benzocaine family members and may partly explain the unique pro-chondrogenic activity of butamben (Fig. 3.4B). The dose range whereby butamben stimulates the 4X48 reporter activity and alcian blue staining is in the low micromolar range consistent with previous studies on butamben inhibition of potassium channels (Kv4.2 channel, butamben K_D 0.06 μ M; 35% inhibition) (Winkelman et al., 2005) (Fig. 3.4C, data not shown). In the developing limb, *Kcnd2* and *Sox9* are dynamically expressed and an increase in *Sox9* expression in limb sections 2-3 is preceded by a decrease in *Kcnd2* expression (Fig. 3.4D). Further, knockdown of *Kcnd2* in PLM cultures led to a modest increase in *Sox9* expression and this was comparable to butamben-mediated induction of *Sox9* following a 1 day treatment (Fig. 3.4E, F). To further assess the involvement of potassium channels in chondrogenesis, PLM cultures were treated with the broad-spectrum potassium channel blocker, 4-aminopyridine (4-AP) (Fig. 3.4G). Similar to butamben, 4-AP also stimulated 4X48 activity and cartilage formation as assessed by alcian blue staining (Fig. 3.4G and data not shown). In aggregate, these results suggest that butamben regulates chondrogenesis at least in part through modulation of potassium channel activity.

BMPs are potent regulators of the chondrogenic program, many of which exhibit robust pro-chondrogenic activity. Query of microarray datasets generated from BMP4-treated PLM cultures (Hoffman et al., 2006), revealed that BMP4 treatment reduced the expression of *Kcnd2* ~ 3 fold and this was subsequently

confirmed using qRT-PCR on PLM cultures (Fig. 3.5A, B). To test if modulation of *Kcnd2* by BMP4 was involved in BMP pro-chondrogenic activity, NOGGIN rescue experiments were performed (Fig. 3.5C, D). NOGGIN a BMP2, 4 and 7 antagonist, interferes with chondrogenesis both in PLM cultures and *in vivo* (Pizette and Niswander, 2000; Weston et al., 2000). Addition of NOGGIN (200 ng/ml) reduces 4X48 activity and decreases cartilage nodule formation in control and BMP4-treated cultures. The addition of butamben, partially rescues both 4X48 activity and cartilage formation in the presence of NOGGIN (Fig. 3.5C, D). Together, these results suggest that within the chondrogenic program, a reduction in KCND2 activity promotes expression of the chondroblastic phenotype. Further, KCND2 appears to function downstream of BMP signaling within chondrogenesis. However, it is not clear if BMPs affect chondrogenesis directly through down-regulation of *Kcnd2* or perhaps more likely, BMPs are known stimulators of chondroblast differentiation and *Kcnd2* is simply down-regulated as a consequence of differentiation.

Potassium channels play diverse roles in cell physiology and changes in their activity have been shown to regulate a number of processes including cell proliferation, differentiation, and death (Burg et al., 2008; Lang et al., 2005; Pardo, 2004). Potassium channel activity impacts cell proliferation and inhibitors of K channels have been shown to reduce cell proliferation in a variety of cell types (Pardo, 2004), thus butamben could be enhancing chondroblast differentiation through interfering with cell cycle progression and proliferation in chondroprogenitors, thereby indirectly stimulating differentiation. Alternatively, potassium channels also regulate cell volume and morphology and drug-induced

changes (such as with the cytochalasins) in chondroprogenitor morphology have been shown to trigger chondrocyte differentiation (Woods et al., 2005). Butamben could be acting through either of these two mechanisms, a combination thereof or through additional unknown pathways. Notwithstanding, butamben and the other pro-chondrogenic compounds will serve as useful tools for probing the pathways regulating the chondrogenic program.

3.4 Methods and Materials

3.4.1 Reagents

BMP4, and NOGGIN recombinant proteins were purchased from R&D Systems. NOGGIN was resuspended in sterile PBS containing 0.1% BSA, and added to media at a concentration of 200 ng/ml. BMP4 was resuspended in a reconstitution buffer which consisted of 4 mM hydrochloric acid containing 0.1% bovine serum albumin and was added to media at a concentration of 20 ng/ml. Butamben and 4-aminopyridine were obtained from Sigma, and stock solutions were prepared in DMSO.

3.4.2 Small Molecule Libraries

All small molecule libraries (Prestwick Chemical Library[®], Biomol Natural Products Library) screened were kindly provided by Dr. Michel Roberge (University of British Columbia) and obtained through the Canadian Chemical Biology Network. All compounds were tested at a final concentration of ~ 15 μ M.

3.4.3 Establishment and Transfection of Primary Limb Mesenchymal Cultures

Primary limb mesenchymal (PLM) cultures were established from CD-1 murine embryonic limbs (E11.5) as previously described (Hoffman et al., 2006; Weston et

al., 2002). Limb mesenchyme was dissociated by dispase treatment and a single cell suspension was obtained by filtration through a 40 μ M cell strainer (BD Biosciences). PLM cells were pelleted by centrifugation at 200 X g and resuspended to produce a stock cell suspension at a concentration of 2.0×10^7 cells/ml. Cells were used for transfection (see below) or for establishment of cultures for alcian blue staining. For the latter, 10 μ l of cells were spotted into the well of a 24-well plate, allowed to adhere for 1 h, following which culture medium consisting of 60 % Ham's F12 nutrient mix/40 % Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10 % FBS (Qualified, Invitrogen) was added to each well; this time was considered T=0. Cultures were maintained for a period of up to 4 d; to minimize handling, culture media was replaced on alternate days.

Transfection of PLM cells in 24 or 384-well format was carried out using similar methodology with Effectene reagent. For stabilization purposes, sucrose or trehalose (final concentration 0.4 M) was added to the Effectene EC buffer, and establishment of DNA/transfection mixtures was according to the manufacturers recommendations. Luciferase reporter genes consist of a firefly reporter gene, pGL3(4X48) or RARE, and a *Renilla* (*Renilla reniformis*) luciferase reporter phRL-SV40 to normalize for transfection efficiency. Reporter plasmids containing SOX9 binding sites (pGL3[4X48]) or trimerized retinoic acid response elements, RARE-luc (pW1bRARE₃tkLuc), were previously described (Weston et al., 2002). A ratio of 20:1 of firefly luciferase reporter to *Renilla* luciferase was used in all transfections. Co-transfections with genes-of-interest were set-up in a ratio of 3:1 of expression plasmid to reporter genes (firefly and *Renilla*). For 24-well plates, DNA/transfection

mixtures were combined with PLM cells (2×10^7 cells/ml) and 10 μ l of this mixture was spotted into the centre of the well. Plates were incubated under standard tissue culture conditions for 45 minutes at which time 1.0 ml of primary culture medium was added per well. Co-transfections in 384-well plates were carried out in a similar manner, with the exception that DNA/transfection mixtures were dispensed into wells, followed by media, cells, and an additional aliquot of media as described below.

For compound screening, transfections in 384-well plates were prepared in advance using trehalose containing EC buffer. Briefly, 4 μ l of EffecteneTM-complexed DNA was dispensed per well of the 384 well plate(s). Plates were centrifuged for 1 minute at 200 x g, sealed and stored at -20°C until needed. For transfection, plates were thawed and 45 μ l of primary culture medium was added to each well. PLM cells were isolated as described previously and 10 μ l containing 9×10^4 cells were dispensed into each well. Subsequently, 30 μ l of primary culture medium was pipetted into each well. Plates were incubated at 37°C in a humidified atmosphere and 5% CO_2 for 1 hour at which time culture medium was removed and replaced with 100 μ l of fresh medium. For both plate formats, culture medium was replaced ~ 16 hours post-plating with fresh medium. At this time, compounds were manually pinned (300 nl) into each well using a 96-well dispensing unit. At 24 hours post-treatment, cells were lysed and luciferase activity was determined according to the manufacturer's recommendations (Promega).

For alcian blue staining, culture medium was aspirated and cells were washed once with PBS. Cultures were fixed in 95 % ethanol at -20°C overnight.

Fixative was removed by aspiration and cells were sequentially washed once with PBS, followed by 0.2 M HCl. Cells were stained overnight with a 1 % Alcian Blue solution prepared in 0.2 M HCl.

3.4.4 RNA Collection From E11.5 Mouse Limbs

Using Graefe knives, E11.5 limb buds were serially sectioned and each region was transferred to individual microfuge tubes containing 700µl of RLT lysis buffer (Qiagen RNeasy kit). Limb sections were homogenized in the RLT lysis buffer by repeated pipetting, and RNA was isolated as per the manufacturer's protocol.

3.4.5 Quantitative Real-Time PCR

To follow the expression of transcripts for *Kcnd2*, and *Sox9* quantitative real-time PCR was performed using the 7500 Fast Sequence Detection System (Applied Biosystems). The primer/probe set used for detection of *Sox9* was as described in Weston et al. (2002). For detection of all other transcripts, TaqMan Gene Expression Assays (Applied Biosystems) were used. Total RNA was isolated from primary cultures and limb sections as described above, and an aliquot was reverse transcribed to cDNA using a High Capacity cDNA Archive kit (Applied Biosystems). Quantification was performed using ~10 ng of total RNA and the expression of all genes relative to endogenous *rRNA* was determined using TaqMan Ribosomal Control Reagents (Applied Biosystems).

3.4.6 Microscopy and Image Acquisition

Images of fixed cultures (in 70% ethanol) were collected at room temperature using a dissection microscope (Stemi SV11 Apo, S1.6x objective; Carl Zeiss MicroImaging, Inc.). Color images were acquired with a QImaging Retiga 1300i (12-

bit) camera and using Openlab 4 software (Improvision). Photoshop adjustments (brightness/contrast) were applied uniformly to all images.

3.4.7 siRNA Knockdown in PLM Cultures

Kcnd2 knockdown was performed using siRNAs purchased from Dharmacon (catalogue # L-042846-00) with the corresponding siRNA control (catalogue # D-001810-0X), and transfected into PLM cells using LipofectamineTM RNAiMAX (Invitrogen). PLM cells were transfected in suspension with *siKcnd2* and 10 μ l PLM cultures were established as outlined above. For experiments involving the collection of RNA, siRNA 12-15 transfected PLM cultures were plated per well of a 6-well plate (Nunc), and 2 ml of media were added one hour post-plating. RNA was collected as previously outlined.

3.4.8 Statistical Analysis

With the exception of the small molecule screens, all experiments were performed a minimum of 3 times. Luciferase assays were performed in quadruplicate using 3 distinct populations of primary cells. Quantitative real-time PCR was performed in duplicate and repeated a minimum of 3 times with independent RNA samples. Data were analyzed by one-way analysis of variance, followed by a Bonferroni post-test for multiple comparisons using GraphPad Prism Version 5.00 (GraphPad Software, Inc.). Significance is represented as follows: *, $P < 0.05$; **, $P < 0.01$, and *** $P < 0.001$.

3.5 Acknowledgements

We would like to thank Dr. Michel Roberge (University of British Columbia) for kindly providing us with access to the Prestwick and Biomol compound libraries and

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3.6 Figures

Figure 3.1. Disaccharides Such as Sucrose, and to a Greater Extent Trehalose, Increase Transfection Efficiency of Effectene™ and Facilitate the Storage of Transfection-Ready DNA. **A,** Comparison of transfection efficiency of control versus disaccharide containing mixtures (S, sucrose; T, trehalose); transfection efficiency was determined by transfection of a plasmid containing Renilla luciferase (RL) under the control of a SV40 promoter and measurement of RL-activity. PLM cultures treated with BMP4 (20ng/ml) or vehicle for 24 h showed no difference in reporter gene activity. The addition of 0.4M sucrose or trehalose increased *Renilla* reporter gene detection by approximately 1.5 and 2.3 fold, respectively in fresh preparations. Following 1 month or 3 years of storage at -20°C, DNA-Effectene™ mixtures containing 0.4M sucrose or trehalose maintained most of their transfection activity. Numbers over bars represent ratio of S or T over Ctrl. **B,** Addition of sucrose or trehalose had a negligible effect on PLM culture biology and BMP4 responsiveness. PLM cultures were co-transfected with pGL3(4X48) and phRL-SV40 and treated with BMP4 (20 ng/ml). In all cases, BMP4 increased RLU activity and similar inductions were observed after freezing for 1 month and to a lesser extent after 3 years of storage. All experiments were performed a minimum of 3 times using 3 distinct populations of cells. Error bars represent SD. Significance versus corresponding control ***, $P < 0.001$.

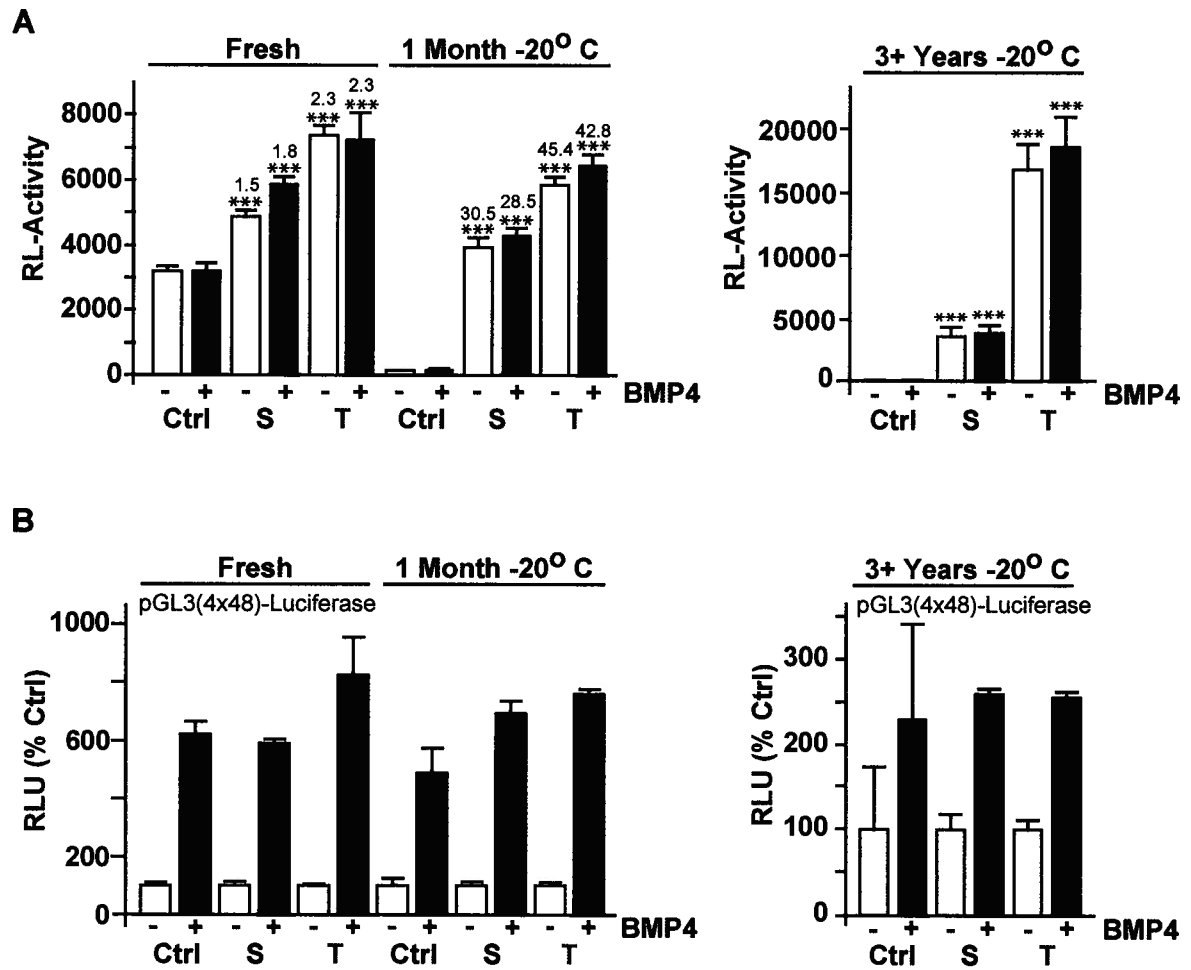
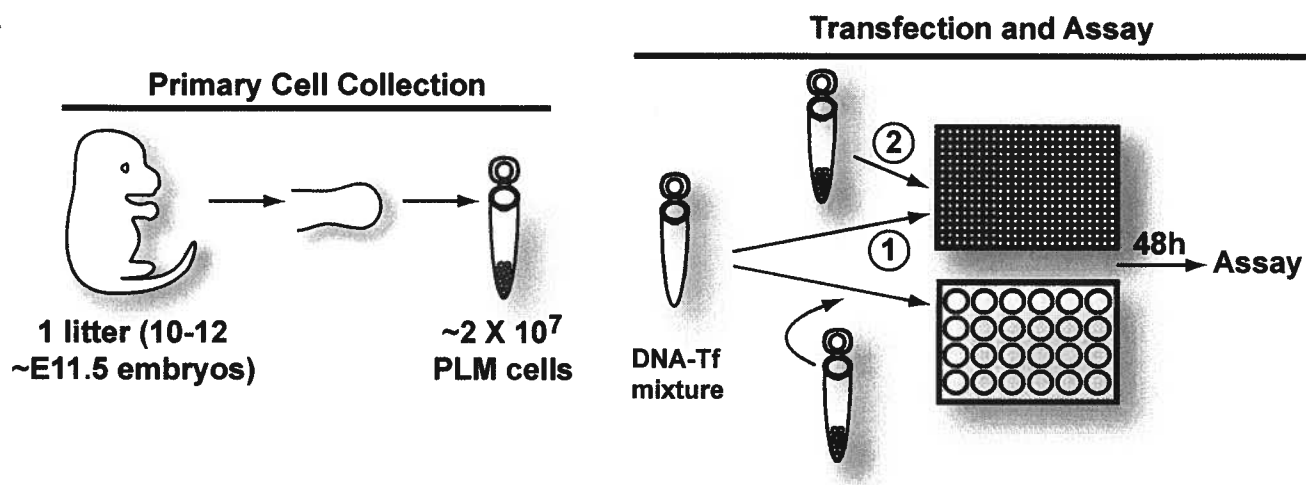


Figure 3.2. Optimization and Validation of 384-well Format for Chondrogenic Assays in PLM Cultures. Schematic representation of the 24 and 384-well transfection strategies. **A**, PLM cells are collected on the day of transfection from several litters of E11.5 mouse embryos. Previously prepared DNA/transfection (Tf) mixtures are dispensed into tubes, cells added and spotted into a 24-well plate. For transfections in 384-well plate, DNA/transfection mixtures are aliquoted into each well followed by cells. Reporter gene activity (luciferase, fluorescent protein) is measured 48 h post-transfection. **B**, Optimization of assays in a 384-well format. Cells were co-transfected with plasmids encoding known chondrogenic modulatory proteins along with pGL3(4X48) and phRL-SV40, and relative reporter gene activity was compared between standard 24-well assays versus various cell densities in 384-well format. Cell densities were as follows: 24-well: 165000; 384-well: 120000, 90000, and 60000 cells per well. Cells were treated with BMP4 (B4) (20 ng/ml) or vehicle control. Data were normalized to vehicle treated control (Ctrl) cultures co-transfected with pCDNA3.1+ and reporter gene. The responses from the 384-well plates for the most part paralleled that observed in 24-well format. Figure inset shows basal relative reporter gene activity under the various plating conditions.

A



B

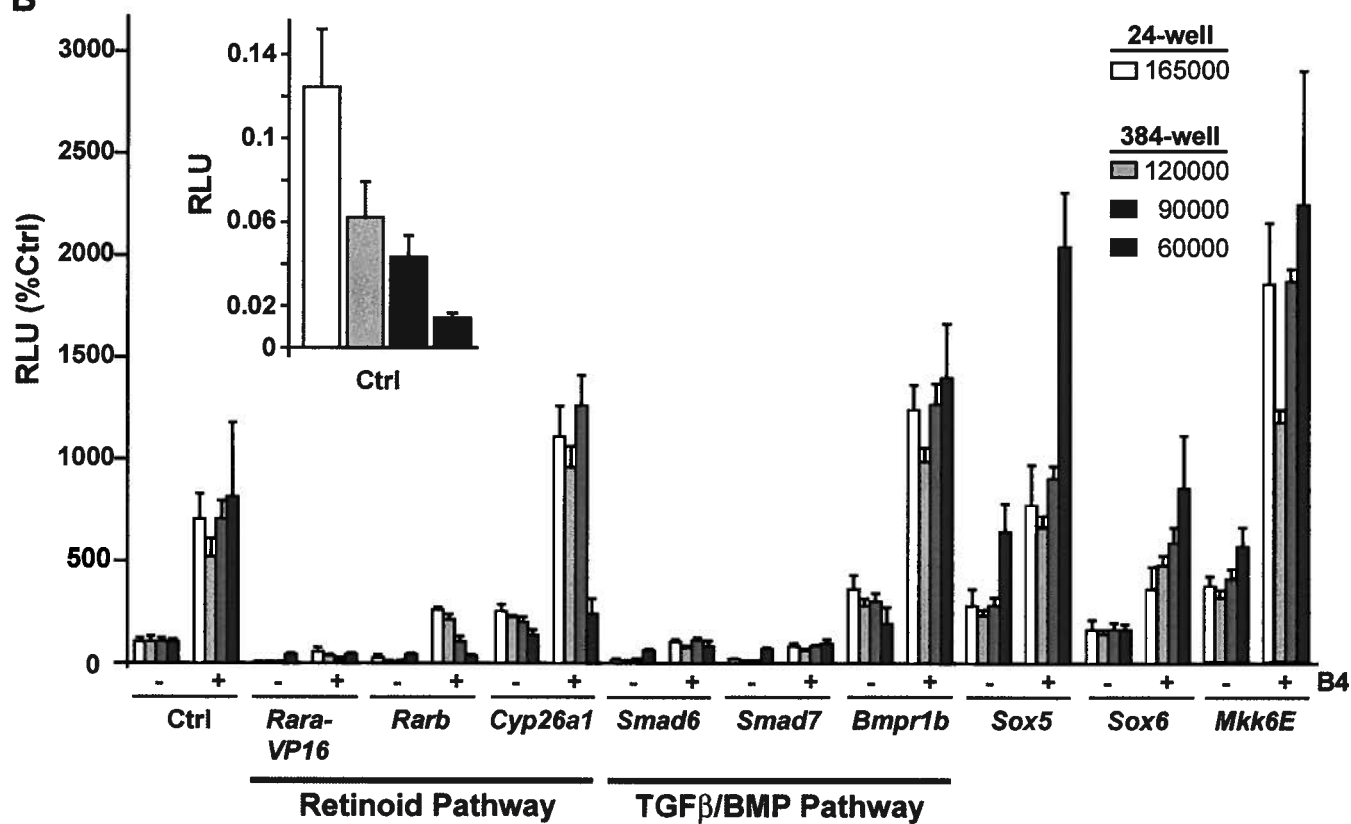
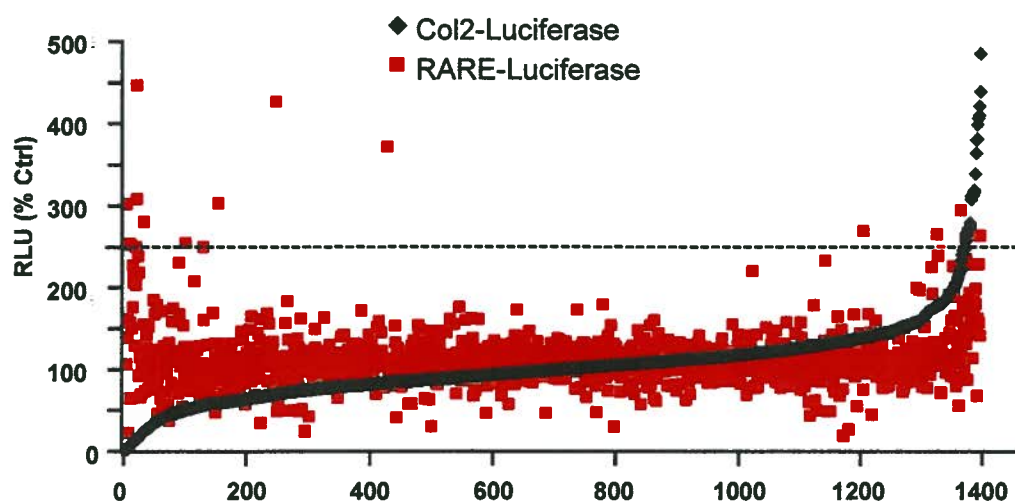


Figure 3.3. Identification of Novel Chondrogenic Modulators using a Chemical Biology Strategy. **A**, 1482 compounds were screened in 384-well format for their ability to modulate a chondrogenic- or retinoid-responsive reporter gene, pGL3(4X48) and RARE, respectively. Using a 2.5 fold cut-off on the chondrogenic responsive reporter gene, 28 compounds were identified; note, cytochalasin B is not shown on this graph. **B**, Table listing the identity of the 28 compounds and their respective abilities to regulate reporter gene activity. **C**, Secondary screening by alcian blue staining to further test prochondrogenic activity of chemical compounds from table in **B**. PLM cultures were plated as high density micromass cultures and treated with “hit” compounds (~15 μ M final concentration) or DMSO vehicle 16 hours post plating. Culture medium was replaced on day 3 and cultures were stained with alcian blue on day 4. The numbers correspond to the compound numbers (#) in **B**. Magnification bar, 1 mm.

A



B

| # | Compound Name | Reporter | | Alcian blue | # | Compound Name | Reporter | | Alcian blue |
|----|---------------------|----------|------|-------------|----|----------------------|----------|------|-------------|
| | | 4X48 | RARE | | | | 4X48 | RARE | |
| 1 | Clorgyline HCl | 269 | 173 | - | 15 | Pinocembrin | 262 | 84 | - |
| 2 | 5'-AMP | 304 | 109 | n/c | 16 | 7-Hydroxyflavone | 306 | 177 | - |
| 3 | Apigenin | 264 | 285 | - | 17 | 6-Methoxyluteolin | 391 | 156 | - |
| 4 | Tiabendazole | 296 | 260 | n/c | 18 | 6,7-Dihydroxyflavone | 422 | 253 | + |
| 5 | Hexetidine | 275 | 82 | - | 19 | Tamarixetine | 395 | 219 | - |
| 6 | Acacetin | 238 | 165 | - | 20 | Genistin | 232 | 118 | - |
| 7 | Phenazopyridine HCl | 262 | 253 | + | 21 | 5-Methoxyflavone | 300 | 219 | - |
| 8 | Butamben | 466 | 135 | + | 22 | Pratol | 366 | 191 | - |
| 9 | Indoprofen | 268 | 186 | n/c | 23 | Rhapontin | 263 | 176 | + |
| 10 | Oxybenzone | 510 | 103 | + | 24 | 5,6-Dehydrokawain | 300 | 153 | - |
| 11 | Chrysin | 386 | 220 | - | 25 | Methysticin | 351 | 172 | + |
| 12 | Kaempferol | 326 | 151 | + | 26 | Harringtonine | 302 | 29 | - |
| 13 | Cyclopiazonic acid | 384 | 65 | - | 27 | Yangonin | 367 | 145 | - |
| 14 | Cytochalasin B | 1658 | 27 | + | 28 | Galangine | 406 | 151 | - |

C

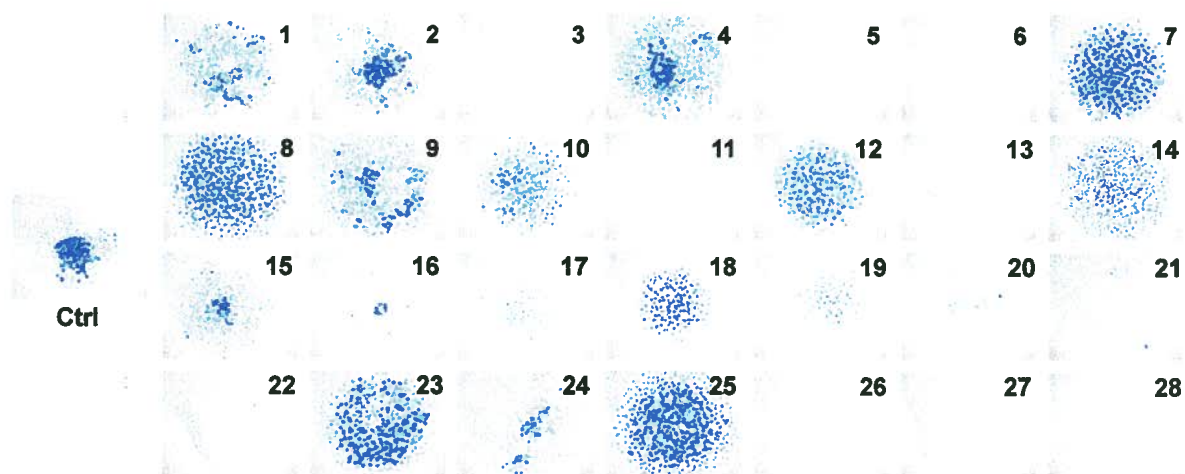


Figure 3.4. The Prochondrogenic Activity of Butamben is Associated With its Potassium Channel Blocking Capability. **A**, With the exception of butamben, benzocaine derivatives or related compounds (15 μ M treatment) exhibit negligible pro-chondrogenic activity as determined by examination of pGL3(4X48) reporter gene activity in PLM cultures. **B**, Structures of benzocaine and the chemically-related compound, butamben. Note the similarity in their structures, however, an additional ethyl group is present on the side-chain of butamben in comparison to benzocaine. **C**, Treatment of PLM cultures with butamben leads to an increase in pGL3(4X48) reporter gene activity in a dose dependent manner. Control cultures (Ctrl) were treated with DMSO vehicle. Transfected PLM cultures were treated with various doses of butamben 16 h post-transfection and reporter gene activity was measured 24 h later. **D**, *Kcnd2* and *Sox9* exhibit dynamic expression patterns in the E11.5 mouse limb bud. qRT-PCR was used to examine gene expression in limb sections (inset) from the distal (1) to proximal (5) region. *Sox9* expression is elevated in more proximal sections and this is congruent with the distal-proximal gradient of increasing chondroblast differentiation. *Kcnd2* is expressed to higher levels in the distal limb and expression declines in more proximal regions. **E**, Treatment with butamben (5 μ M) also increases *Sox9* expression as determined by qRT-PCR in PLM cultures. Cultures were treated for 1 or 3 days and gene expression quantified. **F**, SiRNA-mediated knockdown of *Kcnd2* increases *Sox9* expression in PLM cultures. Knockdown efficiency of < 40% was achieved and this was accompanied by a modest 20% increase in *Sox9* expression. **G**, Treatment of PLM cultures with 4-aminopyridine (4-AP), a broad spectrum potassium channel

blocker, increases pGL3(4X48) reporter gene activity. An experimental plan similar to that described in **C** was used. Significance versus corresponding control; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

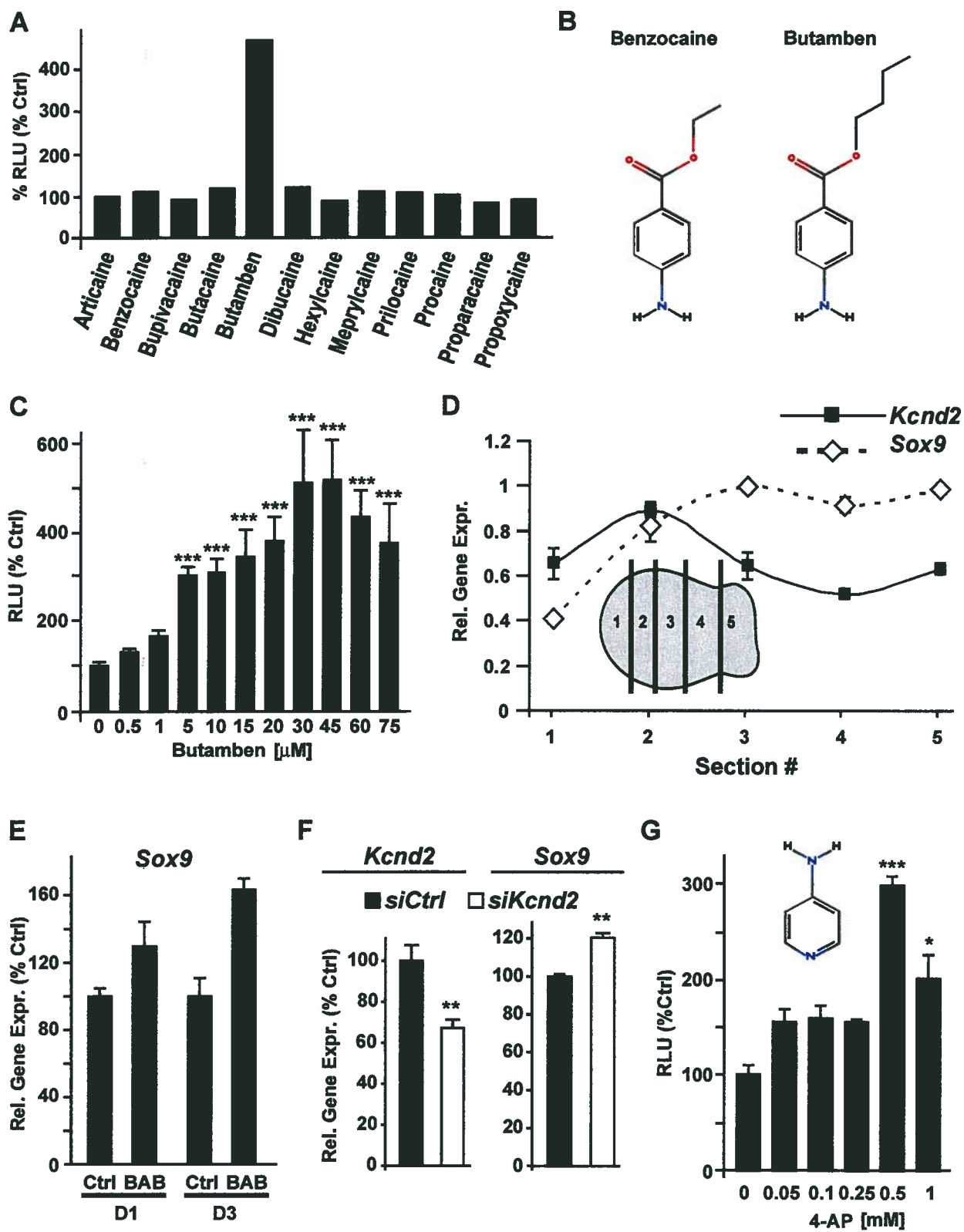
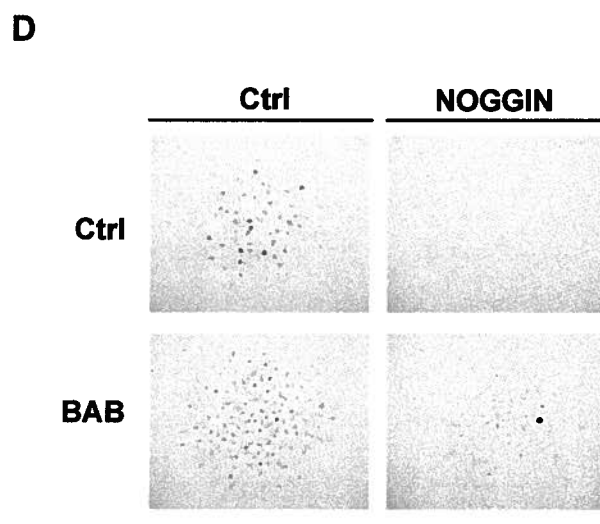
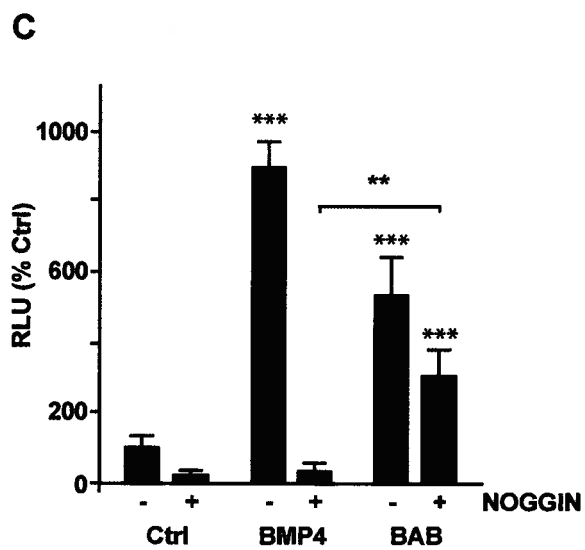
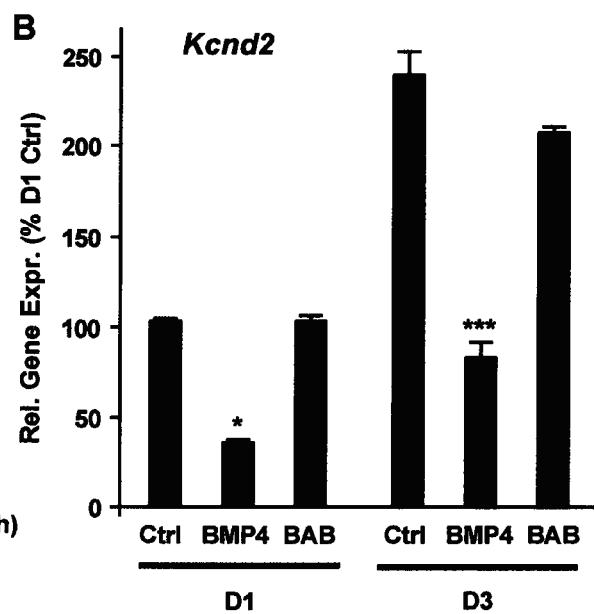
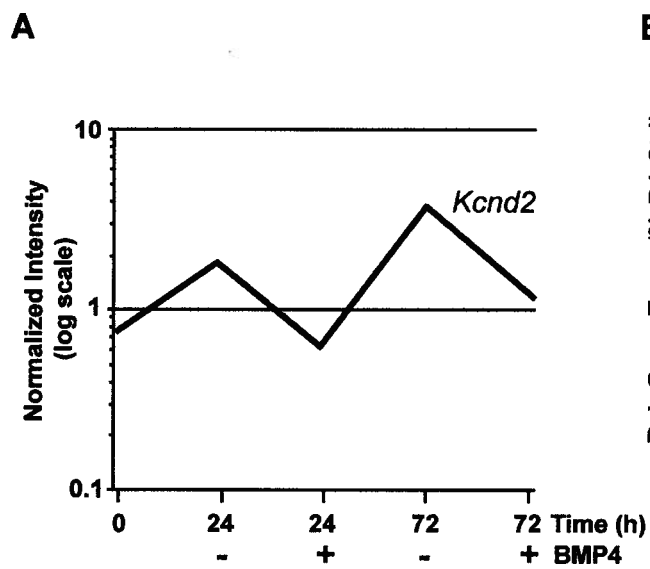


Figure 3.5. BMP4 Regulate Chondrogenesis Through Regulation of *Kcnd2* Expression and Consequently Potassium Channel Activity. **A**, Expression profiling of BMP4-treated PLM cultures reveals that BMP4 downregulates the expression of *Kcnd2*. **B**, qRT-PCR confirms that BMP4 (20ng/ml) decreases *Kcnd2* expression in PLM cultures in comparison to vehicle controls. Treatment with butamben (BAB) (15 μ M) showed no appreciable effect on *Kcnd2* transcript abundance. **C**, The BMP antagonist NOGGIN (200ng/ml) reduced both basal and BMP4-induced pGL3(4X48) reporter activity by 5 and 16-fold, whereas NOGGIN only partially attenuated (0.3 fold) the prochondrogenic activity of BAB (15 μ M). **D**, Alcian blue staining of treated cultures showed that the addition of butamben partially rescues chondrogenesis in the presence of NOGGIN. Error bars represent SD. Significance is shown as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Magnification bar, 0.5 mm.



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Chapter 4

4.1 Discussion

Over the past 30 years, genes and their protein products have been scrutinized for their role in the patterning of the skeletal elements of the limb (Tickle, 2003). The importance of the AER at the leading edge of the developing limb is highlighted by its function in promoting the proliferation and survival of the underlying mesenchyme (Lu et al., 2006). Of the signaling networks mediated by the AER, the FGFs are crucial to the primordial mesenchyme and promote cell survival and mesenchymal cell expansion (Dudley et al., 2002; Niswander and Martin, 1993; Sun et al., 2002a).

To further our understanding of the molecular mechanisms underlying FGF4 action in the limb, *in vitro* cultures of limb mesenchymal cells were employed. Observations that FGF4 transiently increased distal mesenchymal (DM) cell proliferation and thereby led to increased mesenchymal condensation, prompted the investigation of the transient nature of this response to FGF4. By gathering unbiased genome-wide transcriptional data, an ~18-fold increase in the transcriptional abundance of a cyclin dependent kinase inhibitor (*Cdkn2b*) was observed within the first 24 hr following treatment. Similarly, anti-proliferative CDKN2B levels increase in response to FGF4 in an MEK1-dependent manner.

The importance of the AER FGFs is evident in FGF8 loss-of-function mutant mice which present with limb truncations, whereby compound mutations with FGF4, further increase the severity of disrupted limb morphogenesis. These experiments are complemented by AER removal studies (Sun et al., 2000; Sun et al., 2002b). In

both cases, an increase in mesenchymal cell death is observed resulting in truncated or absent limbs. Previous studies have shown that disruption of NF κ B, which is located within the 'progress zone', results in limb defects consistent with FGF mutants (Kanegae et al., 1998). Chapter 2 provides evidence for FGF4 regulation of NF κ B through the activator RIPK4, in a MEK1- dependent manner. Further, inhibition of NF κ B negatively impacts cell survival (Fig. 4.1). Collectively, these findings define an important linkage between the FGF and NF- κ B signaling pathways, and establish a novel role for FGFs in chondrogenesis.

4.2 Context-Dependent Gene Regulation

Previous studies have demonstrated that transcriptional control of *Cdkn2b* in various established cell lines, many of which are tumor derived, is mediated by TGF- β 1 (Li et al., 1998; Li et al., 1995). Examination of an FGF4 treated DM microarray data set, revealed an increase in *Tgf- β 1* which was validated by qRT-PCR. These observations provided support for previous studies and outlined a simplified sequence of events - induction of TGF- β 1 by FGF4 led to the observed induction in CDKN2B. To validate the regulation of this gene in the context of the developing limb mesenchyme, we obtained several luciferase based reporter genes containing fragments of the *Cdkn2b* promoter region. In stark contrast to the previous studies, TGF- β 1 failed to induce reporter gene activity in the limb mesenchyme, whereas FGF4 treatment increased reporter gene activity consistent with previous studies (Li et al., 1998; Li et al., 1995). Additionally, qRT-PCR showed that *Cdkn2b* transcript abundance was unaltered by TGF- β 1 treatment, and the TGF- β 1 specific inhibitor (RBI) failed to block the induction of *Cdkn2b* by FGF4 in this context. In the

developing limb, FGF4 influences the transcription of *Cdkn2b* -independent of TGF- β 1 signaling.

The importance of context as it pertains to genes and their regulation, adds an additional level of complexity. Based on current knowledge, context-dependent gene regulation depends on combinatorial regulation - the principle by which genes are activated or repressed by specific combinations of transcription factors under precise conditions (Mayo et al., 2006; Tuch et al., 2008). The plasticity of gene regulatory networks is of interest because it allows the generation of novel phenotypes. For example, transcription factors that are activated by mitogen-activated protein kinase (MAPK) signaling, as have been shown to be involved downstream of FGF signaling, regulate very different gene expression programs in different cell types (Zeitlinger et al., 2003). Presumably, the binding and activity of these transcription factors depends on other factors, some of which include the combination of other transcription factors, signaling activities, and the cell-type specific chromatin environment (Michelson, 2002; Tuch et al., 2008).

4.3 Identifying Transcription Factors

As a logical next step in characterizing FGF induction of *Cdkn2b*, it will be necessary to understand the crosstalk between DNA and transcription factor(s). Candidate transcription factors (FOXOs, HDAC1, OCT-1, SMRT, SMADs, SP1, and numerous others) (Feng et al., 2000; Hitomi et al., 2007; Katayama et al., 2008) have been proposed to regulate the transcription of *Cdkn2b* however pertaining to the context-dependent nature of transcription, these candidates will need to be tested empirically in PLM cells. In light of the identified MEK1/ERK-dependent

regulation of *Cdkn2b*, it may be of particular interest to examine transcription factors that have been previously identified as being similarly regulated. MEK1/ERK regulated transcription factors include: ETS, ELK-1, STAT1/3, c-MYC/n-MYC, c-FOS (Kang et al., 2005; Lee et al., 2008; Thisse and Thisse, 2005).

The promoter region required for activation of transcription of *Cdkn2b* in PLM cultures has been identified as a 463 bp fragment encompassing the proximal promoter upstream of the transcriptional start site. Using bioinformatic approaches putative DNA binding motifs of transcription factors that may bind this region of DNA can be identified. By constructing nested deletions in this promoter region and then assessing the ability of transcription factors to stimulate reporter gene activity, possible transcription factors mediating *Cdkn2b* expression can be identified. Previous studies in HaCaT cells, have shown that a minimal 113 bp promoter sequence of the *Cdkn2b* promoter contains binding sites for transcription factor SP1. Further these studies have shown that SP1 is sufficient for inducing the transcription of this gene via TGF- β 1 (Li et al., 1998). The importance of context dependent gene transcription needs to be addressed, as in PLM cultures TGF- β 1 does not induce *Cdkn2b*. Furthermore, overexpression of SP1 in PLM cultures does not induce the *Cdkn2b* reporter gene -consisting of 463 bp of the promoter region which includes the proposed SP1 regulatory region (data not shown).

DNA-binding proteins can be purified by DNA-affinity column chromatography and subsequently identified by mass spectroscopy. Thereby, using nested deletions to identify minimal promoter sequences required for *Cdkn2b* induction, transcription factors that bind these sequences can be identified. Briefly, promoter fragments are

bound to DNA-affinity beads, incubated with cell lysates, and subsequently purified DNA-binding proteins are eluted and analyzed by mass spectroscopy to determine their identity. Subsequent protein overexpression and siRNA knockdown of gene expression can then be used to validate the regulatory role of these transcription factors.

FGF4 induced transcription of *Cdkn2b* is regulated by MEK1/ERK signaling. By comparing known downstream transcription factors in the MEK1/ERK pathway with FGF4 treated PLM microarray data, it should be possible to identify likely candidates involved in the regulation of *Cdkn2b*. To determine which of the identified transcription factor(s) are involved, a systematic approach using overexpression (or siRNA knockdown) of candidate transcription factors can be used to determine the effect of FGF4-mediated induction of *Cdkn2b* promoter activity.

Additionally, the yeast one-hybrid system can be used to identify which of the proposed transcription factors bind the promoter region of *Cdkn2b*. By generating fusion proteins consisting of the transcription factors fused to transcription activation domains (Deplancke et al., 2004), the interaction of these proteins with the *Cdkn2b* promoter region can be assessed by reporter gene activity. Identification of the transcription factor(s) downstream of MEK1/ERK signaling that regulate *Cdkn2b* transcription will aid in characterizing this FGF4- induced pathway.

4.4 Implications and Applications of Serum-Free Culture Studies

In Chapter 2, we have shown that in the absence of serum factors, FGF4 can not only induce DM cell survival, but can also support proliferation, condensation and differentiation of these cells – the hallmarks of limb development. Remarkably,

we have shown that at low-density, chondrogenic cells expand to form condensations and eventually cartilage. A pillar of the micromass culture method, is the requirement that limb mesenchymal cells be plated at high density, whereas at low density they quickly lose their chondrogenic potential (Ahrens et al., 1977). This ability of FGF4 to facilitate the recapitulation of the chondrogenic program has shed light on the developmental role of FGF4 in the AER. Previous studies have shown that following removal of the AER, FGF4 can functionally replace the AER and direct the formation of the limb (Sun et al., 2002b). In this instructive role within the limb, FGF4 governs the interplay between signaling networks present within the overlying ectoderm and within the mesenchyme. As suggested by the “two-signal dynamic specification model” (Mariani et al., 2008), FGF4 may play a role in maintaining the distal domain of the limb. Additionally, as the cells begin to condense and differentiate, perhaps the role of FGF4 becomes permissive rather than instructive and simply promotes cell survival and maintenance of the chondrogenic potential. This would be consistent with our serum free studies, in which cells rapidly proliferate under these conditions, and once a particular density is achieved (usually by day 6) pre-cartilage nodules form, indicative of the earliest stages of chondrogenic differentiation.

These newly developed culture conditions provide a unique environment within which to study PLM cultures without the variable effects of animal serum. This method provides a model system to systematically test the importance of other factors. Using this method it should be possible to determine the temporal significance of signaling events, by sequentially adding in known factors involved in

chondrogenesis. Establishment of SFM cultures (treated with FGF4) for a short period (eg. 4 days) followed by supplementation with known factors expressed during limb formation and subsequent analyses, should further facilitate delineation of the molecular mechanisms operating in the chondrogenic program.

4.5 High-Throughput Design to Aid in the Discovery of Chondrogenic Mechanisms

To increase our experimental throughput and to incorporate more unbiased functional screens, efficient methods were developed for following differentiation events in primary cells. We enhanced our transfection efficiency by taking advantage of the lipid stabilizing effects of disaccharides. The addition of trehalose to the proprietary formulation of EffecteneTM provided the most efficient transfection condition, but this stabilization also facilitated the storage of transfection complexes. This novel advance afforded us for the first time, the ability to exploit large-scale PLM cell transfection, and observe the effects of small-molecule compounds on reporter gene output (primarily SOX5/6/9-luciferase) (Fig. 4.2).

4.6 Small-Molecule Screening to Identify Chondrogenic Modulators

Chemical genetics utilizes diverse small-molecule compounds to delineate biological events in a manner similar to the strategies of classical genetics involving mutagenesis – systematically altering one component of a pathway/gene and observing the outcome (usually phenotype). As this discovery-based methodology often involves the screening of thousands of chemical compounds without a specific hypothesis, recently it has only been exploited by the pharmaceutical industry. This seemingly “kitchen sink” approach takes diverse libraries of chemical compounds

and screens them in the hopes of discovering a novel compound that affects the biology of interest.

Determining the precise mechanism of action of a small-molecule (ie. its cellular target) remains quite challenging. New bioactive small-molecules are identified either on the basis of their ability to produce a specific phenotype ('forward chemical genetics') or on their ability to interact with a specific target ('reverse chemical genetics') (Kawasumi and Nghiem, 2007; Lokey, 2003; Mayer, 2003). In the case of forward chemical genetics, the small-molecule's mechanism of action is often unknown, whereas in the case of reverse chemical genetics, the small-molecule's target is presumably known, but its *in vivo* specificity must be established. Cell-permeable small-molecules permit the precise timing of their delivery (and duration), whereas mutational studies as well as RNA interference (RNAi) do not. The importance of having temporal control facilitates the development of new therapeutics (Eggert et al., 2004) whereby control over drug administration and duration of its effect can be monitored.

The most widely used approach for inhibiting biological pathways on a genome-wide basis is RNAi (Eggert et al., 2004). An important feature of this methodology is the high degree of specificity to silence the target gene, and thereby permits a systematic approach to identifying the role of each component/gene in a pathway. Although the transcriptional profile of small-molecule treatment may provide an enormity of data, extrapolating the mechanism of the small-molecule from those data has been a challenge and continues to be so. New comparative approaches relying on databases of transcriptional profiles are likely to facilitate this

process and make it more systematic. As such, by grouping uncharacterized compounds with those of known mechanism on the basis of the similarity of their transcriptional profiles, hypotheses can be generated and tested regarding their mechanism of action. Similarly, data collected from genome-wide RNAi screens can be used to support the discovery of “hits”. These experimental approaches are easily adaptable to the PLM screening technique, and the use of fluorescent protein based reporter genes in combination with automated microscopy platforms (ie. Cellomics KineticScan) will further accelerate advances in data collection (outlined in Fig. 4.2).

4.7 Systems Biology as a Predictive Tool

Systems biology represents an analytical approach to define the relationships among all elements within a biological system. Systems may include simple generalized cellular processes involving few proteins within one cell or a greater complexity involving groups of cells within a tissue working in a coordinated manner (Kawasumi and Nghiem, 2007; Kitano, 2002; Siliang Zhang, 2006). Regardless of the application, the goal of systems biology is to establish the relationship between the components that comprise the system. In other words, systems biology attempts to explain “the whole” in terms of the summation of its “parts”.

By using the integrative approach of systems biology, transcriptional profiling data consisting of thousands of genes can be cross-compared with databases pertaining to identified small-molecule targets, and in our case reporter gene data. From the standpoint of chondrogenesis as outlined in Chapter 3, using either retinoid responsive or SOX5/6/9 reporter genes, a database of small-molecule activities (pro-chondrogenic, anti-chondrogenic) can be easily constructed. To this end, reporter

gene data gathered in Chapter 3 has been compiled. As demonstrated, bioinformatically probing microarray datasets aided in the identification of the molecular basis of one of the many screening “hits”. The pro-chondrogenic activity of butamben -a potassium channel (KCND2, Kv4.2) blocker led to the examination of transcriptional data of BMP4 treated PLM cultures. BMP4 downregulates the transcriptional abundance of *Kcnd2*. Functional analyses confirmed that inhibition of this particular potassium channel’s (Kv4.2) function stimulated SOX5/6/9 activity and cartilage formation. Additionally, a wide-spectrum potassium channel blocker, 4-aminopyridine, also promoted cartilage formation. The importance of inhibiting potassium channels during chondrogenesis has never been investigated.

The ultimate multidisciplinary goal of a systems biology approach in this regard would be to construct mapped relationships between compounds and genes, thereby providing greater resolution of the molecular events that govern various chondrogenic events (eg. proliferation, condensation, differentiation etc.).

4.8 Regulation and Potential Role of Kv4.2 Channels in Chondrogenesis

The Kv4.x family of potassium channels are expressed in many tissues throughout the body and are present in particularly high levels in the brain and heart (Birnbaum et al., 2004). The complex regulation of these channels has been investigated in numerous tissues and organisms. Attempts to determine the mechanism(s) regulating Kv4.2 channels have shown protein kinase C (PKC) to be involved in suppressing this potassium channel by phosphorylation in ventricular myocytes (Apkon and Nerbonne, 1988), dendrites in the hippocampus (Hoffman and

Johnston, 1998), and in *Xenopus* oocytes (Nakamura et al., 1997). Although the role of potassium channels has not been characterized in the developing limb, as demonstrated in Chapter 3, inhibition of Kv4.2 channel activity increases chondrogenesis. Previous studies have shown that PKC stimulates chondrogenesis (Lim et al., 2003; Lim et al., 2000; Sonn and Solursh, 1993), which may in part be through the suppression of Kv4.2 channels as in other tissues.

Inhibition of potassium channels leads to an increase in cellular volume due to the inability of the cells to pump out potassium ions. This increase in osmotic pressure disrupts the cytoskeleton. Previous reports have shown that inhibition of potassium channels induces differentiation of cells due to the stresses imposed on the cytoskeleton by increased cell volume (Goldring et al., 2006; Lang et al., 2007). Interestingly, disruption of the cytoskeleton has also been shown to induce chondrogenesis (Goldring et al., 2006; Lim et al., 2000). As chondrogenic cells begin to differentiate within the condensed mesenchyme, they undergo a state of hypertrophy in which the cell volume increases by ~20 fold (Goldring et al., 2006). Numerous studies have suggested that modulation of potassium channels in a variety of cells promotes cellular differentiation (Biella et al., 2007; Felipe et al., 2006; Iwamoto et al., 2007). Perhaps the most remarkable and convincing experiment showing the involvement of potassium channels in limb morphogenesis, is a recent report stating that modulation of the expression of potassium channels in *Xenopus laevis* embryos results in the generation of additional limbs (Ingber and Levin, 2007). Likely through indirect means, BMP4 causes the downregulation of *Kcnd2* expression, and this is evident in the ability of butamben to partially rescue

the phenotype produced by the BMP-antagonist NOGGIN. In light of these experiments, the often overlooked role of ion signaling in the context of limb development needs to be examined to delineate a broader view of cellular events such as cytoskeletal rearrangement and effects on cellular volume.

4.9 Impact of Research

Clarification of the function of developmental factors involved in chondrogenesis provides a more complete understanding of the sequence of events beginning with limb bud initiation and terminating with the identifiable adult limb. Through necessity, novel techniques and approaches have been developed throughout the course of this research. These include: **1)** quantitative information via qRT-PCR of regions which surround bead implants in the developing limb to provide *in vivo* confirmation of molecular events observed *in vitro*. This technique has provided a fast, quantitative alternative to whole mount *in situ* hybridization (WISH), and permits the detection of subtle changes in gene expression that may otherwise be overlooked as background signal in a conventional WISH. **2)** Establishment of parameters for low-density serum free PLM cultures that can recapitulate the chondrogenic program under the influence of FGF4. To date, the ability to culture PLM cells under low-density, serum free conditions that permit cartilage formation has not been reported. As such, the ability to determine the function of FGF or other signaling molecules in isolation (and combinations) has not been assessed until now. As an extension of this method, genes of interest (GOI) or siRNAs can be transfected into PLM cells under these conditions to assess their impact on chondrogenesis. **3)** Enhancement of EffecteneTM based transfections to improve

transfection efficiency and facilitate the storage of transfection-ready DNA complexes. PLM cells are challenging to transfect which is in part correlated to the density at which PLM cultures are established. As such, the modified transfection reagent alleviates one constraint imposed by the PLM micromass culture method (>2 fold increase in transfection efficiency). Further, as PLM cells are laboriously harvested and transfected on the same day, the ability to prepare DNA transfection complexes well in advance of their use, facilitates large-scale experimentation. **4)** Development and application of 384-well based PLM cell transfection schema to facilitate large-scale gene and chemical compound screens. Isolation of PLM cells and their subsequent transfection requires an efficient strategy to extract as much information from these valuable populations of cells. Similarly, empirical testing of GOIs requires an efficient means of assessing their possible chondrogenic modulatory function. This is the only technology currently available to screen genes and small-molecule compound libraries for potential modulators of chondrogenesis. **5)** Identification of small-molecule compounds that stimulate SOX5/6/9-reporter gene activity, in particular butamben, that may lead to novel therapeutics for diseased/damaged cartilage. Until recently, small-molecule screening has been a tool exclusively utilized by the pharmaceutical industry to identify chemicals of interest using established cell lines. Here we have shown that in an academic environment, using primary cells, “hit” compounds can be identified for their possible role in regulating chondrogenesis. Further, we have shown that data obtained via the chemical screen can be analyzed in a comparative manner against transcriptional

data obtained through microarray analyses, thus providing a more complete understanding of the mechanisms involved in the biological process.

In aggregate, the findings of this research have further elucidated the role of FGF4 in the developing limb, and provided a new means of identifying and characterizing both genes and small-molecule compounds as they relate to chondrogenesis. Specifically, the requirement of FGF4 to induce mesenchymal proliferation, and survival has been shown, and albeit through the contribution of different genes, both events require downstream activation of the MEK1/ERK pathway. By developing new methodology to circumvent the current limitations of the transfection efficiency of PLM cells, a new high-throughput primary cell based approach has been devised. The data obtained have identified novel small-molecule compounds that stimulate chondrogenesis, and provided insights into the chondrogenic regulatory function of potassium channels.

4.10 Future Directions

Although we have identified CDKN2B as a molecular mechanism that attenuates FGF4-mediated proliferation, we have yet to define the transcription factor(s) responsible for its regulation. As proposed above, an empirical approach will be used to search for likely candidates. Using both open access databases and the available literature as a guide, we can narrow the possibilities.

To investigate and validate the proposed roles of various signaling molecules during chondrogenesis, the serum-free culture conditions will be used to evaluate the impact of signaling proteins. In this regard, it will be of interest to test different members of the FGF family to determine if any overlap in function occurs. In addition

to treatment of SFM cultures with factors, overexpression of genes encoding them and/or other genes of interest will be used to determine their effect on PLM survival and differentiation. Although we can pharmacologically block NF κ B signaling and prevent mesenchymal survival, the absolute requirement of its activator –RIPK4, needs to be determined. To test this requirement, overexpression of *Ripk4* as well as siRNA knockdown will be evaluated for effects on cell survival.

Since the overall goal of this research was to further characterize the chondrogenic program, it is fitting that we have developed a new tool to aid in the discovery and characterization of chondrogenic networks. Using the chemical-genetics approach, additional small-molecule libraries will be screened using the aforementioned reporter genes. It may be of importance to revisit previously screened libraries using additional reporter genes (eg. CDKN2B or NF κ B and others). By generating datasets for numerous reporter genes relevant in chondrogenesis, the overall understanding of the contribution of multiple pathways in the chondrogenic program can be evaluated. To date, we have begun compiling a database consisting of reporter gene data. Additionally, we have amassed numerous microarray datasets. The goal is to use a bioinformatic approach to integrate these data and categorize our findings, such that initially pro- and anti-chondrogenic effectors can be grouped, and subsequently, subgroups will be generated based on the underlying mechanisms of action. Additionally, using the 384 well system, a large scale siRNA library screen will likely prove invaluable as specific targets will be the focus of analysis and these data will also be incorporated into our database. With reference to Chapter 3, using this methodology both BMP4

and butamben would have been initially grouped for prochondrogenic activity, and then subgrouped based on their ability to inhibit potassium channel Kv4.2 activity. Interestingly, this gives rise to the possibility of bioinformatically prescreening and perhaps using customized targeted small-molecule libraries prior to experimentation. Despite the apparent appeal of this approach, actual biological events may dictate the requirement of a more empirical approach, especially when the targets of the small-molecules are unknown. Similarly, although enhancing/inhibiting the activity of a biological process may yield a desired phenotype, the downstream effects need to be addressed. As such, the downstream effects of inhibiting Kv4.2 (KCND2) activity need to be identified.

The establishment of a cross-referenced database, consisting of both transcriptional and reporter gene datasets will undoubtedly increase our global understanding of signaling events during chondrogenesis.

4.11 Concluding Remarks

The combined approach of biology and technology has arisen out of necessity. As the focus of chapter 2, we showed a step-wise progression from an observed biological phenotype (FGF4 induced transient proliferation of PLM cells) which led to a hypothesis (involvement of a cell cycle inhibitor), followed by the identification of a gene of interest (using bioinformatics). Subsequent validation studies confirmed the transcription, translation and determined the mechanism by which this GOI was regulated (MEK1/ERK). The prime focus was on one gene, however consequently in this pursuit we identified numerous others. The approach of Chapter 3 was discovery-based with the hypothesis that of the chemical

compounds screened, modulators of chondrogenesis would be identified. The need existed for a new way of approaching the dilemma of having an overabundance of transcriptional data obtained through bioinformatic data mining of microarrays. As such, we developed a high-throughput transfection technology to aid in characterizing the chondrogenic role of hundreds, if not thousands of genes either alone or in combination. This technology was easily adaptable to small-molecule screening, and led us to numerous lead compounds. This technological approach identified a particular biological event (inhibition of Kv4.2 channel function), which had not been previously identified as involved in cartilage formation. It is through both traditional and novel approaches that we have gained new insights into the basic molecular programs regulating limb skeletogenesis.

“Technology will continue to drive biology, and biology will continue to drive technology. The emergence of noteworthy techniques and pivotal findings requires that the funding and facilities to pursue imaginative ideas be available and that those along the whole spectrum of knowledge be encouraged to participate together. And those who are trained in this spirit may make the most remarkable contributions.”(Fields, 2001)

4.12 Figures

Figure 4.1. Schematic Summary of FGF4 Mediated Signal Transduction in PLM Cultures as Evidenced in Chapter 2. FGF4 bind to its receptor and induces downstream targets to activate the MAP kinase signaling cascade. MEK/ERK signaling activates transcription of *Cdkn2b* and *Ripk4*, which can be blocked by U0126 - a MEK inhibitor. CDKN2B accumulation in the cytosol inhibits mesenchymal cell proliferation by binding to CDK 4 or 6 and inducing allosteric changes that abrogate the binding of CDKs to Cyclin D. RIPK4 activates NF κ B, which subsequently translocates to the nucleus and promotes cell survival.

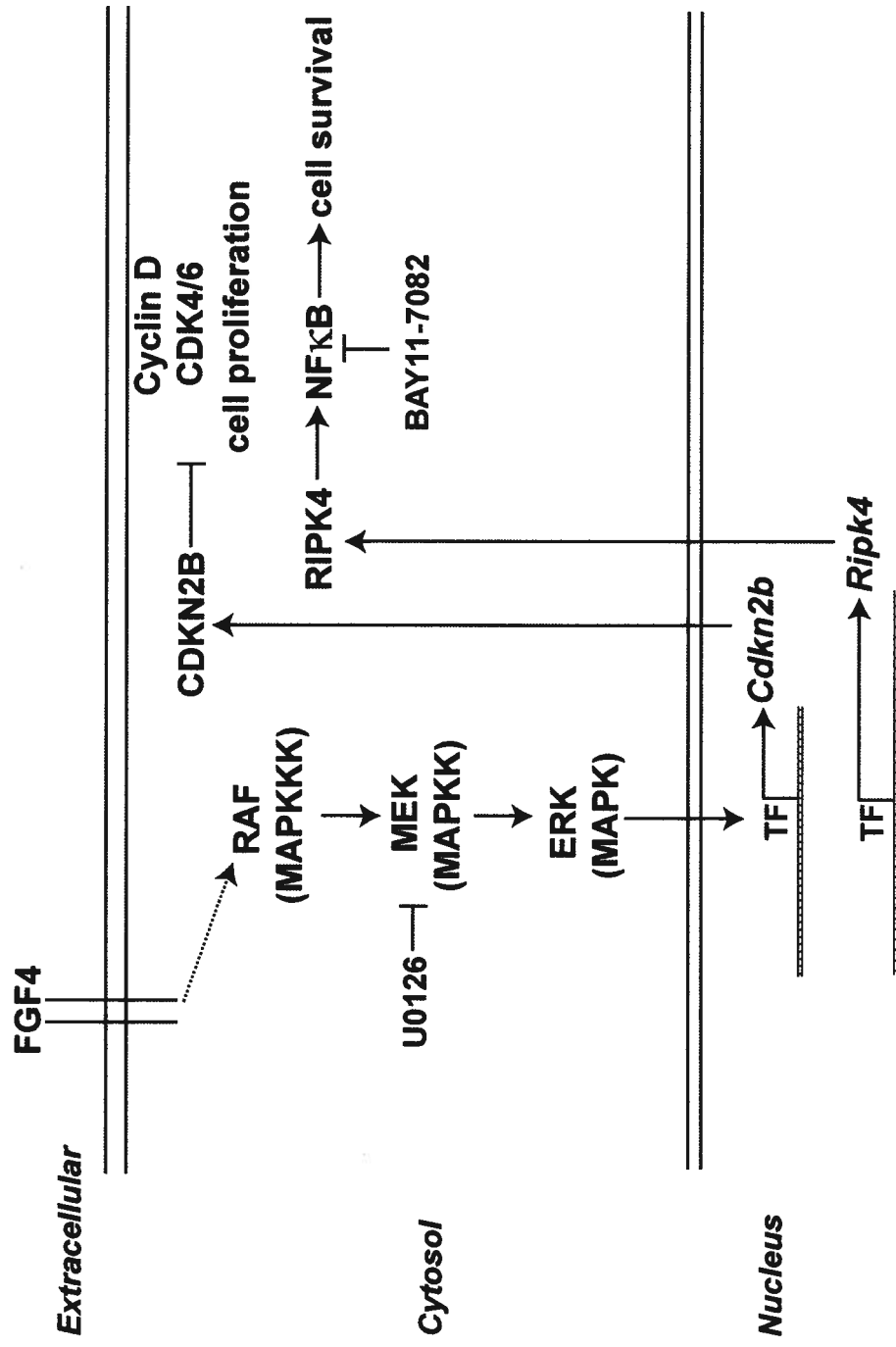
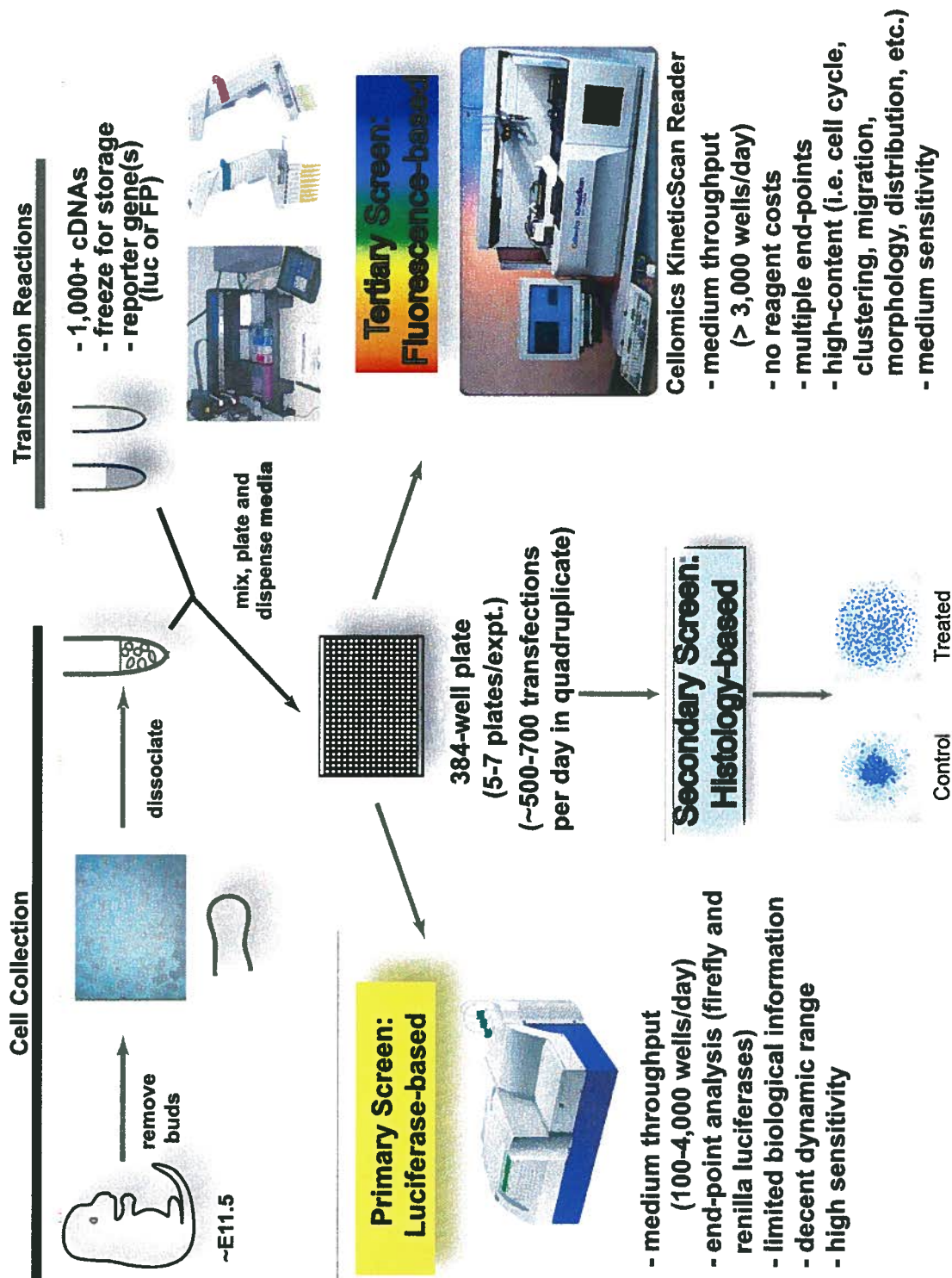


Figure 4.2. Overview of the Strategies Developed to Efficiently Assess the Role of Genes and Compounds. Primary limb mesenchymal (PLM) cells are harvested from E11.5 murine embryos. Previously prepared transfections are spotted into 384-well plates followed by primary cells and culture medium. The luciferase-based screen provides a high degree of sensitivity and dynamic range, thus enabling reliable detection of very small changes in reporter gene activity. Potential “hits” that modulate reporter gene activity at least 2.5 fold are further evaluated for chondrogenic function. The secondary screen, relies on histological staining using alcian blue, to validate pro/anti-chondrogenic factors. The tertiary screen is performed using the Cellomics KineticScan Reader (KSR) which provides a greater amount of biological information via a fluorescent reporter; however, sensitivity and dynamic range are reduced.

Overview of Screening Strategies Developed to Efficiently Assess the Role of Genes Identified Through Bioinformatics



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