A chemical genetics strategy exposes novel modulators of chondrogenesis that act by blocking a potassium channel, \textit{KcnD2}, & reveals a potential role for potassium channels in limb development

by

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Abstract

Much of the vertebrate skeleton is formed through endochondral ossification. In this process, a chondrogenic template is laid down, which is subsequently replaced by bone. The first step involves condensation of mesenchymal cells and their differentiation into chondroblasts that initiate elaboration of the chondrogenic template. At later stages, chondrocytes undergo hypertrophy, and produce a matrix for bone formation. To enhance our understanding of molecular programs regulating this process a chemical genetics approach was employed. Our strategies involved the development of screens using primary cultures of murine limb bud-derived mesenchymal (PLM) cells. Chondroblast differentiation is associated with increased SOX5, 6 and 9 activity; while hypertrophic differentiation is associated with reduced SOX5, 6 and 9 activity. Therefore, a SOX5/6/9-responsive reporter gene was used to follow expression of the chondroblast phenotype. Compound libraries representing more than 1400 compounds were screened; 28 compounds were found to increase reporter gene activity greater than 2.5 fold. In secondary screens, 7 of 28 positive compounds stimulated cartilage formation, as assessed by alcian blue staining. Two compounds identified, Butamben (butyl 4-aminobenzoate; BAB) and Phenazopyridine hydrochloride (PHCl), exhibited strong pro-chondrogenic activity and morphologically similar alcian blue staining. BAB is a member of the benzocaine family of analgesics and functions by inhibiting sodium channel activity. However, BAB has also been shown to have potassium channel-blocking activity. Specifically, BAB inhibits the activity of Kcnd2; which through transcriptional profiling was also found to be down-regulated by bone morphogenetic protein-4 (BMP4). We speculated BAB and PHCl may be able to modulate chondrogenesis by acting on potassium channels. To confirm this idea we examined molecular activities of PLM cultures treated with BAB and PHCl at two stages of chondrogenesis: 1. pre-chondrocyte to chondroblast and 2. chondrocyte to hypertrophic chondrocyte. Results confirm, BAB and PHCl increase expression of chondrogenic markers and reduce expression of hypertrophic markers. In addition patch clamp analysis revealed both BAB and PHCl are able to block, at least partially, KCND2 channel activity. We confirmed the dynamic expression pattern of Kcnd2 by qPCR and radioactive section in situ hybridization. Together, these results reveal an unanticipated and novel role for Kcnd2 in chondrogenesis.
Preface

The chondrogenic screen and initial work on this project was completed by Kamal Garcha, a Ph.D. graduate from the Underhill lab. From his work, I was able to identify BAB and PHCl as modulatory compounds that stimulated chondrogenesis. Furthermore Dr. Underhill’s Lab had previously generated microarray data that showed \textit{Kcnd2} was dynamically expressed in the primary limb mesenchymal cultures.

Patch Clamp analysis of PLM cells was performed by Robert Fougere, Ph.D. candidate, at the UBC Life Sciences Center in Dr. David Fedida’s lab. I was responsible for the dissection of embryonic mouse limbs and preparation of PLM cells for analysis and well as the preparation of the PLM culture media and compounds, BAB and PHCl.

Radioactive \textit{in situ} hybridization was performed by Katherine Fu, Ph.D., at the UBC Life Sciences Center in Dr. Joy Richman’s Lab. I was responsible for dissecting, fixing and sectioning tissues the preparation of TESPA coated slides, baking sections to the coated slides, preparing template for \textit{in situ} probe as well as the imaging of slides post hybridization.

Murine tissues used for sectioning were processed at the UBC Pathology Lab, UBC Hospital. I was responsible for dissecting, fixing and dehydrating tissues priors to wax impregnation processing, as well as the embedding of the tissues after processing.

Since this study involved the use of cells from animals and complex research methodology ethical and biosafety approval was obtained from the Animal Care & Biosafety Committees at UBC; Animal Ethics Certificate: A07-0094, Biosafety Certificate: B07-109.
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<td>ALP</td>
<td>Alkaline phosphatase</td>
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<tr>
<td>BAB</td>
<td>Butamben</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>COL</td>
<td>Collagen</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>IHH</td>
<td>Indian hedgehog</td>
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<td>LUC</td>
<td>Luciferase</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PHCl</td>
<td>Phenazopyridine hydrochloride</td>
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<td>PLM</td>
<td>Primary limb mesenchyme</td>
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<td>qPCR</td>
<td>Quantitative real-time PCR</td>
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<td>RA</td>
<td>Retinoic acid</td>
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<tr>
<td>RLU</td>
<td>Relative light units</td>
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<tr>
<td>SHH</td>
<td>Sonic hedgehog</td>
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<td>SOX</td>
<td>SRY-box containing</td>
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<td>WNT</td>
<td>Wingless</td>
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Chapter 1 - Introduction

Cartilage and the Skeleton

The vertebrate skeleton, comprised of bone and cartilage, provides the structural framework for the musculoskeletal system which allows movement of the body. Cartilage is a connective tissue comprised of chondrocytes suspended within an extracellular matrix; the extracellular matrix contains proteoglycans and collagens (Lefebvre, V. & Smits, P. 2005). Cartilage plays a fundamental role in the development of the skeleton, as the mineralized bones within the limb are formed from a cartilage intermediate by a process known as endochondral ossification (Zhang, X., et al. 2004). In fact, much of the vertebrate skeleton including the appendicular and axial skeleton as well as the sternum, pelvis, and elements of the cranium, form through endochondral ossification, whereby mesenchymal cells differentiate to chondrocytes and form a cartilaginous anlagen in the embryo that provides the skeletal template, which is eventually replaced by bone (Lefebvre, V. & Smits, P. 2005). In addition, the chondrocytes that make up the cartilage in the growth plate of long bones control the longitudinal growth of the skeleton (Karsenty, G., et al. 2002). Cartilaginous structures found throughout the body persist throughout life and provide support, in not only the articular joints, but also in the respiratory and auditory tracts and are essential for breathing, locomotion and hearing (Lefebvre, V. & Smits, P. 2005).

Many painful and debilitating diseases such as osteoarthritis can affect the maintenance and synthesis of bones and cartilage, of the skeleton, causing irreparable damage (Sandell, L. & Aigner, T. 2001). A better understanding of the underlying molecular biology of chondrogenesis will help tease out the pathways that determine bone and cartilage formation, as well as aid in the
identification of possible targets for treatments of painful diseases associated with the degeneration of these tissues. This project aims to study the molecular mechanisms underlying the commitment and differentiation of mesenchymal cells to a chondrogenic fate using the embryonic murine limb as a developmental model as well as two compounds, Butamben (BAB) and Phenazopyridine hydrochloride (PHCl) to manipulate the chondrogenic program.

**Cartilage and Osteoarthritis**

Osteoarthritis is characterized by the loss of cartilage in the joints which includes inappropriate chondrocyte dedifferentiation and hypertrophy of chondrocytes, both of which are associated with a decrease in the activity of the SOX5, 6 and 9 transcription factors. SOX9 belongs to the Sry-related high mobility group box gene family and is required for both commitment and differentiation of chondrocytes. Its expression is in fact increased at the early mesenchymal stage and remains highly expressed in prechondrocytes and chondroblasts; Sox9 expression is reduced as chondrocytes differentiate to hypertrophic chondrocytes (Figure 1.1) (Lefebvre, V. & Smits, P. 2005)

Two phases define osteoarthritis: 1. a biosynthetic phase, in which chondrocytes attempt to repair the damaged extracellular matrix by synthesizing structural and functional proteins and macromolecules such as growth factors and bone morphogenetic proteins (BMPs) and 2. a degradative phase, in which the chondrocytes produce enzymes such as aggrecanases (ADAMTS) and matrix metalloproteinases (MMPs) that actively digest the extracellular matrix and inhibit matrix synthesis (Sandell, L. & Aigner, T. 2001). Ultimately the biosynthetic phase is unable to maintain the rapid pace of the degradative phase, which results in the degeneration
of the cartilage tissue. As cartilage has limited regenerative potential, much of the current therapeutic focus has been aimed at compounds that slow the degenerative process.

**Stages of Chondrogenesis**

Skeletal development is a complex process, considering that unlike most organs it is not confined to a single structure, but more than 200 elements in unique shapes, sizes and orientations, throughout the body (Karsenty, G., et al. 2002). Multi-potent mesenchymal progenitor cells, derived from mesoderm, are able to differentiate into bone, cartilage, tendons, marrow stroma and fat (Pearse et al., 2007). These mesenchymal progenitor cells are able to differentiate into skeletogenic cells by either endochondral or intramembranous ossification and require skeletal patterning to determine the size, shape and location of skeletal elements. As previously described, in endochondral ossification the mesenchymal progenitor cells differentiate into chondrocytes and produce a cartilaginous template that is eventually replaced by bone. In intramembranous ossification, a cartilaginous template is not required; the mesenchymal progenitor cells differentiate directly into osteoprogenitors that ultimately develop into mature bone. Limbs are formed through endochondral ossification and the regulation of limb chondrogenesis requires multiple pathways and factors including members of the bone morphogenetic protein (BMP), fibroblast growth factor (FGF), sonic hedgehog (SHH), retinoid signalling, and Wingless (WNT) families, to control patterning and differentiation (Barna, M. & Niswander, L. 2007). Each step of limb chondrogenesis is controlled by specific factors including transcriptional activators and repressors, and can be characterized by specific
histological features, cellular activities and gene expression profiles which correspond to changes in the composition of the extracellular matrix, cell morphology, proliferation and differentiation.

To initiate chondrogenesis, multipotent mesenchymal cells commit to a chondrogenic fate and subsequently condense, approximately midway through gestation in the mouse embryo, forming the ‘membranous skeleton’ (Lefebvre, V. & Smits, P. 2005, Weston, A., et al. 2002). In the case of limb development, mesenchymal progenitor cells migrate to presumptive sites of limb formation and become tightly packed; pre-cartilage matrix and cell adhesion molecules such as cadherin type 2 \( (Cdh2) \), N-CAM \( (Ncam1) \), tenasin C \( (Tnc) \), hyaluronan and fibronectin mediate this process (Hall, B. & Miyake, T. 2000). Subsequently, prechondrocytes emerge reducing expression of mesenchymal and condensation markers such as collagen type 1 \( (Col1a1) \) and the aforementioned cell adhesion molecules (Figure 1.1) (Lefebvre, V. & Smits, P. 2005).

Prechondrocytes within the cartilaginous nodules differentiate into chondroblasts and subsequently form mature chondrocytes; this process is characterized by a switch from a fibroblast-like morphology to a spherical shape and an increase in the synthesis and secretion of extracellular matrix (Figure 1.1) (Lefebvre, V. & Smits, P. 2005). The abundant extracellular matrix consists primarily of collagen type II \( (Col2a1) \) and the large proteoglycan aggregan \( (Acan) \) as well as other glycoproteins, link proteins and cartilage type IX and XI proteins (Figure 1.1) (Adams, S., et al. 2007; Lefebvre, V. & Smits, P. 2005; Weston, A., et al. 2002). As the prechondrocytes differentiate an increase in the expression of cartilage markers such as collagen type II \( (Col2a1) \) and as well \( Sox9 \) play essential roles in the commitment to the chondrogenic lineage (Figure 1.1) (Lefebvre, V. & Smits, P. 2005). As previously stated \( Sox9 \) expression is
high in both prechondrocytes and chondroblasts. In addition to Sox9 expression, chondroblasts exhibit high expression of two closely related SOX family members, Sox5 and Sox6 (Figure 1.1) (Lefebvre, V. & Smits, P. 2005). Studies have shown that Sox5, Sox6 and Sox9 cooperate to activate Col2a1, by binding to an enhancer region in the first intron of Col2a1 (Lefebvre, V. & Smits, P. 2005). During this period, cartilage derived retinoic acid sensitive protein (Cdrap) expression is initiated by differentiating chondroblasts (Figure 1.1) (Bosserhoe, A., et al. 1997).

As the ovoid chondrocytes in the growth plate differentiate into prehypertrophic chondrocytes and ultimately hypertrophic chondrocytes they undergo a major phenotypic switch, their cytoplasmic volume dramatically increases and they exit the cell cycle (Figure 1.1) (Lefebvre, V. & Smits, P. 2005). Prehypertrophic chondrocytes exhibit higher expression of Col2a1 and Acan as well as other early chondroblast extracellular matrix genes compared to the expression levels in chondroblasts (Figure 1.1) (Lefebvre, V. & Smits, P.). Prehypertrophic chondrocytes also begin to synthesize collagen type X (Col10a1), Indian hedgehog (Ihh) and parathyroid hormone-related peptide receptor (Pthr1) (Figure 1.1) (Lefebvre, V. & Smits, P. 2005). Additionally, Runx2, active in mesenchymal cells but inactive in chondroblasts, is expressed in prehypertrophic chondrocytes and expressed in hypertrophic and terminal chondrocytes; Runx2 may play a role in activating Ihh expression in prehypertrophic chondrocytes (Figure 1.1) (Lefebvre, V. & Smits, P. 2005).

Interestingly, the composition of the extracellular matrix of hypertrophic chondrocytes is characterized by abundant collagen type X (Col10a1), high levels of alkaline phosphatase activity (ALP), as well as synthesis of vascular endothelial growth factor (Vegf) and matrix
metalloproteinases (Mmp13) while Ihh, Pthr1 and other early chondroblast and pre-hypertrophic chondrocyte matrix genes cease to be expressed (Figure 1.1) (Adams, S., et al. 2007; Lefebvre, V. & Smits, P. 2005; Weston, A., et al. 2002). In addition, aggrecanases, such as a disintegrin and metalloproteinase with thrombospondin motifs (AdamTs4 & AdamTs5) are expressed in hypertrophic chondrocytes and act to cleave aggrecan destabilizing the extracellular matrix surrounding chondrocytes (Figure 1.1) (Fosang, A., et al. 2008).

Hypertrophic chondrocytes undergo a final differentiation to terminal chondrocytes at which time the expression of Col10a1 is reduced and expression of osteoblast associated markers such as ALP, osteopontin and Mmp13 is increased. Terminal chondrocytes then undergo apoptosis, at which time bone forming cells are able to invade their lacunae to establish primary ossification centers (Lefebvre, V. & Smits, P. 2005; Weston, A., et al. 2002). Vascularization of the cartilage allows osteoblasts to invade and form trabecular bone (Maes et al. 2010). The extracellular matrix surrounding hypertrophic chondrocytes becomes mineralized and acts as a template for trabecular bone formation.
Figure 1.1 Chondrogenesis model

Chondrogenesis is a stepwise program that begins when mesenchymal cells expressing Sox9 and Runx2 commit to the chondrogenic lineage, shortly thereafter these cells coalesce to form precartilaginous condensations; this is driven in part by the expression of cell to cell adhesion molecules such as Cdh2, Ncam1 and cell to matrix adhesion molecules such as Tnc as well as an increase in Sox9. Prechondrocytes then differentiate to chondroblasts. This step is characterized by an increase in expression of chondrocyte markers Sox5, Sox6, Sox9, Acan, Col2 and Cdra. As chondroblasts differentiate into prehypertrophic chondrocytes and then hypertrophic chondrocytes expression of Runx2, Col10a1, Mmp13, AdamTs4, AdamTs5 and Ihh is observed. BMPs stimulates chondrocyte differentiation and hypertrophy and have been shown to act on cells at each stage of chondrogenic program. 310 is a retinoic acid receptor (RAR) antagonist and is used as a control compound that stimulates chondrocyte differentiation and inhibits hypertrophic differentiation.
mesenchymal cell → prechondrocyte → chondroblast → hypertrophic chondrocyte

- **Sox9**
- **Runx2**
- **Cdh2**
- **Ncam1**
- **Tnc**
- **Sox9**
- **Sox5**
- **Sox6**
- **Acan**
- **Col2**
- **CD-Rap**
- **Runx2**
- **Col10a1**
- **Mmp13**
- **AdamT54**
- **AdamT55**
- **Ihh**
BMPs in chondrogenesis

The regulation of limb chondrogenesis involves a myriad of factors from several signalling pathways including members from the BMP and growth and differentiation (GDF) family, retinoic acid (RA), sonic hedgehog (SHH) and the wingless (WNT) group of factors that act together to specify the positional identity and fate of multipotent mesenchymal cells, and to regulate their progression through the chondrogenic program. BMPs are members of the transforming growth factor beta (TGFβ) superfamily of proteins and are potent prochondrogenic factors. It is well documented that BMPs play important roles in endochondral bone formation and they, as well as their receptors, are present in the chondrocytes and adjacent perichondrium (Kobayashi, T., et al. 2005). Studies have established a critical role for BMP signalling during the earliest stages of the chondrogenic program when mesenchymal cells coalesce and establish precartilaginous condensations (Zhang, X., et al. 2004).

BMPs first become apparent in the murine embryonic limb bud at age E10.5 and this coincides with the appearance of proximal mesenchymal condensations and regions of Sox9 expression (Karamboulas, K., et al. 2010). Following condensation, mesodermal cells within the condensations differentiate into chondrocytes during outgrowth such that the proximal mesenchymal cells differentiate prior to more distal mesenchymal populations. Previous studies in the Underhill lab have revealed that BMPs inhibit cartilage formation in more immature cells from the distal hind limb while promoting cartilage formation in slightly more mature mesenchymal populations such as those cells of the fore limb (Karamboulas, K., et al. 2010). Both the signals that promote and inhibit chondrogenesis are important determinants of skeletal development during limb outgrowth.
In addition, BMPs exhibit potent prochondrogenic activity and may promote the expansion of cartilage and bone by both stimulating the recruitment of mesenchymal cells to a chondrogenic fate and promoting chondroblast differentiation (Figure 1.1) (Hoffman, A., et al. 2006). Hoffman, A., et al. 2006, demonstrated that the pro-chondrogenic BMP’s regulate chondrogenesis in part by decreasing the activity of the retinoid signalling pathway. Furthermore, antagonism of this pathway has been shown to promote chondroblast differentiation and cartilage formation.

**Experimental Model**

Many of the stages of chondrogenesis and the molecules that coordinate the program have been characterized in the developing limb model system, as the limb bud alone is able to establish signalling centers that provide many of the factors that coordinate the size, shape and patterning of the limb skeleton (Weston, A., et al. 2003). Namely, this model was used to establish that retinoid signalling functions downstream of the BMP signalling pathway and is important in chondrogenesis (Hoffman, et al. 2006). BMP4 along with the retinoic acid receptor (RAR) antagonist, 310, were control factors used for comparison – BMP4 stimulates chondrocyte differentiation and hypertrophy; while 310 stimulates chondrocyte differentiation but inhibits hypertrophy.
To effectively model the chondrogenic program in vitro this study employs a micromass culture technique that involves the culture of primary cells derived from embryonic age (E) 11.5 murine limb buds (Figure 1.2). Limb buds are harvested, and by using the tissue dissociation enzyme dispase the mesenchymal cells are selectively dissociated, whereas the epithelial cells remain as a sheet. Following filtering, the enriched limb mesenchymal cells are plated at high density (> 1 X 10^7 cells/ml) in micromass cultures. Shortly after plating, the cells condense forming aggregates that subsequently differentiate into chondroblasts forming cartilage nodules that become apparent after a few days in culture (Hoffman, L., et al. 2006). These in vitro primary micromass cultures undergo differentiation and both accurately and reliably recapitulate the in vivo sequence of events (Barna, M. & Niswander, L. 2007). Furthermore, RNA extracted from these cultures and analyzed with real time quantitative PCR (Q-PCR) provides a reliable picture of gene expression at the different stages of chondrogenesis.

Furthermore, the Underhill lab previously generated a low-throughput reporter gene-based assay to investigate the chondrogenic program using PLM cultures (Hoffman, L., et al. 2006; Weston, A., et al. 2002). The assay exploits the observation that at the onset of chondroblast differentiation the expression of Sox5, 6 and 9 is increased and as the cells differentiate and undergo hypertrophy the expression of Sox5, 6 and 9 is reduced. To assess chondrogenic activity the assay follows these events using a SOX-responsive reporter, COL2-Luc. The reporter contains 4 reiterated binding sites for SOX5, 6 and 9 (4x48 base pairs), derived from the Col2a1 gene, upstream of a minimal collagen type II promoter (-89 to +6), coupled to a firefly luciferase gene – and has proved to be a reliable means of assessing chondroblast differentiation (Hoffman, L., et al. 2006; Kan, A., et al. 2009; Weston, A., et al. 2002).
Figure 1.2 Primary limb mesenchymal cell collection

Limb buds were collected from CD-1 mice, embryonic age (E) 11.5, dissociated into single cells and resuspended at a concentration of approximately $2 \times 10^7$ cells/ml, and plated as high density micromass cultures. Cultures were incubated at 37°C for 1 hr to allow cells to adhere to the plate, and then primary media was added. Compounds were prepared in primary media and added 24hrs after culture initiation – media and compounds were changed every other day. PLM cultures were used for histological staining, reporter gene (luciferase) and Q-PCR assays from 1 to 15 days (D).
Primary Cell Collection

1 litter (10-12 ~ E11.5 embryos) → ~ 2 x 10^7 PLM cells

DNA-Tf mixture

24h

Compound(s) Added

Cell Culture for Histological staining, RNA Extraction & Transfection Assays

*10D

Histological Staining with Alcian Blue +/or Alkaline Phosphatase

*1-15D

RNA extraction & Q-PCR

*1-9D

Luciferase

*Compounds & media replaced every 48h
**Chondrogenic screen & identification of potential K+ channel modulators**

To enable the screening of chemical libraries for chondrogenic activity the Underhill lab subsequently developed a higher throughput assay based on using primary limb mesenchyme (PLM) cultures in combination with plating and screening in 384-well format. As previously indicated, PLM micromass cultures closely recapitulate *in vivo* chondrogenesis; and within 2-3 days in culture, chondrogenic progenitors differentiate into matrix-secreting chondroblasts and form cartilage nodules (Hoffman, L., *et al.* 2006). The chondrogenic screen, in which PLM cells were transfected with the reporter gene Col2-LUC, was used to identify compounds that stimulate chondrogenesis. To date, over 4000 compounds have been screened, in 384-well format, for their ability to modulate the activity of a chondrogenic-responsive reporter gene (Col2-LUC) (Figure 1.3A). From a compound screen, comprised of approximately 1,400 known drugs and natural products, 28 compounds that stimulate chondrogenic activity were identified (Figure 1.3B). Secondary screening by alcian blue staining of PLM micromass cultures indicated seven of the twenty-eight compounds exhibited chondrogenic activity as evidenced by an increase in alcian blue staining (Figure 1.3C). Alcian blue is a histological dye that stains the glycosaminoglycans in cartilage blue. PLM cells treated with two of those seven compounds, Butamben (butyl 4-aminobenzoate; BAB) and Phenazopyridine hydrochloride (PHCl), stimulated not only Col2-LUC reporter gene activity, but also produced micromass cultures with numerous cartilaginous nodules and morphologically similar alcian blue staining (Figure 1.3C).
Figure 1.3 Chondrogenic screen

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-responsive reporter gene, Col2-LUC. 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 compound identity of the 28 compounds, their respective abilities to regulate reporter gene activity and the results of secondary screening by alcian blue staining. C) Secondary screening by alcian blue staining to further test prochondrogenic activity of 28 chemical compounds identified in screen. PLM cultures were plated as high density micromass cultures and treated with “hit” compounds (~15 µM 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.
BAB, a butyl ester of aminobenzoic acid, is an analgesic compound (Winkleman, D., et al. 2005). It is a member of the benzocaine family of analgesics that act by inhibiting sodium channel activity and have been used in humans as a topical anaesthetic spray for over 30 years. Winkleman et al. 2005, uncovered that BAB also blocks potassium channels, specifically Kcnd2, otherwise known as Kv4.2, in addition to sodium channels. BAB is known to act on sodium channels when used at a concentration of approximately 100 mM; surprisingly at concentrations of 500 nm or more BAB also acts to inhibit the activity of potassium channels such as Kcnd2. BAB is structurally similar to benzocaine and some of its derivatives (Figure 1.4B), yet BAB was determined to have a dose dependent prochondrogenic effect on PLM cultures as assessed by Col2-LUC reporter gene activity (Figure 1.4A) and increased Sox9 expression (Figure 1.4D); while, other benzocaine derivatives exhibited negligible prochondrogenic activity (Figure 1.4C).

PHCl has also been used as an analgesic or local anaesthetic, primarily taken orally to treat urinary tract pain associated with cysts or inflammation. However, the mechanism of action and the molecular target for PHCl remains elusive. PLM cultures treated with PHCl exhibited high levels of Col2-LUC activity during the screen and very similar chondrocyte morphology to PLM micromass cultures treated with BAB in the secondary screens (Figure 1.3A/C). Structurally BAB and PHCl are quite different, yet their activity in PLM cultures, appear to be quite similar (Figure 1.4B).

Noggin is a BMP antagonist and when added to PLM micromass cultures, with or without BMP4, disrupts cartilage formation and reduces COL2-Luc reporter gene activity (Figure 1.4E/F).
However, Noggin was only partially effective in PLM cultures treated with BAB; in other words, BAB was able to rescue cartilage formation in the presence of Noggin. Similar results were also observed with PHCl, as it was also able to rescue PLM chondrocytes in the presence of Noggin.
Figure 1.4 BAB stimulates chondrogenesis and increases Sox9 expression

The prochondrogenic activity of BAB appears to be associated with its potassium channel blocking capability. A) Treatment of PLM cultures with BAB leads to an increase in Col2-LUC 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 BAB 16 hrs post-transfection and reporter gene activity was measured 24 hrs later. B) Structures of benzocaine; the chemically-related compound, BAB and PHCl. Note the similarity in the structures of BAB and benzocaine; BAB however, has an additional ethyl group present on the side-chain in comparison to benzocaine. PHCl is not structurally similar to BAB, but does exhibit similar chondrogenic activity to BAB. C) With the exception of BAB, benzocaine derivatives or related compounds exhibit negligible pro-chondrogenic activity as determined by examination of Col2-LUC reporter gene activity in PLM cultures. D) Sox9 expression increases with BAB treatment after only 1 day in culture, a greater increase in expression is observed after 3 days in culture. Control cultures (Ctrl) were treated with DMSO vehicle. E) The BMP antagonist NOGGIN (200ng/ml), reduced both basal and BMP4 induced COL2-Luc reporter activity by 5 and 16 fold, respectively, whereas NOGGIN only partially attenuated, 0.3 fold, the prochondrogenic activity of BAB (15µM). F) PHCl as well as BAB can rescue cartilage formation in the presence of NOGGIN – NOGGIN (200ng/ml) again reduced both basal and BMP4 induced COL2-Luc reporter activity, and only partially attenuated the prochondrogenic activity of PHCl (15µM). Error bars represent SD. Significance is shown as *, P < 0.05; **, P < 0.01; ***, P < 0.001.
A

Benzocaine  Butamben (BAB)  Phenazopyridine Hydrochloride (PHCI)

B

C

D

E

F
Potassium channels

Ion channel proteins, including calcium, sodium and potassium channels, are highly specific and specialized to rapidly transport inorganic ions through the cell membranes along an electrochemical gradient (Wohlrab, D., et al. 2004). Ion channels have been found to be involved in the initiation and regulation of many biological processes such as cell volume, maintenance of membrane potential and signal transduction as well as cell differentiation, proliferation and death (Wohlrab, D., et al. 2004).

Potassium channels are a diverse family of integral membrane proteins present in nearly all mammalian cells that participate in a range of physiological responses (Mobasher, A., et al. 2007). Pardo, L., et al. 2004, investigated the roles of potassium channels in cell proliferation and confirmed they play a role in membrane potential and cell volume. Their various roles in neurons including: shaping action potentials, controlling membrane excitability, neuronal firing patterns, neurotransmitter release, cell volume, cell proliferation and gating kinetics is well documented (Pertez, A., et al. 1999), yet their role in prechondrocytic or chondrocytic cells remains undetermined.

In 1990 Grandolfo, M., et al., speculated that K+ channels may be involved in regulation of the membrane potential and important in the maturation of chondrocytes (Grandolfo, M., et al. 1990 & 1992). It has previously been shown that human osteoarthritic chondrocytes possess a resting membrane potential that can be influenced by various ion channel modulators (Clark, R., et al. 2010 & Wohlrab, D., et al. 2004). Despite the numerous studies on ion channels very little is
known about the function of ion channels in chondrogenesis; information vital to understanding their physiology in chondrocytes (Clark, R., et al. 2010).

KCND2, otherwise known as Kv4.2, is a voltage gated potassium channel, known to be expressed in mammalian cells; for the purposes of this thesis KCND2 will be used. BAB appears to operate as a pore blocker, as it interferes with potassium efflux through KCND2. As BAB was able to stimulate chondrogenesis at a dose (~1-10 µM) consistent with its potassium channel blocking activity, we hypothesized that the pro-chondrogenic activity of BAB is associated with its potassium channel-modulatory activity. To confirm the idea that potassium channels play a role in chondrocyte differentiation the Underhill lab treated PLM cultures with 4-aminopyridine (4-AP) a broad spectrum potassium channel blocker, and found that it also increases Col2-LUC reporter gene activity in a dose dependent manner (Figure 1.5C).

Additionally, previously generated microarray data from the Underhill Lab demonstrated that Kcnd2 is dynamically expressed in PLM cells and treatment with BMP4 reveals that BMP4 downregulates the expression of Kcnd2 (Figure 1.5A). Decreased expression of Kcnd2 is observed following both 24 and 72 hrs of BMP4 treatment, it is during this time that BMP4 stimulates chondroblast differentiation and hypertrophy of existing chondrocytes. Furthermore, Q-PCR data confirm that BMP4 decreases Kcnd2 expression; BAB however, showed no appreciable effect on transcript abundance over 3 days (Figure 1.5B).
Figure 1.5 *Kcnd2* expression in PLM cells

Expression profiling of BMP4-treated PLM cultures reveals that BMP4 downregulated the expression of *Kcnd2*. **A)** Microarray data showing expression of *Kcnd2* over 72 hrs with and without BMP4. **B)** Graphical representation of *Kcnd2* expression with and without BMP4 derived from microarray data. RT-qPCR confirms that BMP4 (20ng/ml) decreases *Kcnd2* expression in PLM cultures in comparison to vehicle controls. Treatment with BAB (15 µM) showed no appreciable effect on *Kcnd2* transcript abundance. **C)** Treatment of PLM cultures with 4-aminopyridine (4-AP), a broad spectrum potassium channel blocker, increases Col2-LUC reporter gene activity. Error bars represent SD. Significance is shown as *, P < 0.05; ***, P < 0.001.
Hypothesis

A chemical genetics strategy enabled us to identify two new pro-chondrogenic compounds with which to both manipulate the chondrogenic program and to better understand the processes underlying chondrogenesis. The objective of this thesis project was to better characterize the activity of BAB and PHCL, and determine if this information could provide insights into their mechanism(s) of action. As reparative processes in the adult at least partially recapitulate their embryonic development, understanding the mechanisms that regulate chondrocyte differentiation and maturation will provide valuable information in developing treatment for osteoarthritis and other diseases affecting the degeneration of cartilage.

We hypothesize that voltage gated potassium channels, specifically \textit{Kcn}d2, play a critical role in chondroblast differentiation and/or their maintenance.

As preliminary data indicate that BAB and PHCl are able to modulate chondrocyte morphology and BAB has been shown to act a pore blocker of KCND2 we believe these compounds may be influencing cartilage development through the same mechanism, by blocking or inhibiting potassium channels, particularly KCND2. Therefore, we decided to use these compounds as tools to investigate this hypothesis.
This project aims to test our hypothesis as follows:

1. Characterize the ability of BAB and PHCL to regulate chondroblast differentiation by:  
   a. Histological analysis of PLM cultures treated with BAB, PHCl and combinations of controls BMP-4 and 310,  
   b. Examination of gene expression of PLM cultures, treated with and without BAB and PHCl as well as controls like BMP4, for 1-4 days in culture, by QPCR of chondrogenic markers and  
   c. Assess the chondrogenic activity of PLM cells treated with and without BAB and PHCl, for 24-72 hrs in culture, by a reporter gene assay, similar to that of the chondrogenic screen.

2. Investigate the ability of BAB and PHCL to regulate chondrocyte hypertrophy by:  
   a. Histological analysis of PLM cultures treated with BAB, PHCl and combinations of controls BMP-4 and 310  
   b. Examination of gene expression of PLM cultures, treated with and without BAB and PHCl as well as controls like BMP4, for 5-10 days in culture, by QPCR of hypertrophic markers and  
   c. Assess the chondrogenic activity of PLM cells treated with and without BAB and PHCl, for 6-9 days in culture, by a reporter gene assay.

3. Confirm the activity of KCND2 in PLM cells by patch clamp analysis and then examine the ability of BAB and PHCl to block KCND2 channel activity.

4. Examine the spatial and temporal expression of Kcnd2 by:  
   a. section in situ-hybridization of embryonic murine limbs and  
   b. gene expression analysis of PLM cultures treated with chondrogenic modulators (BMP4, 310 and RA), from 1 to 10 days in culture, by QPCR of murine Kcnd2.
Chapter 2 - Materials & Methods

Reagents

BAB (Sigma-Aldrich) and PHCl (Sigma-Aldrich) were dissolved in dimethyl sulfoxide to produce a 1000X stock (DMSO; BDH); compounds were made fresh weekly and stored at -20°C. BAB and PHCl were diluted to desired concentration in PLM culture media. BMP4 (R&D Systems) was resuspended in reconstitution buffer which consisted of 4 mM hydrochloric acid and 0.1% bovine serum albumin; BMP4 was added to PLM media for a final concentration of 10 ng/ml. AGN194310 (310, Allergan) was resuspended in ethanol and added to PLM culture media to a final concentration of 100 nM.

Dissection & Tissue Culture

Primary limb mesenchymal (PLM) cultures were prepared from the fore and hind limb buds of E11.5 embryos from wild type CD-1 mice (Charles River) using the following methodology. Following collection, the limb buds were genetly cut into quarters, and limb mesenchyme and ectoderm of the limbs was separated by proteolytic digestion in Pucks Saline solution containing dispase (12mg/ml; Invitrogen) and 10% fetal bovine serum (FBS; GIBCO BRL), with gentle shaking for 45 to 60 min. After proteolytic digestion, cells were filtered through a Cell Strainer (40μm; Falcon) to obtain a single cell suspension in PLM culture media (40% Dulbecco's modified Eagle's medium and 60% F12 supplemented with fetal bovine serum (GIBCO BRL) to 10% as well as 1% glutamine, 1% penicillin and 1% streptomyacin). Cells were pelleted by centrifugation at 200xG and resuspended at a concentration of 2.0x10^7 cells/ml.
PLM cells were used for transfection and patch clamp analysis or establishment of cultures for Q-PCR and histological assays. To establish PLM cultures, 10 µl aliquots of cells were plated as high density micromasses. A single micromass was plated in each well of a 24 well plate (Nunc); 5-10 micromasses were plated in each well of 6 well culture dishes (Nunc). Following plating, cells were allowed to adhere to the plate at 37°C for 1 hr, then covered with pre-warmed PLM culture media; this was considered Day 0 (D0), unless otherwise specified. Cells were typically treated 24 hrs after culture initiation and cultures were maintained for up to 15 days. For experiments lasting more than 4 days in culture, 0.25 mM ascorbic acid and 1 mM β-glycerol phosphate were added on day 3 (D3). For all experiments, culture media and treatment(s) were replaced every 2 days.

For alcian blue & alkaline phosphatase staining, culture medium was aspirated and PLM cultures were washed twice with phosphate buffer solution (PBS). Cultures stained with alcian blue were fixed in 95% ethanol at 20°C overnight; cultures stained with alkaline phosphatase were fixed in 10% formalin at 20°C, for 10 min. Cultures stained with both alcian blue and alkaline phosphatase were stained first with alkaline phosphatase and subsequently fixed with 10% formalin at 20°C, for 10 min. Fixative was removed by aspiration and cells were washed with PBS twice. Cultures stained with alcian blue were then washed with 0.2 M HCl and stained overnight with a 1% alcian blue solution prepared in 0.2 M HCl. Cultures stained with alkaline phosphatase were stained for 1 hr in darkness at 20°C; alkaline phosphatase solution was comprised of 100 mM Tris pH 8.0, 0.1 mg/ml of Naphtol and 0.65 mg/ml of Fast Red. Alcian blue stained cultures were stored in 70% ethanol; alkaline phosphatase stained cultures were
stored in PBS; cultures stained with both alcian blue and alkaline phosphatase were stored in PBS.

**Plasmid constructs & transfection of PLM cells**

Transfection of PLM cells in 24-well format was carried out with Effectene (Qiagen) reagent as per manufacturer’s instructions. Effectene buffer contained trehalose (0.4M) to improve transfection efficiency. Luciferase reporter genes employed included a firefly luciferase reporter gene, containing four sequential Sox9 binding sites (4x48) upstream of a minimal type II collagen promoter coupled to a luciferase gene, Col2-LUC, and a *Renilla* reporter gene, RL-SV40, to normalize for transfection efficiency. A ratio of 10:1 of Col2-LUC to RL-SV40 was used for all transfections. DNA/transfection mixtures were applied to and gently mixed with PLM cells (2x10⁷ cells/ml) and 10µl aliquots were spotted as high density micromass cultures to the center of each well; this was considered D0, unless otherwise specified. Cultures were incubated at 37°C in a 5% CO₂ atmosphere for 1hr to allow cells to adhere to the plate before pre-warmed (37°C) PLM culture media was added. Cultures were treated with compounds 24 hours after culture initiation; media was replaced every other day.

Lysates for luciferase analysis were collected using the Dual Luciferase Assay system following the manufacturer’s instructions (Promega). Briefly, micromass cultures were washed twice in PBS and lysed in 100µl of passive lysis buffer (Promega) for 20 min with gentle shaking. Each sample was analyzed in triplicate or quadruplicate; firefly luciferase and *Renilla* luciferase activities were determined using 20 µl of lysate per well in 96 well plates. Luciferase activity
was measured by addition of 50 µl of Luciferase assay reagent (Promega) and 50 µl of Stop & Glo reagent (Promega) in a 96 well plate reading luminometer (Molecular Devices).

**RNA Extraction, cDNA Archiving & Quantitative Real-time PCR**

RNA was isolated from PLM high-density micromass cultures using the QIAGEN RNeasy method. Cultures were homogenized by addition of RLT lysis buffer (Qiagen) and triturated by repeated pipetting. Lysate was applied to shredder columns (Qiagen) and RNA was extracted as per the manufacturer’s protocol. Concentration and quality of total RNA eluted was determined using the Nanodrop 1000 spectrophotometer. For quantitative real-time PCR, 1-2 µg of total RNA was reverse transcribed to cDNA using a high capacity cDNA archiving kit (Applied Biosystems, ABI) as per the manufacturer’s instructions.

Quantitative real-time PCR on a 7500 Fast Real-Time PCR system (ABI) was used to follow expression of gene transcripts for *Acan*, *AdamTs4*, *AdamTs5*, *Cdrap*, *Col2a1*, *Col10a1*, *Ihh*, *Kcnd2*, *Mmp13*, *Runx2*, *Sox6* and *Sox9*. For detection of transcripts TaqMan Gene Expression Assays (ABI) were used. Primers and probes were obtained from the TaqMan gene expression collection (ABI) or Integrated DNA Technologies (IDT); see Table 2.1 and Table 2.2 for primer and probe sequences or catalogue numbers. Quantification was performed using ~10ng of total RNA and the expression of all genes relative to endogenous rRNA, 18S, was determined using TaqMan ribosomal control reagents (ABI).
### Table 2.1 Custom Primer & Probe Sets used for Q-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
<th>System</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acan</td>
<td>AGGTTGCTATGGT GACAAGG</td>
<td>TGGAGGTTGAATTT CTCTGGG</td>
<td>6FAM/TCGTGAGG/ZEN/AGATGGAGGGTGA/1ABkFQ</td>
<td>IDT</td>
</tr>
<tr>
<td>AdamTs4</td>
<td>CTGGGATATGCTGG ATGTTG</td>
<td>AGTGCATGGCTTGG AGTTATC</td>
<td>6FAM/TCGTATTTGGAAGATGATG/GGTG/3IABkFQ</td>
<td>IDT</td>
</tr>
<tr>
<td>AdamTs5</td>
<td>TGTGAGAACTGG ATGTTGACG</td>
<td>CCTCAAACACCTCAG TATAACCC</td>
<td>6FAM/ATGACAAGTGTG GAGTGTGC/GGA/3IABkFQ</td>
<td>IDT</td>
</tr>
<tr>
<td>Cdrap</td>
<td>CTGATCGAGCTAT GCCCAAG</td>
<td>CACGACATGGAGA TAGGATG</td>
<td>6FAM/TCGTGAGGAA/ZEN GCTGTC/GGA/3IABkFQ</td>
<td>IDT</td>
</tr>
<tr>
<td>Col2a1</td>
<td>GGCTCCCAACAC CGCTAAC</td>
<td>GATGTTCTGGGAGC CCTCACT</td>
<td>6FAM/CAGATGACTTTCAC TCGTC/MGBNFQ</td>
<td>ABI</td>
</tr>
<tr>
<td>Ihh</td>
<td>CCCAACLACAACAT CCGCAACT</td>
<td>TCACCCGACTTCCA CAC</td>
<td>6FAM/CCGACCGCC/ZEN TCATGACC/3IABkFQ</td>
<td>IDT</td>
</tr>
<tr>
<td>Runx2</td>
<td>TCCCGGGAACC AAGAA</td>
<td>GCGATCAGAGAACA AACTAGGTA</td>
<td>6FAM/CAGATGACTTTCAC TCGTC/MGBNFQ</td>
<td>IDT</td>
</tr>
<tr>
<td>Sox9</td>
<td>CATCACCCGCTCG CAATAC</td>
<td>CGGGCTGCCTGACT GTAGTA</td>
<td>6FAM/ACCATCAGAAACTCCGCT/MGBNFQ</td>
<td>ABI</td>
</tr>
</tbody>
</table>

### Table 2.2 On-Demand Primer & Probe Sets used for Q-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Catalogue #</th>
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<tbody>
<tr>
<td>Acan</td>
<td>Mm00545794-m1</td>
<td>ABI</td>
</tr>
<tr>
<td>Col10a1</td>
<td>Mm00487041-m1</td>
<td>ABI</td>
</tr>
<tr>
<td>Kcmd2</td>
<td>Mm30356567-m1</td>
<td>ABI</td>
</tr>
<tr>
<td>Mmp13</td>
<td>Mm009498428-m1</td>
<td>ABI</td>
</tr>
<tr>
<td>Sox6</td>
<td>Mm00488393-m1</td>
<td>ABI</td>
</tr>
</tbody>
</table>
Probe preparation for in situ hybridization

Col2 and Col10a1 riboprobes for in situ hybridization were generated using plasmids containing a fragment of the Col2 or Col10a1 cDNA. The plasmids were linearized and used as templates for riboprobe synthesis; BamHI was used to linearize the Col2 plasmid, Xho1 was used to linearize the Col10a1 plasmid. Linearized plasmids were separated by gel electrophoresis, gel purified and the concentration was determined using a Nanodrop 1000 spectrophotometer. Transcription reactions required 1µg of template cDNA, 2 µl of transcription reaction buffer, 2 µl of dig RNA labelling mix, 2 µl of RNA polymerase and RNase free H2O was added to a final volume of 20 µl; reaction time 2 hrs at 37°C. T7 RNA polymerase was used to generate the Col2 antisense probe; T3 RNA polymerase was used to generate the Col10a1 antisense probe. To precipitate the probes, 2.5 µl of 4M LiCl, 2 µl 0.2M EDTA and 75 µl of 100% EtOH was added to and mixed with each transcription reaction; reactions were incubated at -80°C for 10 min. Reactions were centrifuged for 5 min at 13,000 RPM, 4°C, after which the supernatant was discarded. Following which 22 µl H2O, 2.5 µl 4M LiCl and 75 µl of 100% EtOH, were added and mixed to reprecipitate the probe; mixture was incubated at -80°C for 10 min. Mixtures were centrifuged for 5 min at 13,000 RPM, 4°C, after which supernatant was discarded. The pellet was washed in 75% ice cold EtOH, the supernatant was discarded and the pellet was allowed to air dry. Hybridization solution containing: 10 mM Tris pH 7.5, 600 mM NaCl, 1 mM EDTA, 0.25% SDS, 10% Dextran Sulfate, 1X Denhartdt’s, 200 µg/ml yeast tRNA, 50% formamide, was warmed to 55°C and the pellet was resuspended in 100 µl of hybridization solution and stored at -20°C.
The *Kcnd2* riboprobe for *in situ* hybridization was prepared from a PCR generated template. PCR amplification of *Kcnd2* cDNA required 2-5µg linearized plasmid 20% 10X PCR buffer, 10mM dNTP’s, 5U/μl Taq polymerase and custom primers containing Sp6 or T7 sequences (IDT);

Sp6 Sense KCND2 primer:
5’-GCGATTTAGGTGACACTATAGAAGACGCCATTGGGTGGATGCCTGT-’3

T7 Antisense KCND2 primer:
5’-GGATCCTAATACGACTCACTATAGGGAGCGGGATGCTGGTGAACTTGC-’3

PCR conditions: 94°C for 30 sec, 35 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, followed by 72°C for 1 min, hold at 4°C. An aliquot of 1μl of PCR product was run on 1% agarose gel for confirmation of *Kcnd2* fragment – *Kcnd2* fragment was 1029bp – and the rest of the PCR reaction was run on a gel and the appropriate sized-band gel purified. The concentration of the PCR product was determined using a Nanodrop 1000 spectrophotometer. As described above, transcription reactions required 1μg of template cDNA, 2μl of transcription reaction buffer, 2 μl of dig RNA labelling mix, 2 μl of RNA polymerase and RNase free H₂O to 20 μl; reaction time 2 hrs at 37°C. T7 RNA polymerase was used to generate the *Kcnd2* antisense strand; Sp6 RNA polymerase was used to generate the *Kcnd2* sense strand. The probe was pelleted as above and stored at -20°C in 100μl of hybridization solution.

Linearized *Kcnd2* PCR template was provided to the Richman Lab for preparation of a probe for radioactive *in situ*-hybridization. The transcription reaction was comprised of 5 μl of 5X reaction buffer, 0.5 μl of 1M DTT, 1.2 μl of 10 mM GTP, 1.2 μl of 10 mM ATP, 1.2 μl of 10 mM CTP, 1 μl of 50 mM UTP, 2-3 μg of linearized DNA template, 10 μl of 10 μM $^{35}$S-UTP
(1000Ci/mm mol; Amersham, SJ1303), 0.5 µl of RNasin and 1 µl of T7 RNA polymerase. All reagents, except RNasin and RNA polymerase were mixed at 20°C. RNasin and RNA polymerase were added and the mixture was incubated at 37°C for 40 min. Next, 1 µl of T7 RNA polymerase was added to the reaction and the mixture was incubated at 37°C for an additional 60 min. To remove template, 0.5 µl of RNasin, 1 µl of 10 mg/ml tRNA, 0.5 µl 1 M DTT and 0.5 µl DNase I was added and mixture was incubated for 10 min at 37°C. To pellet probe, 95 µl of ddH₂O, 10 µl 5 M LiCl and 300 µl of 100% EtOH were added and the mixture was subsequently centrifuged for 15 min at 13,000 RPM, -80°C. The pellet was washed with 10 mM DTT in 70% EtOH. Excess EtOH was aspirated and the pellet was allowed to air dry. The pellet was resuspended in 50 mM DTT in 50 µl ddH₂O. Probe concentration was in the range of 5 X 10⁷ - 1.2 X 10⁸ cpm.

As the Kcnd2 probe was relatively long, ~1029 bases, it was hydrolysed to reduce length and improve tissue penetration. To hydrolyse, 50 µl of hydrolysis buffer containing: 80 mM NaHCO₃, 120 mM NaCO₃ pH 10.2, and 10 mM DTT, was added to the probe, mixed and incubated for 30 sec at 60°C. To stop hydrolysis 50 µl of neutralizing buffer was added; neutralizing buffer contained 0.2 M sodium acetate, 1% glacial acetic acid and 10 mM DTT. To precipitate the hydrolyzed probe, 15 µl of 3 M sodium acetate pH 5.2 was added, followed by 100% EtOH and the mixture was precipitated at -80°C for 10 min and then centrifuged for 15 min at 13,000 RPM. The pellet was washed with 10 mM DTT in 70% EtOH. Excess EtOH was aspirated and the pellet was allowed to air dry. The pellet was resuspended in 50 mM DTT in 50 µl ddH₂O. The probe was stored at -80°C for up to 4 weeks.
All probes were used separately and did not match any known sequence in GenBank except those of the intended genes.

**In Situ hybridization**

Embryo’s were dissected at embryonic ages 11.5, 12.5, 13.5, 14.5, 15.5 in PSA and fixed overnight at 4°C in 4% paraformaldehyde (PFA) in DEPC-PBS; all solutions prior to hybridization were prepared with DEPC (Sigma) treated H2O and autoclaved to eliminate RNase activity. Following fixation embryo’s were washed twice in PBS and dehydrated in 100% ethanol. The tissue was processed (impregnated with wax) by the pathology department at University Hospital (UBC). Tissues were embedded in paraffin wax at 60°C and were allowed to set overnight at room temperature.

Sections for dig labelled *in situ* hybridization were cut at 10-25 μm using a microtome (Leica RM2255). Sections were placed on positively charges slides and dried overnight at 42°C. Slides were stored at 20°C until use. Sections for radioactively labelled *in situ* hybridization were cut at 7 μm using a microtome (Leica RM2255). Sections were placed on TESPA (3-triethoxysilypropylamine; Sigma-Aldrich) coated slides and dried overnight at 42°C. Slides were stored at 4°C until use.

Colorimetric *in situ* hybridization slides were rinsed twice in xylene for 5 min to remove wax and rehydrated to PBS with sequential washes with 100%, 75%, 50% and 20% EtOH then washed twice with PBS. Slides were fixed in 4% PFA in PBS for 10 min and then washed three times in PBS containing 0.1% Tween-20 (PBT). Slides were then placed in proteinase K solution.
containing: 10 µg/ml proteinase K (Sigma-Aldrich) in PBS for 10 min at 37°C then rinsed twice in PBS for 5 min. Slides underwent a second fixation in 4% PFA in PBS for 20 min, then were rinsed three times in PBS. Slides were acetylated in 0.1 M triethanolamine with 0.025% acetic anhydride for 10 min, rinsed three times in PBS for 5 min and air dried for 30 min at 20°C. Hybridization solution was warmed to 65°C and 100 µl of hybridization solution containing 1 µl probe was added to each slide. Slides were covered with silicone coverslips and incubated at 65°C overnight. Coverslips were removed by washing in 5X SSC. Slides were then washed in 1X SSC in 50% formamide for 30 min at 65°C. Next they were washed in TNE containing: 50 mM Tris pH 7.5, 2.5 M NaCl, and 5 mM EDTA, for 10 min and 37°C. TNE was removed and replaced with TNE containing RNase A at 20 mg/ml for 30 min at 37°C, then washed in TNE for 10 min, at 37°C. Slides were washed once in 2X SSC for 20 min at 65°C then washed twice in 0.2X SSC for 20 min each at 65°C. To prepare slides for antibody incubation and detection they were rinsed twice in maleic acid buffer (MAB) containing 0.1% tween-20 (MABT) for 5 min at 20°C; MAB contains 200 mM Maleic acid, 300mM NaCl and the pH was adjusted to pH 7.5 using 10 M NaOH. Slides were blocked in 20% heat inactivated sheep serum (HISS) with MABT and incubated at 4°C with anti-dig antibody (conjugated to alkaline phosphatase) overnight in a humidified environment. Slides were washed four times in MABT for 5 min each at 20°C. Subsequently slides were washed in alkaline phosphatase buffer (NTMT) pH 9.5 for 10 min at 20°C; NTMT contained: 100 mM NaCl, 100 mM Tris pH 9.5, 50 mM MgCl₂ and 0.1% Tween-20. Slides were stained in NTMT with nitro blue tetrazolium chloride (NBT; Roche) and 5-bromo-4-chloro-3-indoyle phosphate (BCIP; Roche) until the desired staining was achieved. Slides were rinsed once in NTMT and twice in PBS for 5 min. Slides were fixed following
staining in 4% PFA with PBS and 0.1% glutaraldehyde for 20 min and rinsed twice in PBS and once in H₂O. Slides were mounted in glycerol based mounting medium.

Radioactive *in situ*-hybridization was performed by the Richman lab at the UBC Life Science Center. Briefly, slides were warmed from 4°C to 20°C then dewaxed in xylene for 15 min. Slides were rehydrated sequentially in 95, 90, 80, 60, and 30% EtOH in ddH₂O. Slides were washed twice in ddH₂O for 2 min then placed in 0.02M HCl for 20 min. Next the slides were soaked in 2X SSC for 5 min. Slides were then placed in proteinase K solution containing: 5 µg/ml proteinase K (Sigma-Aldrich), 100 mM Tris pH 7.5 and 50 mM EDTA for 10 min at 37°C. Slides were rinsed in sterile PBS with 2 mg/ml glycine for 2 min then twice in sterile PBS. Slides were fixed in 4% PFA in PBS for 20 min then rinsed in PBS twice. Slides were acetylated in 0.1 M triethanolamine with 0.025% acetic anhydride for 10 min, rinsed in PBS for 5 min and H₂O for 2 min. Slides were then dehydrated sequentially in 30, 60, 80, 90 and 95% EtOH for 2 min each and air dried for 1 hour. For hybridization, slides were washed with agitation in wash solution for 15 min at 55°C; wash solution contained: 2X SSC, 50% formamide and 10mM DTT for 15 min. Slides were washed a second time with agitation for 20 min and the temperature was raised to 65°C. The slides were allowed to hybridize overnight at 65°C. To remove DTT, slides were then rinsed twice for 15 min in RNase buffer at 37°C. RNase buffer contained: 500 mM NaCl, 10 mM Tris pH 8.0 and 1 mM EDTA. Next, slides were incubated at 37°C in 40 µg/ml pancreatic RNase A in RNase buffer for 30 min and then rinsed in RNase buffer for 15 min. Slides were washed twice in wash solution for 20 min at 65°C then washed once in solution containing 0.1X SSC and 10 mM DTT for 20 min at 65°C. Next slides were rinsed for 5 min in 0.1X SSC and dehydrated sequentially in 300 mM ammonium acetate in 70% EtOH, 95% and
100% EtOH then air dried for 1 hour. Slides were dipped under an Ilford 902 safelight in Ilford K5 emulsion diluted 6:8 with 2% glycerol in ddH2O. Excess emulsion was allowed to drain and slides were air dried overnight. Slides were stored in a light proof container filled with silica gel at 4°C and were developed 1 week later. To develop, slides were warmed to 20°C for 60 min and developed for 5 min with a Kodak D19 developer. Slides were rinsed with H2O and fixed for 5 min with Kodak Unifix then washed for 3 hours and 35 min under running H2O and rinsed in ddH2O before staining; the stain contained 0.3% hematoxylin. The slides were rinsed in 30% EtOH and allowed to air-dry overnight, then soaking in xylene for 10 min and mounted with Entellan mounting medium.

**Patch Clamp Analysis**

Patch Clamp analysis was carried out by Dr. D. Fedida’s laboratory (UBC Life Sciences Center). The Underhill lab provided Media, compounds and cells. Embryo’s were dissected and PLM cells were collected as described above. Isolated cells were plated on sterile glass cover slips and diluted with PLM culture media to 2.0x10^5 cells/ml in 25 mm wells and maintained at 37°C in a 5% CO2 atmosphere. Patch clamp recordings were obtained within 36 hours of isolation. Bath solution consisted of 135 mM NaCl, 5 mM KCl, 1 mM MgCl2, 2.8 mM NaAcetate and 10 mM HEPES adjusted to pH 7.4 with NaOH. The pipette solution consisted of 130 mM KCl, 5 mM EGTA, 1 mM MgCl2, 10 mM HEPES, 4 Na2ATP and 0.1 mM GTP adjusted to pH 7.2 with KOH. All solutions were maintained at ambient room temperature, 20-23°C, during recordings. Whole-cell current observations and data analysis were recorded with Axopatch 200B amplifier and pClamp 8 software (MDS Analytical Technologies). Thin-walled borosilicate glass (World
Precision Instruments) was used to fabricate patch electrodes; glass was fire polished to a finished resistance of ~1-3 megohm. All whole-cell recordings underwent capacity compensation and access resistance was below 5 megohm. Data were sampled at a frequency of 20 kHz and filtered with a 4-pole Bessel filter with an $f_c$ of 5 kHz. Membrane potentials were not corrected for small junction potentials that arose between bath and pipette solutions.

For BAB and PHCl block assays, a +40 mV pulse, cycling at 1 Hz, was used and the series resistance compensation was set at 80%. BAB and PHCl concentrations were increased in a stepwise manner. Peak currents were normalized to the currents recorded prior to the addition of compounds. When necessary, linear rundown was corrected by offline subtraction. Availability curves were fit using a single Boltzman function.

**Microscopy & Image analysis**

Images of fixed cultures (in 70% ethanol or PBS) and *in situ* hybridization limb sections were collected using a dissection microscope (Stemi SV11 Apo, S1.6x objective; Carl Zeiss MicroImaging, Inc.). Colour images were acquired with a QImaging Retiga 1300i (12-bit) camera and using Openlab 4 software (Improvision). Dark field images were taken with a Leica DFC425-C camera. Photoshop adjustments (brightness/contrast) were applied uniformly to all images within a panel.
Statistical Analysis

With the exception of radioactive *in situ* hybridization, all experiments including histological assays, luciferase assays and real time qPCR were performed a minimum of three times using separate preparations of PLM cells. Luciferase assays were performed in either triplicate or quadruplicate. Real time qPCR data were analyzed in duplicate and repeated a minimum of three times with independent RNA samples. QPCR data were averaged and normalized to 18S rRNA. Both Q-PCR and Luciferase data were analyzed by one-way analysis of variance (ANOVA), followed by a Tukey post-test for multiple comparisons using Graphpad Prism software. One representative experiment is shown for all luciferase and Q-PCR results. Error bars represent standard deviation (SD). Significance is represented as follows: *, P<0.05; **, P<0.01; ***, P<0.001.
Chapter 3 - Results

Pre-Chondrocyte to Chondroblast

In the developing mouse limb chondrogenesis is initiated approximately midway through gestation (Lefebvre, V. & Smits, P. 2005). BAB and PHCl were identified as chondrogenic-modulatory compounds from a chondrogenic screen and subsequent assays were focused on identifying at which stage of chondrogenesis they were active. To determine the mechanism by which BAB and PHCl stimulate chondrogenesis we concentrated on determining the reporter gene activity, cellular morphology and gene expression profiles at two crucial stages of chondrogenesis: pre-chondrocyte to chondroblast and chondrocyte to hypertrophic chondrocyte.

To assess the impact of BAB and PHCL on chondroblast differentiation, assays were carried out over the first few days of culture, as chondroblast differentiation typically takes place during day 2-4 of culture. To establish the optimal concentration of BAB and PHCL for these studies, dose-response plots were generated. For these purposes, PLM cells were transfected with the chondrogenic responsive reporter gene Col2-LUC and treated with various concentrations of BAB and PHCl and luciferase activity was measured 48 hrs post-transfection or 24 hrs post-compound addition. From these experiments, it appeared that the optimal concentration of BAB and PHCl was 10-20 µM. When concentrations of < 10 µM of BAB were used, reporter gene activity was reduced, while at higher concentrations the increase in reporter gene activity was modest (Figure 3.1A). Concentrations of PHCL up to and including 20 µM led to increased reporter gene activity, whereas higher concentrations reduced reporter activity (Figure 3.1C). As
noted above, BMPs enhance chondroblast differentiation and the ability of the compounds to modulate this activity was tested in Col2-LUC reporter gene assays. In both cases, the combination of either PHCL or BAB increased BMP4-induced reporter gene activity ~ 2-fold (Figure 3.1 B, D). Interestingly, at low concentrations (5 and 1 µM for BAB and PHCL, respectively) in which BAB or PHCL had a modest effect on reporter activity alone, they potentiated BMP4 activity. Histological analyses of PLM micromass cultures after 8 days, stained with alcian blue, further demonstrated that all conditions led to variable increases in the extent of alcian blue with the treated cultures typically exhibiting greater density of staining (Figure 3.1E).

To further characterize the apparent pro-chondrogenic activity of BAB and PHCL they were compared to known chondrogenic modulators BMP4 and 310. Both BMP4 and 310 have previously been shown to stimulate chondroblast differentiation (Hoffman, L., et al. 2003). For these experiments, compounds/factors were added at Day 0 and luciferase activity was measured 1 and 3 days following compound addition, i.e. during the chondroblast differentiation window. In comparison to treatment with BMP4 or 310, the addition of BAB or PHCL led to similar increases in reporter gene activity within 1 day. However, by 3 days BAB and PHCL exhibited a significantly increased reporter gene activity in comparison to either 310 or BMP4-treated cultures (Figure 3.2). Interestingly, the addition of BAB or PHCL in combination with either BMP4 or 310 had a negligible or modest impact on reporter gene activity in comparison to the BAB or PHCL alone treated cultures.
To further examine the impact of BAB and PHCL treatment on chondroblast differentiation, various markers reflective of chondroblasts were examined by qPCR. For the most part all markers were increased at early time points (D1 and D2) following BAB or PHCL treatment, whereas with the exception of Col2al, there was no appreciable increase observed in either Acan or Sox6 expression in day 4 cultures (Figure 3.3). The increased expression of Sox6 at day 1 indicates that the compounds either accelerate chondroblast differentiation or enhance recruitment of non-chondrogenic cells to the chondrocytic lineage.
Figure 3.1 BAB & PHCl exhibit robust pro-chondrogenic activity

/(C) PLM cells were transfected with the Col2-LUC reporter gene on Day 0 and treated with compounds at varying concentrations 24 hrs after culture initiation. Luciferase activity was measured 48 hrs post treatment. The optimal concentrations of BAB and PHCl were determined to be ~ 10 µM. Reporter gene activity increased linearly with an increase in the concentration of BAB. Conversely, PHCl treatment increased reporter gene activity when used at a concentration of 20 µM or less; when concentrations of PHCl greater than 20 µM were used reduced reporter gene activity was observed.  

/(B/D) PLM cells were transfected with the Col2-LUC reporter gene on Day 0 and treated with BAB (10 µM) or PHCl (10 µM) with and without BMP4 (10 ng/ml) 24 hrs after culture initiation. Luciferase activity was measured 48 hrs post treatment. When BAB or PHCl were used in combination with BMP4 an additive increase in reporter gene activity was observed. Error bars represent SD. Significance is shown as *, P < 0.05; **, P < 0.01; ***, P < 0.001.  

/(E) PLM cultures treated with BAB (10 µM) and PHCl (10 µM) with and without controls, 310 (100 nM) and BMP4 (10 ng/ml), for 10 days and stained with alcian blue demonstrated further that BAB and PHCl are potent chondrogenic stimulators. Magnification bar, 1 mm.
The pro-chondrogenic activity of BAB and PHCl were compared to BMP4 and 310. PLM cells were transfected on Day 0 with the chondrogenic responsive reporter gene, Col2-LUC; treatments were added on Day 1 and every other day thereafter. Luciferase activity was measured 24 and 72 hrs following compound/factor addition. The activity of BAB and PHCl was comparable to, and somewhat greater than that of BMP4 and 310. Error bars represent SD. Significance is shown as *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 3.3 BAB & PHCl increase expression of chondrogenic markers

PLM cells treated with BAB or PHCl show increased expression of chondrogenic markers; shown here are the profiles for expression of Acan, Col2α1 and Sox6. PLM cells were treated 24 hrs after culture initiation this was considered Day 0 (D0) and lysed 24 hrs (D1), 48 hrs (D2) and 72 hrs (D4) post treatment. Error bars represent SD. Significance is shown as *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Chondrocyte to Hypertrophic Chondrocyte

The expression and activity of the SOX5, 6 and 9 trio peaks in chondroblasts and chondrocytes, and is reduced in prechondrogenic cells and chondrocytes undergoing hypertrophy. The Col2-LUC reporter gene provides a read-out on the activity of the SOX trio, but does not discriminate between the impact of the compounds on stimulating chondroblast differentiation nor reduced hypertrophy, situations in which SOX trio activity should be increased. As BAB and PHCL both increase reporter gene activity and early markers of chondroblast differentiation, we next sought to address their impact on later stages in the chondrogenic program, those associated with chondrocyte hypertrophy. In micromass cultures, chondrocyte hypertrophy typically begins around day 5 in a limited number of cells and over time many more cells become hypertrophic. Markers of hypertrophy become markedly expressed by day 8 and later. Initial studies aimed to assess the impact of BAB and PHCL on Col2-LUC reporter gene activity in longer-term cultures (Day 6 and 9) following treatment during (D1) or after (D3) chondroblast differentiation. Analysis at D6 following either D1 or D3 treatment revealed a significant increase in reporter gene activity with either treatment (Figure 3.4A). At D9, the impact was greatly reduced with later stage treatments (D3) having limited effect on reporter activity in comparison to control cultures (Figure 3.4A). Histological analyses of PLM micromass cultures treated with BAB and PHCL with and without controls factors, 310 and BMP4, demonstrated further that BAB and PHCL are potent chondrocyte stimulators as shown by the dense alcian blue staining (Figure 3.4B). In addition, alkaline phosphatase staining is reduced, primarily in the center of the micromass cultures when treated with BAB and PHCL, similar to the activity of 310 indicating that BAB and PHCL may be acting to inhibit or delay hypertrophic chondrocyte differentiation (Figure 3.4B).
To further examine the impact of BAB and PHCL on chondrocyte hypertrophy various chondrogenic markers and hypertrophy markers were examined in long-term cultures (5 or 10 days). During hypertrophy, chondrogenic markers are reduced, whereas hypertrophic marker expression is increased. Compounds and/or factors that inhibit hypertrophy should cause sustained or increased expression of chondrogenic markers and diminished expression of hypertrophic markers. At day 5 and 10, BAB and PHCL treatment generally leads to increased expression of *Acan* and *Cdrap*, and to a lesser extent *Sox6* and *Sox9* (Figure 3.5). Under these conditions, BAB and PHCL have a negligible impact on BMP4-mediated induction of these genes. In contrast, BAB and PHCL addition markedly inhibited the expression of multiple pre-hypertrophic and hypertrophy associated genes including, *Col10a1, Ihh* and *Mmp13*, and had a minor effect on *Adamts4* and 5, and *Runx2* (Figure 3.6). Consistent with the ability of BMP4 to stimulate hypertrophy, it increased the expression of these various genes. The ability of BMP4 to stimulate hypertrophy, can be used to further evaluate the ability of compounds or factors to inhibit hypertrophy. In this regard, combination treatments with BMP4 and BAB or PHCL significantly decreased BMP4-mediated induction of *Col10a1* and *Mmp13*, but interestingly not the other genes (Figure 3.6). This indicates that treatment with BAB and PHCL inhibits expression of the hypertrophic phenotype.
Figure 3.4 BAB & PHCl stimulate chondrogenic activity at the hypertrophic stage

Treatment of PLM cells with BAB and PHCl results in prolonged chondrogenic responsive reporter gene activity. A) PLM cells were transfected on Day 0 with the chondrogenic responsive reporter gene Col2-LUC; compounds were added at varying concentrations on either Day 1 or Day 3 of culture and every other day thereafter. Luciferase activity was measured on Day 6 and Day 9 of culture. Error bars represent SD. Significance is shown as *, P < 0.05; **, P < 0.01; ***, P < 0.001. B) PLM cultures treated with BAB and PHCl with and without controls, 310 and BMP4, for 10 days, stained with alkaline phosphatase and alcian blue/alkaline phosphatase overlay. Magnification bar, 1 mm.
A

Col2-LUC

Treat D1

Lyse D6

Relative light units (RU, % Ctrl)

(·) BAB PHCI

***

Treat D3

Lyse D6

Relative light units (RU, % Ctrl)

(·) BAB PHCI

***

B

(-) 310 B4 310/B4

(-) BAB PHCI

BAB+ PHCI
Figure 3.5 BAB & PHCl sustain increased expression of chondrocyte markers

PLM cells treated with BAB or PHCl with and without BMP4 exhibit increased expression of chondrogenic markers such as *Acan, Cdrap, Sox6*, and *Sox9*. PLM cultures were treated 24 hrs after culture initiation and every other day thereafter; PLM cultures were lysed 5 and 10 days post treatment for collection of RNA. Ascorbic acid (AA) and β-glycerol phosphate (βGP) were added to PLM culture media 3 days post-treatment. Error bars represent SD. Significance is shown as *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 3.6 BAB & PHCl reduce expression of hypertrophic markers

PLM cells treated with BAB or PHCl with and without BMP4 exhibit reduced expression of hypertrophic markers such as *AdamTs4*, *AdamTs5*, *Col10a1*, *Ihh*, *Mmp13* and *Runx2*. PLM cultures were treated 24 hrs after culture initiation and every other day thereafter; PLM cultures were lysed 5 and 10 days post treatment and RNA collected. AA and βGP were added to PLM culture media 3 days post treatment. Error bars represent SD. Significance is shown as *, P < 0.05; **, P < 0.01; ***, P < 0.001.
**Kcnd2 Activity in the Murine Limb**

Previous studies had shown that BAB was a partial KCND2 channel blocker and the chondrogenic activity of BMP4 coincided with the concentrations shown to inhibit KCND2. To further evaluate if BAB was functioning in part through modulation of KCND2 activity, electrophysiological studies were undertaken to determine if KCND2-like channel activity was present in PLM cells. For these purposes, PLM cells were isolated and patch clamping was performed. Results revealed that PLM cells express a fast inactivating transient potassium channel activity, indicative of KCND2 (Figure 3.7A-D). Thereby, KCND2 is an active channel in PLM cells; its peak depolarization was determined to be +60 mV (Figure 3.7A-D). Subsequently, a patch clamp assay was used to confirm BAB was able to block the KCND2 channel, as found by Winkleman, D.B., et al. 2005. Consistent with the previous report, BAB (10 µM) was found to maximally inhibit channel activity by approximately 30%, and increasing the dose had no further impact on channel activity (Figure 3.8A).

BAB and PHCL exhibited remarkably similar activities in the various chondrogenic assays, so we hypothesized that PHCL may operate through a similar mechanism as BAB, namely inhibition of potassium channel activity and in particular KCND2. To date, while PHCL has been used for over 80 years and has exhibited interesting biological activities in a number of systems, no mechanism of action has been defined. To address this possibility, patch clamp analysis was employed in a heterologous system in which Kcnd2 was over-expressed in HEK293 cells. Results indicate that PHCl is a more effective KCND2 channel blocker than BAB, and at
higher concentrations PHCL exhibits > 80 % inhibition of KCND2 channel activity (Figure 3.8B-D).
PLM cells express a fast, inactivating transient potassium current, consistent with that of KCND2. **A)** Currents evoked from PLM cells held at -80 mV subtracted from currents evoked from PLM cells held at -40 mV. Test pulse ranged from -80 mV to +100 mV. **B)** Peak current density evoked from cells held at -80 mV plotted as a function of test potential. **C)** Representative trace of steady-state inactivation protocol. Cells were held at -80 mV and conditioned at potentials ranging from -110 mV to +20 mV. Peak current was recorded during a second test depolarization to +60 mV and plotted as closed circles in **D)** ($V_{1/2} = -59.5 \pm 7.4$ mV, $k = 9.3 \pm 9.2$ (n=4)). Open circles represent conductance-voltage relationship of steady-state currents evoked using the same protocol as in panel A ($V_{1/2} = -23.9 \pm 4.43$ mV, $k = 8.7 \pm 4.5$ (n=3)). Dotted lines in **A** and **C** denote zero current level.
A

100 mV
-80 mV

50 pA | 50 ms

B

peak current (pA/pF)

mV

-80 -60 -40 -20 0 20 40 60 80 100

C

20 mV
-80 mV

60 mV

200 pA | 100 ms

D

Availability

mV

-120 -90 -60 -30 0 30
Figure 3.8 BAB & PHCl are able to block KCND2

Patch Clamp analysis demonstrates that BAB and PHCl block, at least partially, KCND2 channel activity. A) Block of KCND2 by BAB. Diary plot of peak currents evoked using protocol shown in Figure 3.7C. Each point represents a current measurement and graph shows reduction of peak KCND2 current by addition of BAB and subsequent wash. B) Currents evoked by pulses to +40 mV at 1 Hz showing block by addition of PHCL at concentrations indicated. Currents were sampled at the time block reached a steady-state level. C) Diary plot of peak currents evoked using protocol shown in Figure 3.7C. Each point represents a current measurement and graph shows reduction of peak KCND2 current by addition of increasing concentrations of PHCl and subsequent wash. D) Hill plot of peak currents evoked in the presence of increasing concentrations of drug, normalized to control currents evoked prior to addition of drug ($IC_{50} =$ 78.6 μM, Hill coefficient = 1.0 (n=3)).
**Kcnd2 Expression in the Murine Limb**

PLM cells were found to functionally express KCND2-like channel activity. The prochondrogenic compounds, BAB and PHCL, selected from an unbiased screen, inhibit KCND2 channel activity in culture. In addition, microarray data indicate *Kcnd2* is dynamically expressed in PLM cultures and is down-regulated by BMP4. Together these results suggest that KCND2 may play an important role in skeletal development. To further address this possibility we examined the expression of *Kcnd2* in PLM cultures under a variety of conditions and used *in situ* hybridization to localize *Kcnd2* transcripts in the developing murine limb. In the first series of experiments, *Kcnd2* expression in PLM cultures was recorded and the impact of BAB and PHCL treatment on *Kcnd2* expression over 10 days in culture was assessed (Figure 3.9A/B). Control cultures show that *Kcnd2* expression decreases > 2-fold by day 4 and continues decreasing over time (Figure 3.9A). In early cultures (D1-2), BAB and PHCL treatment modestly increased *Kcnd2* expression (< 2-fold Figure 3.9B). However, at later stages (D4-D10) BAB and PHCL treatment increased *Kcnd2* expression > 2-fold (Figure 3.9B). This is not entirely surprising, given that inhibitors often lead to a compensatory increase in the expression of their target or alternatively, the cells are being maintained at a stage with higher *Kcnd2* expression. The former possibility is certainly more congruent with the possibility that BAB and PHCL operate in part through blocking KCND2 channel activity.

As noted above, both 310 and BMP4 stimulate, whereas retinoic acid inhibits chondroblast differentiation. Both 310 and BMP4 treatment significantly reduced *Kcnd2* expression when assessed during the period of chondroblast differentiation (D1-3) (Figure 3.10A). Furthermore,
co-treatments of BMP4 with either 310 or RA, led to decreased expression of \textit{Kcnd2}. At later stages, BMP4 treatment was sufficient for the most part to reduce BAB or PHCL-mediated induction of \textit{Kcnd2} (Fig. 3.10B). In summary, \textit{Kcnd2} appears to be down-regulated under conditions that promote chondroblast differentiation and this is consistent with the pro-chondrogenic activity of BAB and PHCL, which inhibit KCND2 function.

The distribution of various gene transcripts in the developing murine limb was determined using \textit{in situ} hybridization with DIG labelled riboprobes. To identify regions indicative of chondrocytes and hypertrophic chondrocytes, \textit{in situ} hybridization was performed with \textit{Col2a1} and \textit{Col10a1}, respectively (Figure 3.11A/B). The \textit{Col2a1} image shows strong specific expression in the cartilaginous tissue (Figure 3.11A). The \textit{Col10a1} shows weak specific staining in the hypertrophic cartilaginous tissue (Figure 3.11B); most specific staining was apparent in the ribcage (data not shown). The \textit{Kcnd2} \textit{in situ} hybridization showed very weak specific staining within the digits; limited expression was also observed within the intercostal rib chondrocytes and the cranium (Figure 3.11C).

To increase the sensitivity of section \textit{in situ} hybridization, we worked with Dr. Joy Richman’s lab on a radioactive \textit{in situ} hybridization assay. \textit{In situ} hybridization with a radioactively labelled \textit{Kcnd2} probe produced a stronger expression pattern within and around the chondrocytes of the murine limb and in around the joint capsule (Figure 3.11).
Figure 3.9 *Kcnd2* expression throughout chondrogenesis

PLM cells treated with BAB or PHCl show increased expression of *Kcnd2* over 10 day time course. PLM cultures were treated 24 hrs after culture initiation and every other day thereafter, and RNA was collected and gene expression analyzed using qPCR. A) Controls indicate *Kcnd2* decreases over 10 days in culture. B) BAB and PHCl increase *Kcnd2* expression 2-3 fold over 10 days in culture. AA and βGP were added to PLM culture media 3 days post treatment. Error bars represent SD. Significance is shown as *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 3.10 BAB & PHCl modulate Kcnd2 expression in PLM cultures

A) PLM cultures were lysed 24 hrs and 72 hrs post treatment. PLM cells treated with BAB, PHCl or RA show increased expression of Kcnd2, whereas treatment with 310 or Bmp4 (B4) reduces KCND2 expression. 310/B4 treatment had an additive effect reducing Kcnd2 expression more than either treatment alone; B4/RA treatment reduces Kcnd2 expression but not as drastically as B4 treatment alone. B) PLM cultures were lysed 5 and 10 days post treatment. Bmp4 reduces Kcnd2 expression while BAB and PHCl continue to increase Kcnd2 expression. BAB and PHCl are able to at least partially rescue Kcnd2 expression when treated in combination with Bmp4, especially at 5 days of culture post treatment. At 10 days of culture post treatment the combination of BAB or PHCl with Bmp4 is able to increase expression to a level higher than treatment with Bmp4 alone but not above control levels. AA and βGP were added to PLM culture media 3 days post treatment. Error bars represent SD. Significance is shown as *, P < 0.05; **, P < 0.01; ***, P < 0.001.
A

B
Figure 3.11 *Kcnd2* expression and spatial arrangement in the murine limb

KCND2 is expressed in and around chondrocytes of the murine limb and within the joint regions. Section *in situ* hybridization shows limited but specific expression of A) *Col2a1* (*Col2*); arrow shows *Col2a1* expression in chondrocytes; B) *Col10a1* (*Col10*); arrow shows expression in hypertrophic chondrocytes; C) *Kcnd2* is difficult to detect using DIG-labelled probe. Section *in situ* probes were DIG labelled. *Col2a1* limb shown is from E15.5 wild type CD-1 litters; *Col10* and *Kcnd2* limbs are from E14.5 wild type CD-1 litters. Sections are 10µm. Magnification bar, 1 mm D) Radioactive *in situ*-hybridization images of E14.5 CD-1 murine limb is shown, left to right, in bright-field (BF), dark-field (DF), and BF-DF overlayed. BF-DF overlay image shows *Kcnd2* expression in red. Arrow shows regions of specific *Kcnd2* expression within and flanking the cartilages, also note expression around the joints (most apparent on digit 4, 2nd from the bottom). Sections are 7µm. Magnification bar, 1 mm.
Chapter 4 - Discussion

Summary of Findings: Pre-Chondrocyte to Chondrocyte

As the immature pre-chondrogenic cells differentiate into chondroblasts this is associated with increased Col2-LUC reporter gene activity and elevated expression of chondrogenic markers such as Sox5, Sox6, Sox9, Acan, Cdrap and Col2a1. Reporter gene assays indicate that BAB and PHCl are robust stimulators of chondrogenesis. The chondrogenic activity of BAB and PHCl, used alone or in combination with BMP4 or 310, is greater than or comparable to the activity of BMP4 or 310 alone (Figure 3.1B/D & 3.2).

Histological assays where PLM cultures were stained with alcian blue to detect cartilage-associated proteoglycans indicate that treatment with BAB and PHCl produce morphologically similar cultures with a greater number of cartilaginous nodules focused at the center of the cultures. When BAB and PHCl are used together the PLM cultures are more similar to treatment with BMP4; cultures are thick with limited nodules along the outskirts of the micromass (Figure 3.1E).

Quantitative PCR results demonstrate that treatment with BAB and PHCl increase expression of several chondrogenic markers. Specifically, BAB treatment led to a significant increase in the expression of Acan, Col2a1, and Sox6; PHCl only significantly increased expression of Col2a1. Under these conditions, BAB appears to be a more effective modulator of these chondrogenic markers compared to PHCl (Figure 3.3).
Summary of Findings: Chondrocyte to Hypertrophic Chondrocyte

As chondrocytes differentiate to prehypertrophic chondrocytes and then hypertrophic chondrocytes cultures we expect to observe a decrease in Col2-LUC reporter gene activity. While this is true and the basal reporter gene activity is lower as the PLM cultures mature, BAB and PHCl treated cultures still demonstrate increased reporter gene activity well above the level of control cultures for up to 9 days regardless of the day of initial treatment (Figure 3.4A). This result indicates that BAB and PHCl are effective modulators of chondrogenesis even on more mature PLM cultures and this suggests that these compounds may be therapeutically effective on osteoarthritic chondrocytes undergoing dedifferentiation or hypertrophy.

Histological assays where PLM cultures were stained with alcian blue and alkaline phosphatase demonstrate that treatment with BAB and BAB+PHCl reduced the alkaline phosphatase staining especially toward the center of the micromass. Conversely, treatment with PHCl alone did not reduce alkaline phosphatase staining but the cultures showed dark alcian blue staining. When BAB and PHCl are used alone or together the PLM cultures are more similar to treatment with 310; cultures are thick with limited nodules along the outskirts of the micromass (Figure 3.4B).

PLM cultures begin differentiating to hypertrophic chondrocytes around day 5-6 in culture; at which point we expect to see a reduction in expression of chondrogenic markers: *Acan, Cdrap, Col2a1, Sox5, Sox6, and Sox9*, as well as increase in pre- and hypertrophic markers: *AdamTs4, AdamTs5, Col10a1, Ihh, Mmp13* and *Runx2*. qPCR data show that treatment with BAB and PHCl with and without BMP4 increased expression of chondrogenic markers. Hypertrophic
markers Col10a1 and Mmp13 were significantly reduced when treated with BAB or PHCl. Interestingly, BMP4 treatment results in a dramatic increase in Mmp13 and BAB and PHCl are able to at least partially reduce Mmp13 expression. Interestingly, BAB and PHCl had a modest effect on Ihh expression and in the presence of BMP4, were unable to attenuate BMP4-induced Ihh expression. Ihh is one of the earliest markers of the hypertrophic program, indicating that BAB and PHCl may act downstream of Ihh to impact expression of the hypertrophic phenotype.

**BAB and PHCl activity in Chondrogenesis**

As shown above, PLM cultures treated with BAB and PHCl exhibit dramatic and morphological changes in both alcian blue and alkaline phosphatase staining in comparison to control cultures. In addition, BAB and PHCl increase reporter gene activity as early as 1 day after treatment for up to 9 days in culture. Finally, we have shown that BAB and PHCl increase and sustain expression of chondrocyte markers, while simultaneously reducing expression of hypertrophic markers. Together, these results further demonstrate that BAB and PHCl exhibit robust pro-chondrogenic activity while they inhibit, at least partially progression to hypertrophic chondrocytes (Figure 4.1).
Mesenchymal cell development to hypertrophic chondrocytes and eventually bone progresses through a series of steps. BMPs act on cells at each stage of chondrocyte development. 310 (RAR antagonist) is an important stimulator of chondroblasts but inhibits progression to hypertrophic chondrocytes. BAB and PHCl act on pre-chondrocytes to stimulate chondrogenic markers, producing thick chondrocyte cultures and reduce expression of hypertrophic markers.
KCND2 Activity in the Murine Limb

Patch clamp analysis revealed that KCND2 is active in PLM cells. However as KCND2 channels were relatively sparse and PLM cells flatten and begin to produce cartilaginous nodules within just a few hours in tissue culture, therefore HEK293 cells were used for the patch clamp blockage assays.

It has previously been shown that BAB was able to block KCND2 and based on molecular and histological data we suspected that BAB may be specifically blocking the KCND2 channel while PHCl might have broader blocking potential and affect additional potassium channels simultaneously. Our results however, indicate that BAB is a partial blocker of KCND2 while PHCl is able to completely block KCND2 (Figure 3.8 A-D). The partial blocking ability of BAB is sufficient to modulate chondrogenesis and may produce fewer side effects or be more easily tolerated by the cells than PHCl. As PHCl is able to completely block the KCND2 channel this may explain why when used at concentrations greater than 20 µM is not well tolerated by the PLM cells (Figure 3.1C & 3.8 B-D).

In addition to KCND2, a number of other potassium channels have been identified both within chondrocytes and mesenchymal cells that have the ability to become chondrocytes (Barrett-Jolley, R., et al., 2010; Clark, R., et al., 2010; Sundelacruz, S., et al., 2008; Varga, Z., et al., 2011). However, none of these studies examined the expression repertoire of potassium channels in murine embryonic mesenchymal cells, as carried out herein. Articular chondrocytes
have been shown to express several Kv channels, and in recently published studies, Clark et al. (2010) showed that Kv1.6 (Kcnab) was abundantly expressed in mouse chondrocytes isolated from articular cartilage and that this channel may play a role in “repolarizing the chondrocyte following depolarizing stimuli that might occur in conditions of membrane stretch”. They also identified several other channels including Kv2.1, 3.3 and 4.1, while Kv4.2 and 4.3 appeared to be detectable, but at a low level. A second study examining expression of channels in chick limb mesenchyme identified both Kv1.1, 1.3 and Kv4.1 channels, and they found treatment with the K⁺ channel blocker tetraethyl-ammonium, both reduced cell proliferation and the expression of genes associated with chondrocyte differentiation, culminating in reduced cartilage nodule formation (Varga, Z., et al., 2011). These findings are in contrast to the results described herein, and this discrepancy in part may relate to differences in the experimental systems, but more likely reflects the ability of BAB and PHCl to impact a subset of potassium channels, such as Kv4.2, whereas TEA is a relatively broad spectrum K⁺ channel inhibitor.

**Kcnd2 Expression in the Murine Limb**

Quantitative PCR data confirm the dynamic expression of Kcnd2 in PLM cultures and that BAB and PHCl are able to modulate Kcnd2 expression even in the presence of BMP4 (Figure 3.9 & 3.10A & B). While Kcnd2 expression still decreases as PLM cultures mature, treatment with BAB and PHCl nearly doubles the transient expression. BAB and PHCl are most effective in the presence of BMP4 in the D5 cultures compared to the D10 cultures; PHCl is able to nearly completely counteract the effect of BMP4 at this time point.
Additionally in situ hybridization confirmed expression of Kcnd2 in the murine limb around the chondrogenic digits. Initially dig-labelled in situ hybridization demonstrated weak staining in all chondrogenic tissues but this may have been confused with the background staining. Radioactively labelled in situ hybridization indicated Kcnd2 is expressed around the chondrogenic tissues.
Chapter 5 - Limitations

As experimental models are simply replicas of the system in question there are always limitations that must be considered. Some of the limitations considered in this thesis are discussed below:

Embryos from wild type CD-1 time pregnant mice were used to generate PLM cultures and sections for *in situ* hybridization. While every effort was made to ensure the litters were the same age and the limb sections isolated were uniform, some variation between the embryos and limb sections is inevitable. Furthermore, tissue culture of the PLM cells for 1 to 15 days is unable to replicate the exact conditions of the cells in the developing limb. Tissue culture cells do not have access to a circulatory system so hormones and other factors naturally present are missing. In addition, appropriate cell-to-cell contact in tissue culture may be absent; however the micromass cultures do allow for increased cell-to-cell contact and cartilaginous nodules are able to form rapidly.

To determine gene expression of chondrogenic and hypertrophic markers as well as *Kcnd2* expression qPCR was used. Although qPCR is able to determine in real time the transcript abundance it does not always correlate directly with protein abundance or activity. Studies indicate that mRNA transcript abundance alone may not be sufficient to describe the biological system (Gygi, S., *et al.* 1992). Post-transcriptional mechanisms controlling the protein translation rate, the half-lives of specific proteins or mRNAs, as well as the intracellular location and
molecular associations of the protein products of expressed genes also play roles in the biological state and must be considered (Gygi, S., et al. 1992).

Patch clamp analysis demonstrated that KCND2 was an active channel in PLM cells, however the abundance of voltage gated channels was limited and PLM cells flatten and adhere to the cover slip within a few hours in tissue culture. As such, KCND2 expressing human embryonic kidney (HEK293) cells were used for patch clamp block assays with BAB and PHCl. We inferred from the results of the block assays how effective BAB and PHCl are on KCND2 in PLM cells.

Initially in situ hybridization was carried out using Dig labelled probes however these probes were not sensitive enough to detect Kcnd2 expression. An opportunity arose where by the Richman lab had extra radioactive label so we were able to examine a few slides with a radioactive labelled Kcnd2 probe. While the radioactive in situ hybridization assay was preformed with controls the tissue on the control slides did not originate from murine limb sections.
Chapter 6 - Conclusions

A novel high throughput assay that is highly predictive for identifying compounds with chondrogenic modulatory activity identified two chondrogenic stimulators, BAB and PHCl. The compound screen coupled with bioinformatic analyses enabled identification of a new chondrogenic regulatory pathway that involves potassium channel activity. Previously generated microarray data demonstrated that one channel in particular, Kcnd2, is down regulated by BMP4 and exhibits a dynamic expression pattern in the developing skeleton. The idea that potassium channels play a role in chondrocytes morphology and chondrogenesis was tested by molecular and histological analyses of PLM cells treated with BAB and PHCl and determining the spatial and temporal expression of the potassium channel Kcnd2 in the murine limb.

We found that BAB and/or PHCl are able to increase expression of chondrocyte markers well past the chondrogenic stage of chondrogenesis and decrease the expression of hypertrophic markers at the hypertrophic stage of chondrogenesis; this was true for up to 10 days in culture. Through histological analysis of PLM cultures treated with BAB and/or PHCl we observed an increase in the chondrocyte population of PLM cultures. Furthermore, Kcnd2 was found to be active in PLM cultures. Patch clamp analysis indicated that BAB is able to partially block the Kcnd2 channel while PHCl has a much greater channel blocking ability. Kcnd2 was expressed in the embryonic murine limb sections and its expression found to be increased by treatment with BAB and/or PHCl. These studies have revealed that BAB and PHCl are able to modulate chondrogenesis and impact the chondrocyte phenotype by potentially acting on potassium channels, such as KCND2.
Chapter 7 - Future Directions

Moving forward, it may be of interest to closely investigate the mechanisms of action of one or more of the other 5 chondrogenic stimulatory compounds identified in the screen and subsequent assays; especially considering the two compound used for this study, BAB and PHCl, proved to be useful tools in articulating a role for potassium channels in chondrocyte maturation. Compounds such as these may prove to be useful in elucidating other targets and pathways involved in the complex chondrogenic program.

Additionally, Clark, R., et al. 2010, have found potassium channels, other than KCND2 (Kv4.2), such as Kv1.6, are important regulators of membrane potential in articular chondrocytes. Investigating the roles of these potassium channels or channels structurally or functionally similar to Kcnd2 may also provide valuable information on chondrocyte development and maturation.

Kcnd2 knockout animals have been produced and show no conspicuous phenotype in the limb (Guo, W., et al., 2005); however, the tissues of the limb have not yet been carefully studied, in vivo or in vitro, in these animals. In addition to examining the role of Kcnd2, investigating the action of BAB and PHCl in these animals in surgery induced experiments will allow us to determine if these compounds are able to repair degenerative tissues or impart a resistance to osteoarthritis.
References


Guo, W., et al. 2005. Targeted deletion of Kv4.2 eliminates I_{o,f} and results in electrical and molecular remodeling, with no evidence of ventricular hypertrophy or myocardial dysfunction. Vol. 97, No. 12, pg 1342-50.


