The use of a synthetic hedgehog agonist in mouse models of chondrodysplasia

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Table of Contents

Abstract

The role of Indian hedgehog (Ihh) signalling in the regulation of endochondral bone formation is well established. Ihh controls the rate of bone growth by negatively regulating differentiation and positively regulating growth plate chondrocyte proliferation. It has been well documented also that mutations resulting in constitutive activation of signalling through FGFR3 in chondrodysplasia, lead to a significant decrease in this important signalling factor accompanied by reduced proliferation of the chondrocytes and a dwarf phenotype.

In an attempt to rescue the chondrodysplasia phenotype hedgehog agonist Hh-Ag 1.4 was injected subcutaneously into mice with achondroplasia (ACH) or with severe achondroplasia with developmental delay and acanthosis negricans (SADDAN) with mixed results.

Administration of a hedgehog agonist in SADDAN mice led to a significant up-regulation of both *Ptch* and *Gli1*, as measured by quantitative PCR, indicating that Hh-Ag 1.4 does indeed stimulate hedgehog signalling *in vivo*. Also, *in situ* hybridization for *Ihh* seems to show a down regulation of native *Ihh* expression in pre-hypertrophic chondrocytes, possibly due to the activation of the negative PTHrP feedback loop. In our study, Hh-Ag 1.4 treatment resulted in an increased growth plate length and reduced size of the hypertrophic zone. The cortical bone flanking the growth plate in mice injected with Hh-Ag 1.4 was 2-3 times thicker than in control mice, which may be attributed to the positive effect of increased Ihh signalling in osteoblastogenesis. Contrary to our expectations, there was also a noticeable reduction in chondrocyte proliferation in mice treated with the agonist.

Overall, the effect on the growth of long bones was not beneficial and the treatment with high doses of Hh-Ag 1.4 did not result in an amelioration of the chondrodysplastic phenotype.

Résumé

Le rôle de la voie de signalisation Indian hedgehog (Ihh) dans la régulation de l'ossification endochondrale est bien établi. Ihh contrôle le taux de croissance des os par son effet négatif sur la differentiation et son effet positif sur la prolifération des chondrocytes de la plaque de croissance. De plus, il a été démontré que des mutations conduisant à l'activation constitutive du récepteur FGFR3 dans les chondrodysplasies, résultent en une diminution significative de l'expression de cet important facteur de signalisation accompagnée par une diminution du taux de prolifération des chondrocytes et d'un phénotype de nanisme.

En espérant améliorer le phénotype de chondrodysplasie, l'agoniste de hedgehog Hh-Ag-1.4 fut injecté par voie sous-cutanée à des souris présentant un phénotype ACH ou SADDAN mais avec des résultats mitigés.

L'administration de l'agoniste hedgehog à des souris SADDAN a conduit à une augmentation significative de la transcription de *Ptch* et *Gli1*, tel que mesuré par PCR quantitatif, démontrant que Hh-Ag 1.4 stimule la signalisation hedgehog *in vivo*. De plus, la technique d' hybridation *in situ* semble démontrer une diminution de l'expression endogène de *Ihh* dans les chondrocytes hypertrophiques, possiblement une conséquence de l'activation de la boucle de régulation négative du PTHrP. Dans notre étude, le traitement avec Hh-Ag-1.4 a conduit à une augmentation de l'épaisseur de la plaque de croissance et une diminution de la taille de la zone hypertrophique. L'os cortical flanquant la plaque de croissance des souris traitées avec Hh-Ag-1.4 était 2 à 3 fois plus épais que chez les sujets témoins, ce qui peut être attribué à l'effet stimulant de la signalisation Ihh sur l'ostéoblastogénèse. Contrairement à l'effet attendu, nous avons aussi observé une diminution notable de la prolifération des chondrocytes chez les animaux traités avec l'agoniste.

De façon générale, l'effet sur la croissance des os longs n'était pas bénéfique et le traitement avec des doses élevées de Hh-Ag-1.4 n'a pas amélioré le phénotype chondrodysplastique.

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First and foremost I would like to thank my supervisor, Dr. Benoit St-Jacques, without whom none of this would be possible. During my time in his lab I have learned many scientific techniques and expanded my knowledge base considerably. Your guidance and friendship in pursuance of my Masters degree at McGill will always be remembered. I would like to thank Meg Desbarats for genotyping the SADDAN and ACH mice along with answering numerous technical questions over the course of my Masters training. I would like also to thank Alice Arabian and Judy Grover for their aid and consultation on many aspects of my experiments. Special thanks to Dr. St-Arnaud and Dr. Roughly for their advice and insight on the project throughout. I would also like to thank Stephen M. Cohn (M.D., Ph.D.) and David Ornitz (Ph.D.) for the SADDAN and ACH mice respectively. Many thanks to all my colleagues in the laboratory and friends at the Shriners Hospital, you all played an important role. Finally, I would like to thank all of my family and friends for their unwavering support over the last years.

List of Abbreviations

ACH	Achondroplasia
BMP	Bone Morphogenic Protein
BrdU	Bromodeoxyuridine
cDNA	Complementary DNA
cIHH	Chick Indian Hedgehog cDNA Sequence
CNP	C-type Natriuretic Peptide
COL	Collagen
DEPC	Di-ethyl-propyl Carbonate
DHH	Desert Hedgehog
DIG	Digoxygenin
DNA	Deoxyribonucleic Acid
ERK	Extracellular Signal-regulated Kinases
FGF	Fibroblast Growth Factor
FGFR3	Fibroblast Growth Factor Receptor-3
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
GLI	Glioma Associated Oncogene Homolog
GP	Growth Plate
H&E	Hematoxylin and Eosin
HCH	Hypochondroplasia
Hh	Hedgehog
Hh-Ag 1.4	Hedgehog Agonist 1.4 (Curis)
HYP	Hypertrophic Zone
IHH	Indian Hedgehog
MAPK	Mitogen Activated Protein Kinase
OSX	Osterix
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PLCγ	Phospholipase-Cγ
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
PFA	Paraformaldehyde
PTC	Patched
PTHR-1	Parathyroid Hormone Receptor-1
PTHrP	Parathyroid Hormone related Peptide
RNA	Ribonucleic Acid
rtTA	Reverse Tetracycline Trans-activator
RUNX2/CBFA-1	Runt-Related Gene 2/Core Binding Factor α1

SADDAN	Severe Achondroplasia with Developmental Delay and Acanthosis Negricans
SHH	Sonic Hedgehog
SMO	Smoothened
SOX	Sry-Related HMG-Box
STAT	Signal Transducers and Activator of Transcription
TD	Thanatophoric Dysplasia
TRE	Tetracycline Responsive Element
TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick End Labelling
VEGF	Vascular Endothelial Growth Factor
WNT	Wingless-Type MMTV Integration Site Family

List of Tables and Figures

1. Rationale and Objectives

Gene inactivation of *Ihh* by knockout in the mouse leads to pre-natal dwarfism that is as severe as seen in TD human foetuses. *Ihh* null mice display a 50% reduction in the rate of chondrocytes proliferation *in utero* (St-Jacques *et al.* 1999). While *Ihh* negatively controls chondrocyte maturation by up-regulating expression of *PTHrP*, its action on proliferation is thought to be independent of *PTHrP* (Karp *et al.* 2000). Furthermore, *Ihh* signalling independently stimulates the maturation of periarticular chondrocytes into proliferating chondrocytes to regulate growth plate length (Kobayashi *et al.* 2005). *Ihh* is undoubtedly an important regulator of growth plate physiology during development and disruption of *Ihh* signalling results in growth retardation. We hypothesize that restoring normal levels of hedgehog signalling, using a synthetic hedgehog agonist, in mouse models of chondrodysplasia will result in a significant amelioration of this phenotype.

To accomplish this task we have proposed the following set of goals:

1. To inject ACH and SADDAN mice with Hh-Ag 1.4 postnatally for a period of 6 days.
2. To elucidate, via BrdU, real-time PCR, *in situ* Hybridization, and bone histomorphometry whether or not there is a positive effect on the chondrodysplasia phenotype.

2. Introduction

2.1. Bone Formation and Development.

Primitive vertebrates had both a true endoskeleton and an exoskeleton (Carroll, 1988). The skeleton of modern vertebrates is mostly derived from the endoskeleton of these primitive vertebrates, with the exception of the skull and pectoral girdle, which are vestiges of the primitive exoskeleton (St-Jacques and Helms, 2003). While these remnants of the primitive exoskeleton are now thoroughly incorporated into the modern endoskeleton, they form in a manner that is very different (St-Jacques and Helms, 2003).

The bone of the modern skeleton is formed by two different processes. The skull and part of the pectoral girdle form by direct differentiation of mesenchymal cells into osteoblasts via a process known as intramembranous ossification. Bones in the rest of skeleton, derived from the primitive endoskeleton, progress from mesenchyme into cartilage before eventually being replaced by calcified matrix and bone (Patterson, 1977, St-Jacques and Helms, 2003). This process is known as endochondral ossification.

Both processes, endochondral and intramembranous, begin when a previously dispersed population of mesenchymal cells form a "condensation", the earliest sign of the initiation of skeletal elements (Hall and Miyake, 2000). This

aggregation of mesenchymal cells is mediated by a number of secreted proteins and transcription factors responsible for, initiation, proliferation, adhesion, growth, and the setting of boundaries, of the condensation, including: TGFβ-1, N-CAM, N-cadherin, Hox a-2, syndecan, MFH-1, sox-9, Pax-2, BMP-2, and BMP-4 (Ide *et al.* 1994; Koibuchi and Tochinai, 1999; Hall and Miyake, 2000) (**Figure 1**).

A condensation of cells must reach a critical size and the cells within the condensation must interact for the condensation phase to cease and differentiation to be initiated (Hall and Miyake, 2000). The progression from proliferation to differentiation of cells into chondroblasts or osteoblasts requires down-regulation of both N-CAM and the genes controlling proliferation, and an up-regulation of genes associated with differentiation (Bmp-2, Bmp-4, Bmp-5, Msx-1, Msx-2) (Hall and Miyake, 2000). Depending on whether or not the cells have differentiated into chondroblasts or osteoblasts, the condensations will enter into either the endochondral or intramembranous ossification process respectively (**Figure 2**; **Figure 3**).

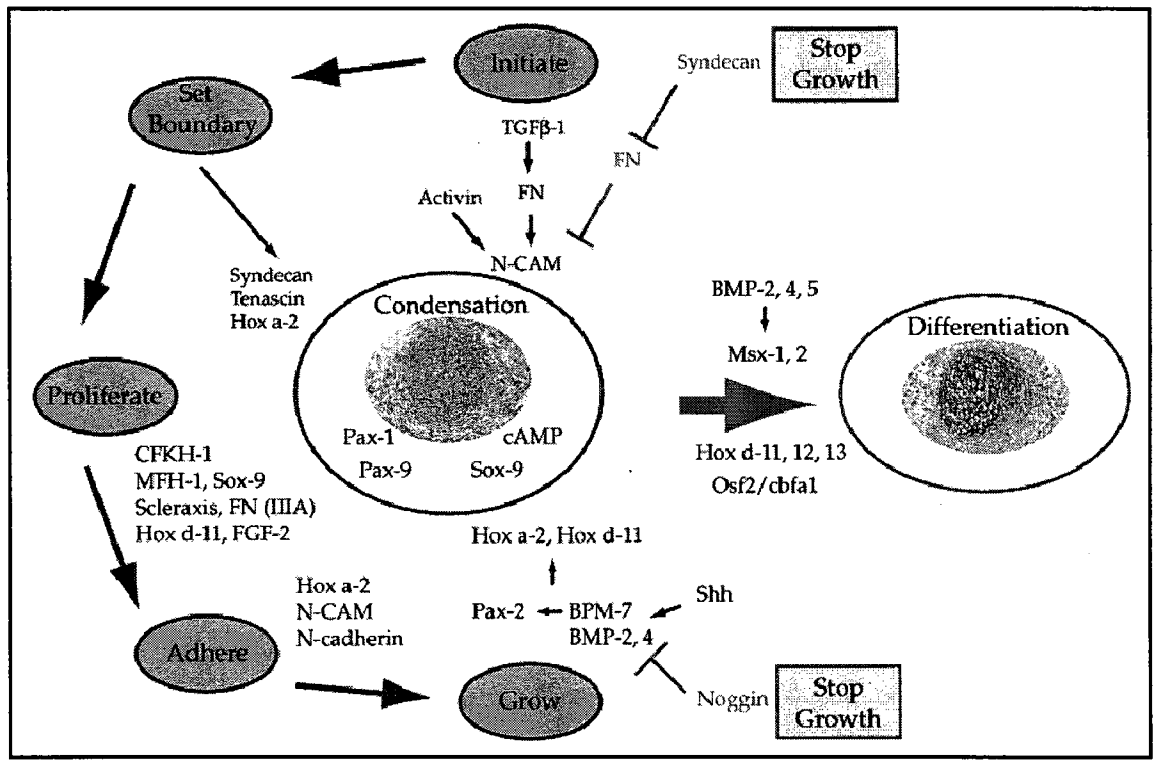

Figure 1: A summary of the major genes and gene products involved in condensation formation (blue) and in the transition from condensation to overt differentiation (green arrow). The condensation is visualised using peanut agglutinin lectin; the elevated levels of cyclic-AMP and major genes expressed at the condensation stage (*Pax-1, Pax-9, Sox-9*) are shown. The differentiating cartilage is visualised with Alcian blue. The major genes associated with five stages in condensation (Initiate; Set Boundary; Proliferate; Adhere, Grow) are shown. Two pathways that stop condensation growth (Stop Growth) are shown in yellow and red. Cessation of condensation leads to differentiation (green arrow), which involves both upregulation of genes to initiate differentiation and downregulation of genes to terminate condensation (Hall and Miyake, 2000).

Endochondral Ossification

The axial and appendicular skeletons as well as the bones at the base of the skull are formed via endochondral ossification (Gilbert, 2003). The endochondral process is preceded by a chondrogenic program in which the core of the chondrogenic condensations differentiate into chondrocytes that secrete and become embedded with a specific isoform of collagen type II matrix as a result of Sox9 induction of the Col type IIa1 enhancers (Eames *et al.* 2003; Sandell *et al.* 1994; Bi *et al.* 1999). Collagen IXa1 (Col9), collagen XIa2 (Col11) and *Aggrecan* are also simultaneously up-regulated as a result of Sox9 induction (Lefebvre *et al.* 1997; Lefebvre and de Crombrugghe, 1998; Bi *et al.* 1999; Healey *et al.* 1999; Mori-Akiyama *et al.* 2003). Indeed, Sox9 has been identified as the first transcription factor that is essential for chondrocyte differentiation and cartilage formation, both crucial to the process of endochondral ossification (Bi *et al.* 1999).

During endochondral ossification, the initial condensation occurs in an avascular environment and remains avascular unlike in intramembranous ossification (St-Jacques and Helms, 2003). The cells from the condensation then differentiate into chondroblasts which secrete the aforementioned cartilage matrix containing collagen types II, IX, XI, and aggrecan (**Figure 2B**). At the edge of these condensations the cells flatten forming a thin membrane of stacked cells (**Figure 2C**). This barrier layer is known as the perichondrium and separates the cells from the

surrounding mesenchyme (Caplan, 1987). As new matrix is produced, the cells in the core of the condensation are surrounded by their own lacuna and are completely enveloped by the perichondrium and become known as chondrocytes.

At this juncture, both the perichondrium and the chondrocytes undergo proliferation, which along with deposition of new matrix causes the growth of the elements (St-Jacques and Helms, 2003). Chondrocytes in the centre then undergo progressive maturation and begins to take on a flattened appearance while organizing themselves into columns along the longitudinal axis (**Figure 2D**) (**Figure 4**: *Left Panel*). Further maturation of these columnar proliferating cells results in hypertrophic chondrocytes, which can be characterized by their large size, round shape, and production of collagen X (St-Jacques and Helms, 2003.

The cartilage matrix changes in the hypertrophic zone, as it begins to release angiogenic factors, like vascular endothelial growth factor (VEGF), and is subsequently invaded by capillaries (Gerber et al. 1999). There is vascularisation of the perichondrium followed closely by the differentiation of the inner perichondrium cells into osteoblasts and the onset of osteogenesis. The primary ossification centre is established as osteoblasts invade the calcified cartilage and secrete the bone matrix of the first trabeculae (St-Jacques and Helms, 2003) (**Figure 2F**).

Within the primary ossification centre, the hypertrophic cartilage is degraded, there is death of the terminally differentiated hypertrophic chondrocytes, osteoblasts replace the disappearing cartilage with trabecular bone, and bone marrow is formed (Olsen *et al.* 2000). At the same time, osteoblasts in the perichondrium secrete a layer of primary compact bone referred to as the bone collar, around the middle portion (diaphysis) of the cartilage (**Figure 2E**).

Intramembranous Ossification

In comparison to endochondral ossification, the process of intramembranous ossification is poorly understood. Intramembranous ossification begins as a process when neural crest-derived mesenchymal cells proliferate and condense into compact nodules. While some of the cells in these nodules become capillaries, others differentiate into osteoblasts, which are the direct precursors to bone formation.

Osteoblast differentiation is the result of many different signalling peptides including, runt-related gene 2/Core binding factor α1 (Runx2/Cbfa1), bone morphogenic proteins 2, 4, and 7 (BMP 2, BMP 4, BMP 7), canonical Wnt/β-catenin, Hh, and osterix (Osx).

Once the mesenchymal cells condense to produce osteoblasts, these cells begin to secrete an osteoid matrix rich in collagen type 1 (Col1). As this matrix is

calcified in the ossification centres, bony spicules radiate outwards from the origin of ossification (St-Jacques and Helms, 2003) (**Figure 3**). These spicules are completely surrounded by the osteoblasts that secreted it, while other osteoblasts are trapped inside the bone and become osteocytes. Osteocytes trapped within the bone communicate to other osteocytes and the bone surface through a network of channel known as canaliculi that are used to exchange nutrients and waste (St-Jacques and Helms, 2003). Osteocytes are also thought to be mechanosensors, which detect micro fractures within the bone, and are crucial to bone remodelling. Less differentiated osteoprogenitor cells in the periphery proliferate and are a source of new osteoblasts, as bone is continually added to form a longer structure known as a trabecula. The primary spicules become connected by secondary trabelculae and form scaffolding that is characteristic of cancellous woven bone (**Figure 3**). Most of the woven bone is eventually replaced by mature lamellar bone created by multiple layers of uniformly oriented collagen fibres. Sustained deposition of fresh bone lamellae transforms the spongy cancellous bone into a more dense compact bone; the bone collar (**Figure 3**).

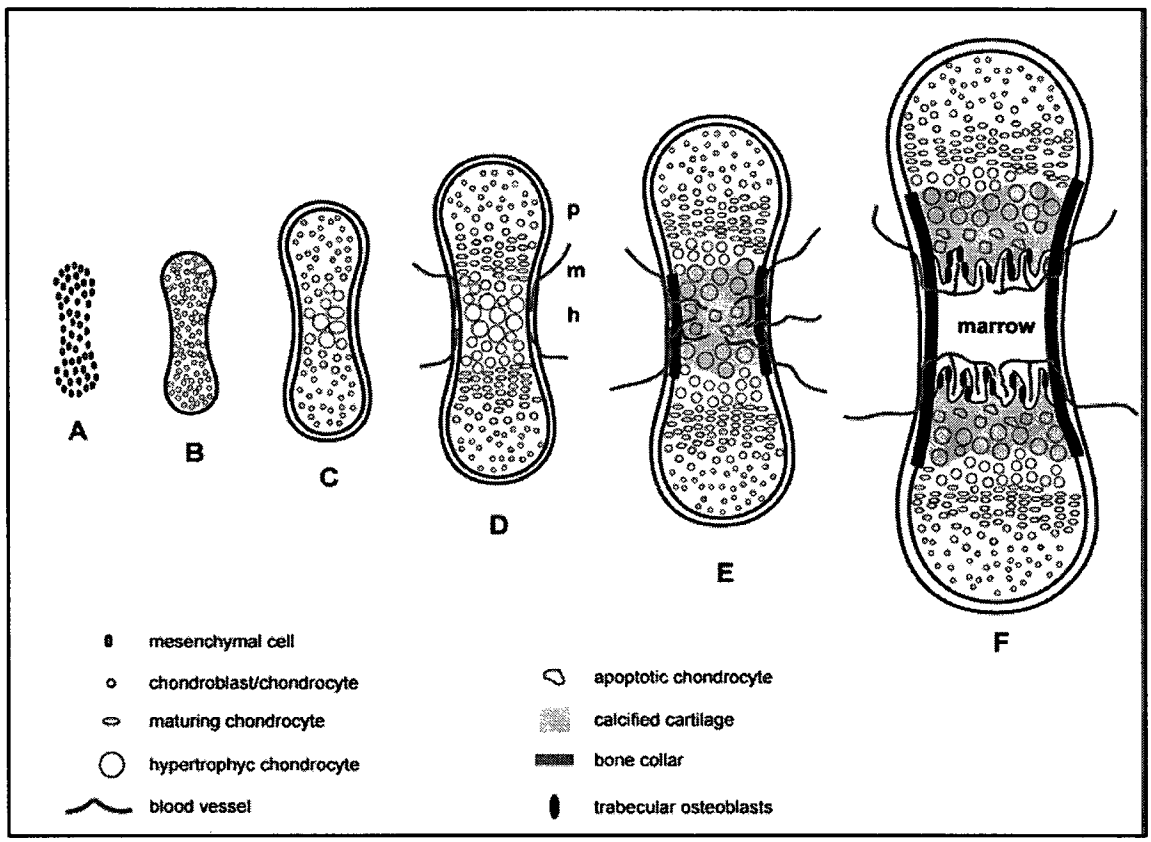

Figure 2: Schematic of endochondral bone formation. (**A**) A mesenchymal condensation begins to form and is followed by (**B**) the formation of the perichondrium and chondroblast differentiation. (**C**). There is rapid proliferation of the perichondrial cells while the chondrocytes further mature into hypertrophic at the centre of the element. (**D**) There is the formation of a structured growth plate and the beginning of vascular invasion of the perichondrium. (**E**) Formation of the bone collar around the cartilage centre, and progression of the vasculature into the hypertrophic zone. There is also apoptosis of the terminally differentiated hypertrophic chondrocytes. (**F**) Calcified cartilage is reabsorbed by chondroclasts, leaving a template for the osteoblasts to synthesize matrix, thus establishing the primary ossification centre. **pe** = perichondrium; **p** = proliferative zone; **m** = maturation zone; **h** = hypertrophic zone; **ma** = marrow space (St-Jacques and Helms, 2003).

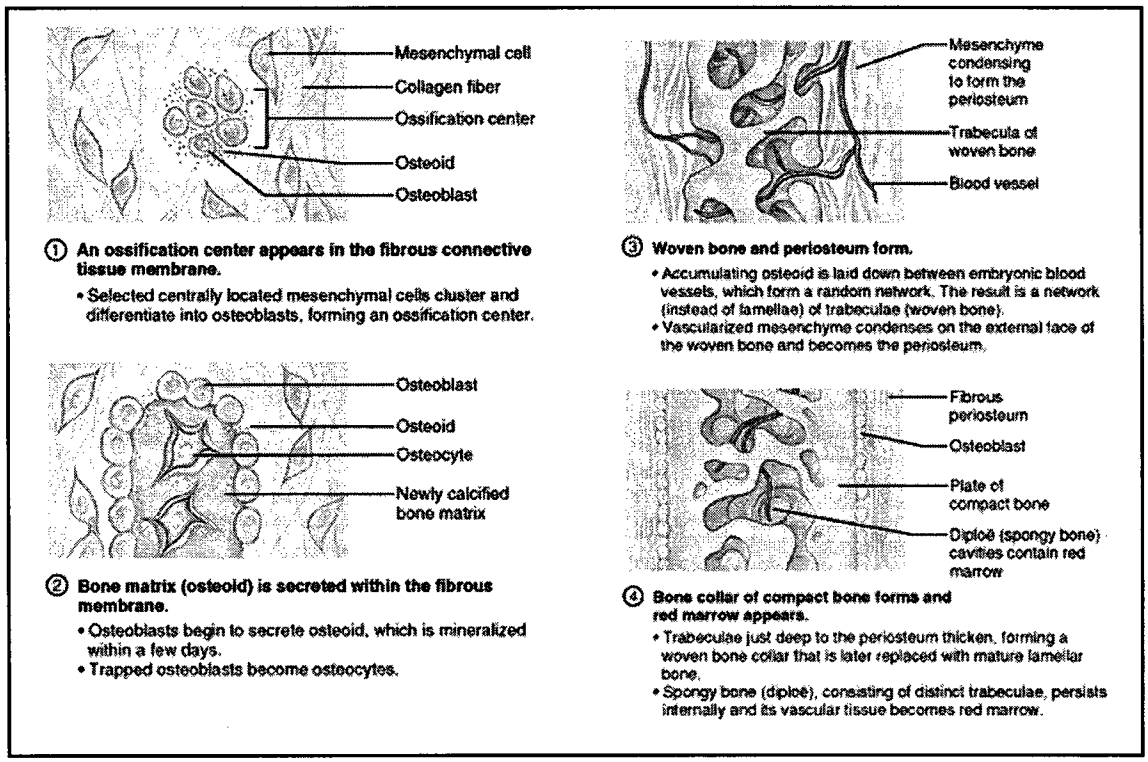

Figure 3: Schematic of Intramembranous Ossification. © Benjamin Cummings 2005.

Osteoblast Differentiation

The osteoprogenitor cells are induced by several peptides and transcription factors into becoming osteoblasts. One such transcription factor Runx2/Cbfa1 has been shown to be essential for osteoblast differentiation (Komori *et al.* 1997; Ducy *et al.* 1997) and for osteoblast function beyond differentiation (Ducy *et al.* 1999; Liu *et al.* 2001). Indeed, mice null for the Runx2/Cbfa1 transcription factor completely lack bone and die at birth (Komori *et al.* 1997; Otto *et al.* 1997). Similarly, mice lacking the Osx gene thought to be downstream of Runx2/Cbfa1 also do not develop bone suggesting this gene also plays an essential role in osteoblast differentiation (Naskashima *et al.* 2002). While Osx is downstream it is believed that BMPs 2, 4 and 7, lie upstream of the Runx2/Cbfa1 transcription factor and are also crucial for early osteoblast differentiation, and like Runx2/Cbfa1 are able to induce ectopic bone formation (Ducy *et al.* 1997; Gilbert, 2003).

Indian hedgehog (IHH) is also an important signalling peptide in differentiating osteoblasts. *Ihh* null mice completely lack osteoblasts in the endochondral skeleton, due in part to a Runx2 deficiency (St-Jacques *et al.* 1999). Long *et al.* (2004) showed that cells lacking Smoothened (Smo), thus Hh signalling, failed to undergo osteoblast differentiation. It was recently demonstrated that this signalling occurs through the canonical Wnt pathway (Hu *et al.* 2005) eventually leading to activation of Runx2/Cbfa1 and Osx.

2.2. FGFR3 Signalling and Chondrodysplasia

Fibroblast growth factor (FGF) signalling is a crucial factor in the regulation of growth plate chondrocyte physiology. The importance of this signalling pathway was revealed by the discovery that achondroplasia (ACH) is the result of an activating autosomal dominant mutation in the transmembrane domain of the fibroblast growth factor receptor 3 gene (*FGFR3*) (Shiang *et al.* 1994). ACH is the most common form of dwarfism in humans and is characterised by reduced growth of long bones, cranio-somatic disproportions, and frontal bossing of the cranium.

FGFR3 is one of the four related High-affinity tyrosine-kinase FGF receptor genes, and is expressed by proliferating and maturing chondrocytes of the growth plate (Ornitz and Marie, 2002). The mutation (G380R) that results in the ACH phenotype is a gain-of-function mutation that causes constitutive activation of signal transduction by the FGFR3 receptor without the presence of a ligand. The chondrodysplasia hypochondroplasia (HCH), severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN), and thanatophoric dysplasias type I (TDI) and type II (TDII), are also due to different activating mutations in *FGFR3* (Ornitz and Marie, 2002) (See **Table 1**). The phenotypes of HCH, ACH, SADDAN, TDI, and TDII exhibit progressively increasing clinical severity, correlating well with the degree of activation of FGFR3. This genotype-phenotype correlation is corroborated by a multitude of mouse models containing

activating mutations in *Fgfr3* (Ornitz and Marie, 2002; Naski et al. 1998; Chen et al. 1999, 2001; Li et al. 1999; Wang et al. 1999; Segev et al. 2000; Iwata et al. 2001).

Members of the FGFR family of receptors activate several intracellular signal transduction pathways by way of phosphorylation of the p38 and ERK1/2 mitogen activated kinases (MAPKs), phospholipase-Cγ (PLCγ), and members of the signal transducer and activator of transcription (STAT) family of factors (Eswarakumar *et al.* 2005). It is likely that it is through one of these complex signal transduction intermediates that activation of FGFR3 causes its downstream effects. Yet, it remains unclear how activation of these pathways leads to the growth plate dysfunctions seen in the chondrodysplasias, including reduced chondrocyte proliferation, reduced matrix synthesis, and delayed onset of endochondral ossification.

Constitutive phosphorylation of p38 MAPKs in the mouse, results in dwarfism characterised by both a decrease in chondrocyte proliferation and delayed chondrocyte hypertrophy as seen in ACH (Zhang *et al.* 2006). Conversely, constitutive ERK1/2 activation results in reduced chondrocyte maturation without a noted change in proliferation (Murakami *et al.* 2004). Lastly, STAT activation leads to reduced chondrocyte proliferation (Su *et al.* 1997; Sahni *et al.* 1999, 2001; Hart *et al.* 2000). C-type natriuretic peptide (CNP), another signalling peptide is also essential for normal skeletal growth (Chusho *et al.* 2001; Yasoda *et al.* 1998), and acts by inhibiting ERK1/2 MAPKs phosphorylation (Yasoda *et al.* 2004; Ozasa *et al.* 2005). In

a recent study by, Yasoda *et al.* 2004, it was discovered that forced expression of CNP in the growth plates of ACH mice lead to a partial rescue of the dwarf phenotype by correcting the decreased extracellular matrix synthesis but had no effect on the decreased rate of chondrocyte proliferation or the delayed maturation of chondrocytes. There are therefore at least three processes that may be possible therapeutic targets to correct the chondrodysplasias caused by FGFR3 activation: chondrocyte proliferation, matrix synthesis, and chondrocyte hypertrophy.

2.3. Indian Hedgehog

Indian Hedgehog (IHH) is a member of the Hedgehog (Hh) family which also includes Sonic Hedgehog (SHH) and Desert Hedgehog (DHH) (Ingham *et al.* 2001). The Hh family of secreted signalling peptides is critical for normal development of the embryo from early patterning to late organogenesis (Ingham *et al.* 2001). *Ihh* is expressed in a number of mammalian embryonic tissues and organs, including the pancreas, thymus, uterus, visceral endoderm, kidney, liver, and retinal epithelium (Marigo *et al.* 1995; Levine *et al.* 1997; Byrd *et al.* 2002; Takamoto *et al.* 2002; Hebrok *et al.* 2000; Valentini *et al.* 1997; Sacedón *et al.* 2003), but apart from the gut, *Ihh* expression domains do not overlap with either *Shh* or *Dhh* (Bitgood and McMahon, 1995).

Mouse model	Method/mechanism	Phenotype	Reference
Ectopic FGF2 expression	PGK promoter transgenic expression	Enlarged occipital bones, skeletal dwarfism	Coffin et al. 1995
Ectopic FGF2 expression	Adenoviral expression in suture	Coronal suture synostosis	Greenwald et al. 2001
Ectopic FGF9 expression	Type II collagen promoter transgenic expression	Achondroplasia-like dwarfism	Garofalo et al. 1999
Increased FGF3/FGF4 expression	Retrovirus insertion	Crouzon-like dysmorphology	Carlton et al. 1998
FGF2-deficient mice	Knockout mutation	Inhibition of bone formation/bone mass	Montero et al. 2000
FGF18-deficient mice	Knockout mutation	Delayed suture closure, expanded growth plate	Liu et al. 2002; Ohbayashi et al. 2002
Dominant-negative FGFR1	Adenoviral expression in suture	Inhibition of calvarial suture fusion	Greenwald et al. 2001
FGFR3-deficient mice	Knockout mutation	Skeletal overgrowth	Colvin et al. 1996; Deng et al. 1996
Gain of function, FGFR2c	Knockout of exon 9(IIIc), abberant alternative splicing	Coronal synostosis	Hajihosseini et al. 2001; Yu and Ornitz 2001
P250R mutation, FGFR1	Knockin mutation	Craniosynostosis	Zhou et al. 2000
K644M mutation, FGFR3	Knockin mutation	Severe dwarfism	Iwata et al. 2001
S365C mutation, FGFR3	Knockin mutation	Severe dwarfism	Chen et al. 2001
K644E mutation, FGFR3	Knockin mutation	Achondroplasia-like dwarfism	Li et al. 1999
K644E mutation, FGFR3	Knockin mutation	Thanatophoric dysplasia-like dwarfism	Iwata et al. 2000
G380R mutation, FGFR3	Type II collagen or *Fgfr3* promoter transgenic expression, Knockin mutation	Achondroplasia-like dwarfism	Naski et al. 1998; Wang et al. 1999; Segev et al. 2000
G369C mutation, FGFR3	Knockin mutation	Achondroplasia-like dwarfism	Chen et al. 1999

Table 1: Mouse models for FGF signalling in skeletal development (Ornitz and Marie, 2002).

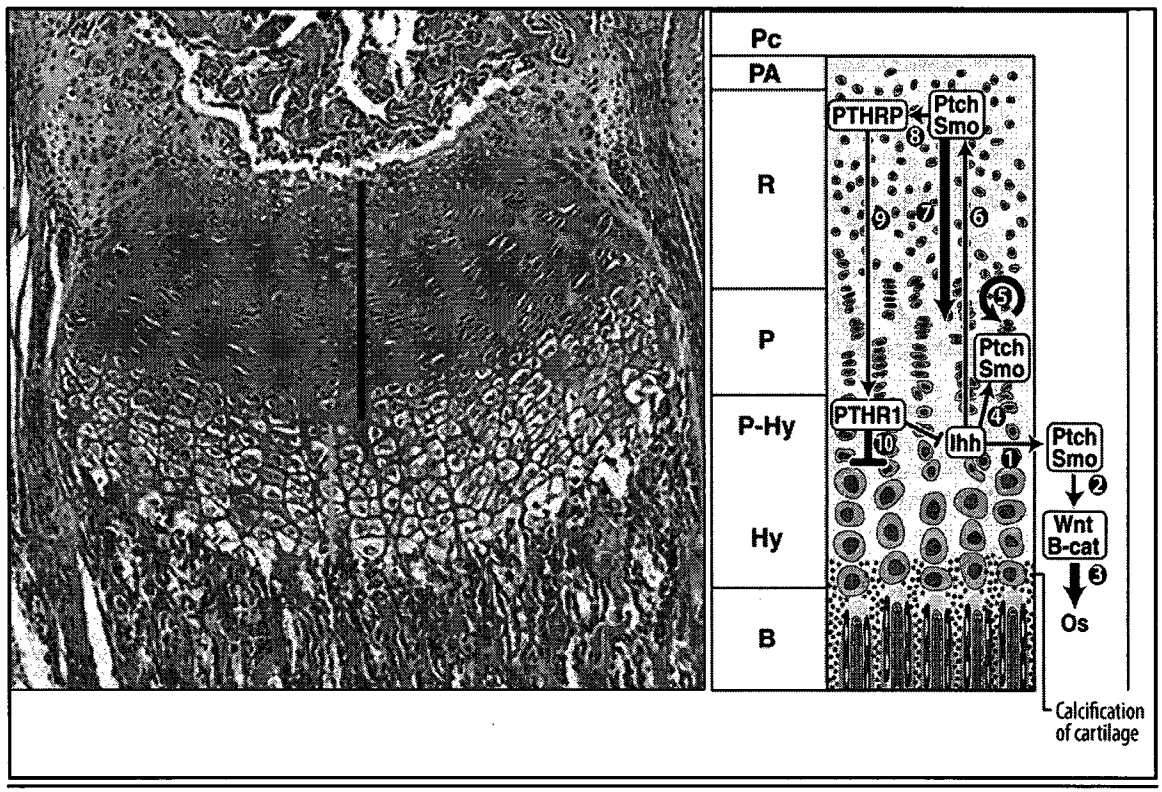

Figure 4: Roles of Ihh in the growth plate. *Left panel*: A longitudinal section through the wrist growth plate of a P15.5 day old mouse. *Right Panel*: The corresponding cartoon representation of signalling in the growth plate. Ihh is secreted by pre-hypertrophic chondrocytes (**P-Hy**) and diffuses to interact with its receptor Ptch/Smo (**1**) on perichondrial cells (**Pc**). This activates Wnt signalling (**2**) and differentiation of perichondrial cells into osteoblasts (**3**). Ihh also interacts with its receptor (**4**) on proliferating chondrocytes (**P**) and maintains their high proliferation rate (**5**). Activation of Ptch/Smo (**6**) in resting chondrocytes (**R**) induces their transition to a proliferative phenotype (**7**). Finally, Ihh signalling stimulates secretion of PTHrP (**8**) that acts on PTHR1 receptor-expressing cells (**9**) and slows down their maturation into pre-hypertrophic chondrocytes (**10**) thus lowering the amount of Ihh being secreted.

2.4. Indian Hedgehog Signalling in the Growth Plate

There are many signalling pathways at play during the development of bone in the mammalian skeleton. Both endochondral and intramembranous ossification are regulated by a multitude of complex signalling pathways that control the onset, the rate, and the duration of bone formation. It has been shown that *Indian Hedgehog (Ihh)* regulates proliferation and differentiation of chondrocytes in the growth plate and is essential for bone formation (St-Jacques *et al.* 1999).

IHH signals through a receptor complex made up of the multiple transmembrane proteins Patched (PTCH) and Smoothened (SMO) (Ingham *et al.* 2001) (**Figure 5**). The prevailing model for Hh signalling states that in the absence of Hh, PTCH, a 12 transmembrane spanning-domain protein, prevents SMO, a 7 transmembrane spanning-domain protein with homology to G-protein coupled receptors from activating downstream components (Hooper and Scott, 1989; Lum and Beachy, 2004; Alcedo *et al.* 1996). Upon binding of Hh to the complex, inhibition of SMO by PTCH is released leading to activation of the pathway (Lum and Beachy, 2004). Intracellular signal transduction by SMO affects the post-translational processing of three transcription factors of the GLI (Glioma associated oncogene homolog) family. The *Gli1* and *Gli2* genes are transcriptional targets of Hh signalling and their products act as transcriptional activators, while *Gli3* acts as a transcriptional repressor. However, upon activation of the Hh signalling cascade,

Gli3 processing is inhibited and full-length *Gli3* lacks repressor activity (Huangfu and Anderson, 2006). Therefore, the net result of Hh signalling is to stimulate Gli transcriptional activation and prevent Gli transcriptional repression activities (Huangfu and Anderson, 2006).

In the growth plate, *Ihh* expression is limited to the "pre-hypertrophic" chondrocytes (**Figure 4**) (St-Jacques *et al.* 1999). High levels of expression of both *Ptch* and *Smo* are restricted to the perichondrium, as well as in proliferating chondrocytes adjacent to the *Ihh* expressing cells (St-Jacques *et al.* 1999). The IHH peptide, like all hedgehogs, is processed from a 45kD to a 19kD N-terminal domain (Hh-N) and a 25kD C-terminal fragment by autoproteolysis (Porter *et al.* 1995). Porter *et al.* (1995), demonstrated that it is the N-product that is the active species in both local and long-range signalling. While expression is limited to the pre-hypertrophic chondrocytes, the IHH signalling peptide (N-terminal product), diffuses throughout the proliferative, pre-hypertrophic and upper hypertrophic zones (Gritli-Linde *et al.* 2001). The domains of *IHH* and the *PTC/SMO* expression overlap in the post-natal growth plate of mouse and human and remain expressed as long as the growth plate is open (Vortkamp *et al.* 1998; Van der Eerden *et al.* 2000; Kindblom *et al.* 2002; Farquharson *et al.* 2001; Nakase *et al.* 2001).

The IHH signalling pathway plays a crucial role in the regulation of growth plate physiology. Mice null for *Ihh* display a phenotype of severe pre-natal dwarfism

similar to that of TD in human (St-Jacques *et al.* 1999). These null embryos show a 50% decrease in chondrocyte proliferation, as measured by BrdU incorporation, *in utero*. This suggests that the pre-hypertrophic cells signal to the less mature *Ptch/Smo*-expressing chondrocytes to maintain proliferation (St-Jacques *et al.* 1999). In a recent study by Long *et al.* (2001), they removed Smo-activity specifically in the chondrocytes using the *Cre-LoxP* approach. These mice, when compared to *Ihh* null mice, show normal differentiation while proliferation is still reduced (Long *et al.* 2001). This supports the notion that there is a direct role for *Ihh* in the regulation of chondrocyte proliferation. While Ihh also negatively controls chondrocyte maturation by up-regulating expression of parathyroid hormone related peptide gene (*PTHrP*), its action on proliferation is independent of PTHrP (Vortkamp *et al.* 1996; Karp *et al.* 2000). The role of an Ihh/PTHrP regulatory loop in retarding the initiation of chondrocyte hypertrophy is well documented (Kronenberg 2003). However, there is also evidence that once a cell has initiated hypertrophic maturation, Ihh signalling may stimulate terminal differentiation (St-Jacques *et al.* 1999; Stott and Chuong, 1997; Deckelbaum *et al.* 2002; Minina *et al.* 2002; Akiyama *et al.* 1999; Mak *et al.* 2008). Ihh has also been shown to be responsible for the maturation of the periarticular chondrocytes into proliferating chondrocytes to regulate growth plate length (Kobayashi *et al.* 2005) (**Figure 4**). Consequently, IHH is

to be considered an important regulator of many aspects of growth plate physiology and perturbed IHH signalling can result in growth deficiency.

In addition to its role as a regulator of growth plate physiology, IHH has been shown to stimulate the differentiation of mesenchymal stem cells and pre-osteoblastic lines into osteoblasts *in vitro* and *in vivo* (St-Jacques *et al.* 1999; Nakamura *et al.* 1997). It was shown by St-Jacques *et al.* (1999), that the skeletons of *Ihh* null mouse embryos lack osteoblasts. This is because in the absence of an *Ihh* signal, perichondrial cells fail to activate the Wnt/β-catenin pathway, essential for osteoblastogenesis (Hu *et al.* 2005). It has also been shown that a Hedgehog signal is required early in the osteoblasts lineage while Wnt signalling is required later on for the complete differentiation of functional osteoblasts (Rodda and McMahon, 2006). Postnatally, the continued production of Ihh by chondrocytes is essential for sustained trabecular bone formation and bone elongation (Maeda *et al.* 2007).

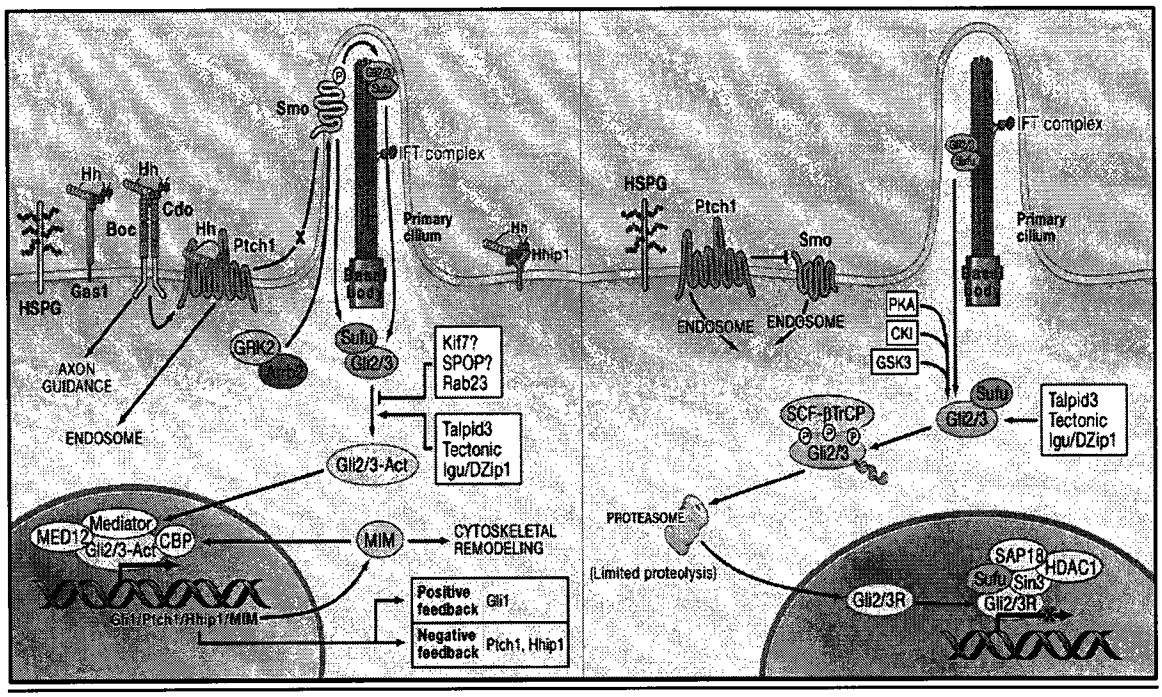

Figure 5: Hedgehog signalling pathway. *Left panel*: Hh-responding cell. Hh elicits transcriptional responses by binding to its receptor Patched (Ptch). The binding of Hh to Ptch alleviates repression of Smoothened (Smo) and allows the translocation of Smo from endosomes to the primary cilium in mice. Smo signal transduction prevents the processing of Gli factors. They translocate to the nucleus in their full-length form (Gli-Act) that acts as an activator of transcription of target genes, including *Patched* and *Gli-1*. *Right panel*: In the absence of Hh-ligand, Ptch inhibits Smo and prevents its translocation to the primary cilium. Transcription factors of the Gli family associate with a cytoskeletal complex. They undergo cleavage into a short form (Gli2/3-R) that translocates to the nucleus and acts as a repressor of transcription target genes (Chen *et al.* 2007).

2.5. Mouse Models of Chondrodysplasia.

There are several mouse models of corresponding human chondrodysplasias including, SADDAN, TD, and ACH, which have been created using transgenic or gene targeting approaches that harbour activating *Fgfr3* mutations. The phenotypes of these mice closely resemble the corresponding human syndromes and show that FGFR3 signalling is indeed a negative regulator of bone growth (Iwata *et al.* 2001; Naski *et al.* 1998; Chen *et al.* 2001). In addition mice homozygous for null alleles of *Fgfr3* display skeletal overgrowth (Colvin *et al.* 1996; Deng *et al.* 1996). It is very likely that part of the inhibitory effect of FGFR3 on chondrocytes is indirect in nature and involves downstream signalling factors. This is due to the finding that other signalling molecules controlling chondrocyte proliferation and maturation downstream of *FGFR3* show significant decreases, including Ihh. A similar decrease in Ihh is also seen in dwarfism caused by constitutive activation of p38 MAPKs (Zhang *et al.* 2006).

Naski *et al.* (1998), produced a mouse model of achondroplasia by creating a transgene containing human coding sequence for *FGFR3* with a G380R substitution (**Figure 6**). The transgene is under control of the rat collagen type II promoter/enhancer sequence and is therefore only expressed in proliferating chondrocytes (Naski *et al.* 1998). This overlaps with the expression domain of the endogenous murine *Fgfr3* gene. Mice heterozygous for the transgene have a skeletal

phenotype similar to the one seen in human achondroplasia, while homozygous mice are not viable. At birth, the mutant mice appear the same as their WT counterparts. However, by week 4 of post natal life they are 30% smaller then WT (Naski *et al.* 1998) (**Figure 6**).

The growth plates of these mice show significantly reduced zones of proliferating and hypertrophic chondrocytes, as seen in human achondroplasia (Naski *et al.* 1998). The reduction in the size of the zone is probably due, at least in part, to the dramatic decrease seen in *Ihh*, *Ptch*, and *Bmp4* expression, which have been shown to control the rate of chondrocyte proliferation (Naski *et al.*1998; St-Jacques *et al.* 1999).

The SADDAN mouse model of chondrodysplasia carries a point mutation (K644M) in the endogenous mouse *Fgfr3* locus (Iwata *et al.* 2001). In this mouse line, gene targeting was used to introduce the K644M mutation in exon-15 of Fgfr3 (**Figure 7**). This locus also contains a neomycin resistance cassette (*neo*) flanked by site-specific recombination sequences (*LoxP* sites) (**Figure 7**). This prevents the expression of the dominant mutant allele and mice heterozygous for this allele are wild-type (*K644Mneo* line). When mice that carry the "floxed"-allele are crossed with the *EIIa*-Cre (Jackson Laboratories, Bar Harbor, ME) line, there is expression of cre-recombinase in every cell type and excision of the *neo* cassette at the *LoxP* sites, leading to expression of the mutant dominant allele and resulting in the

development of the SADDAN phenotype (Iwata *et al.* 2001; Lasko *et al.* 1996). Mice heterozygous for the mutant allele display features that correspond to the human syndrome including severe dwarfism and cranial bossing, while homozygotes die *in utero* (**Figure 7**). Unlike in ACH, the SADDAN phenotype is immediately apparent at birth. As in human SADDAN, the phenotype in mouse is milder than the TD mutant phenotype, despite the *FGFR3* pathway being more highly activated (Li *et al.* 1999; Chen *et al.* 2001). It was noticed in our lab that in some mice Cre-mediated excision was not as efficient (*neo* cassette was still detectable by PCR) leading to an intermediate "mosaic" phenotype (To be further discussed in Results) (**Figure 7**).

Due to the fact that *Ihh* expression is reduced in both models, restoration of normal Ihh signalling in either the SADDAN or ACH models could rescue the growth plate phenotype and consequently the bone growth deficit seen in these syndromes. This would be accomplished by stimulating differentiation of proliferating chondrocytes and maintaining their high rate of mitosis, stimulating matrix synthesis, accelerating maturation of terminally differentiated hypertrophic chondrocytes and stimulating trabecular bone formation at the chondro-osseous junction (**Figure 4**)

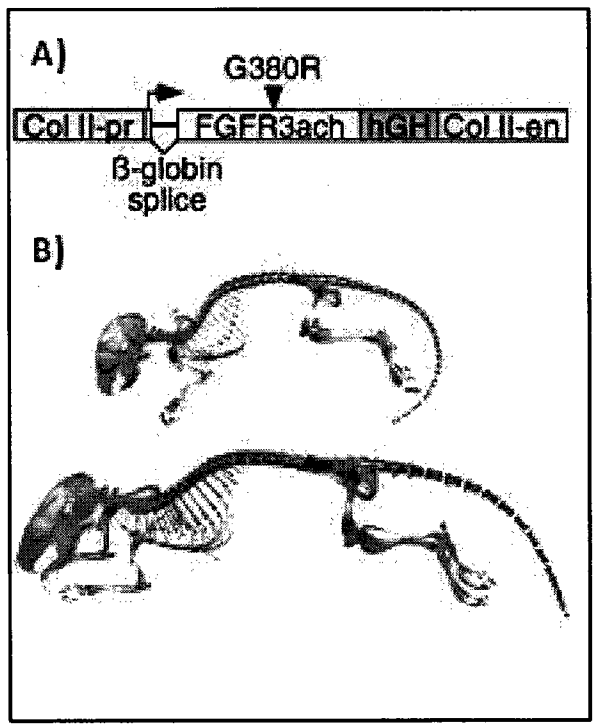

Figure 6: Achondroplasia mouse model. **A)** Transgene containing FGFR3 human cDNA with a G380R substitution under control of a rat collagen type II promoter/enhancer sequence. **B)** Skeletal preparation of wild type (WT, bottom) and achondroplasia transgenic (FGFR3ach, top) mice (from Naski *et al.* 1998).

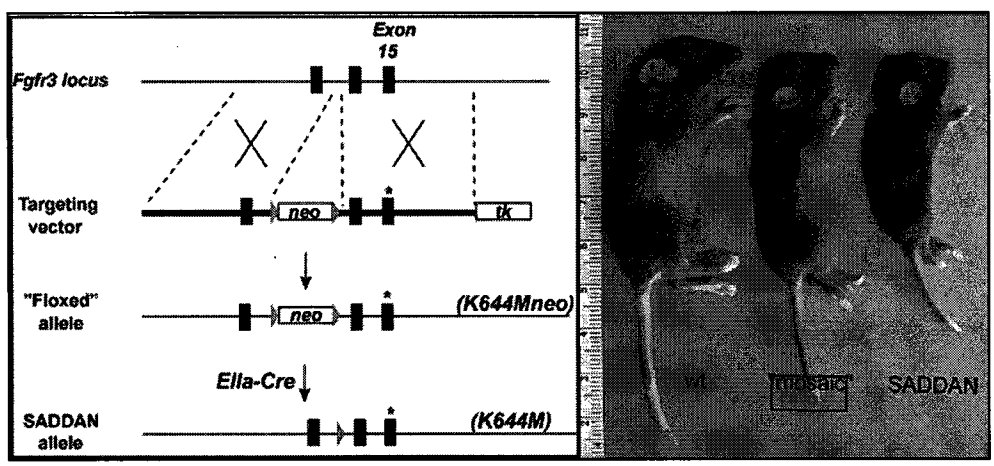

Figure 7: SADDAN mouse model. *Left panel.* Derivation of the SADDAN allele by gene targeting and production of mutant mice by crossing this allele with a Cre recombinase-expressor line of mice. The asterisk denotes the K644M (corresponds to human K650M) point mutation in exon 15 (Iwata *et al.*, 2001). *Right panel.* Phenotypes of 10 day-old mice obtained in our laboratory. Dwarf animals in which the *neo* cassette can no longer be detected by PCR are categorized as SADDAN. Dwarf animals in which the *neo* cassette can be detected by PCR generally present with an intermediate phenotype and are categorized as "mosaic" (St-Jacques Lab, see results).

2.6. Small Molecule Hh-Agonist and Hh-Peptides

Recombinant Hh proteins have been engineered and used in a number of in vitro and in vivo assays. The preparation of highly active peptides is difficult as Hh proteins undergo several complex post translational modifications (Ingham *et al.* 2001).

Experiments have shown that Ptch-GFP appears to be destabilized by Hh protein but not by the Hh-Agonists, and support the idea that Hh protein and Hh agonist act in distinct ways to stimulate the pathway (Frank-Kamenetsky *et al.* 2002).

Small molecule hedgehog agonists (Hh-Ag) have recently been identified by high-throughput screening of combinatorial-chemistry libraries (Frank-Kamenetsky *et al.* 2002; Wu *et al.* 2004). The molecules operate by binding directly to the Smoothened (Smo) component of the Hh response pathway (**Figure 5**). In utero, it rescues aspects of the Hh-signalling defect in *Sonic hedgehog*-null, but not *Smo*-null, mouse embryos (Frank-Kamenetsky et al. 2002). The most potent activators of this pathway belong to a class of chlorobenzothiophene-containing compounds (Frank-Kamenetsky *et al.* 2002).

These molecules have been shown to activate reporter constructs, induce motor neuron differentiation, and rescue *Shh* deficit *in utero*. Two recent studies demonstrated significant activation of Hh signalling and stimulatory effect on neural precursors in the brain of adult mice (Oral delivery) and of pups (subcutaneous

injection). As a result of these studies, it is thought that Hh signalling could be stimulated by small agonist molecules *in vivo* and provide a potential therapeutic value in diseases such as chondrodysplasia where Hh signalling has been compromised.

3. Materials and Methods

All manipulations that required the use of commercial reagents and kits were performed according to the manufacturer's protocol, except where indicated.

3.1. Hedgehog Agonist 1.4 Treatment Schedule for SADDAN and ACH Mice

Both SADDAN and ACH mice were subjected to a series of hedgehog agonist 1.4 (Curis) injections beginning at postnatal day 10 (P10). Subcutaneous injections of Hh 1.4 in saline, at a concentration of 10mg/kg body weight, were made into the fold of skin behind the necks of the mice on days P10, P12, and P14 in SADDAN mice. A concentration of 2.5 mg/kg was used in ACH mice on days P10, P11, P12, P13, and P14. Both groups of mice were then sacrificed on P15. Control animals received an equivalent amount of saline only.

3.2. Tissue Collection, Decalcification, and Embedding

After the treatment schedule the P15.5 day old mice were injected with BrdU labelling reagent (Zymed, now Invitrogen), left for 2 hours, and then sacrificed either by cervical dislocation or CO_2 asphyxiation. The skinned and eviscerated carcasses

were fixed in 4% paraformaldehyde in PBS without Ca^{2+} or Mg^{2+}. The distal growth plates of the radius and ulna were dissected in ice cold PBS and fixed overnight in 4% PFA/PBS (without Ca^{2+} or Mg^{2+}) at 4°C. Tissues were then decalcified by gentle shaking for 30 minutes at 4°C in Immunocal (Decal Corp., Tallman, NY). The duration of the decalcification process adversely affects the quality/quantity of RNA present in the samples and was kept to a minimum.

Samples were then washed in PBS (2 X 10 min.) and dehydrated to 100% ethanol (25%, 50%, 75%, 100%, 100%, X 60 min.). The samples were cleared in CitriSolv (Fisher) (2 X 15 min.) and then embedded in paraffin according to standard protocol. After sectioning in the longitudinal plane, slides were left to dry overnight and then placed on a hot plate (60°C for 30 min.) and left until melted down.

The kidneys, hearts, thymus, and remaining long bone growth plates were collected and placed in RNAlater (Ambion). RNA was then extracted using Trizol reagent (Invitrogen). Liver and tail tissue was collected for genotyping.

3.3. Hematoxylin and Eosin Staining (H&E)

To visualize both the bony and cartilaginous parts of the mouse growth plate, sections were dewaxed in CitriSolve (Fisher) (2X 8 minutes), and rehydrated using a graded ethanol series (2X 100% 5 min., 2X 70% 5 min., ddH_20 10 min.). Sections were

then stained with Harris Modified Hematoxylin with Acetic Acid (Fisher) and an alcohol based Eosin (Accustain, Sigma-Aldrich) according to established protocol. Samples were then dehydrated in a graded ethanol series and xylene and mounted with Permount (Fisher).

The length of growth plates were measured manually on printouts of the H & E sections using a ruler. A line was drawn from the perichondrium/resting chondrocyte border to the top of the hypertrophic zone in the middle of the growth plate. This measurement was taken as growth plate length. Another line was drawn across the entire hypertrophic zone. (**Figure 5**). A ratio of GP/HYP was then calculated.

3.4. BrdU Labeling

SADDAN and ACH mice treated with Hh-Ag 1.4 were given an intraperitoneal injection of an aqueous solution of 5-bromo-2'-deoxyuridine and 5-flouro-2'-deoxyuridine (BrdU, Zymed Laboratories) at a concentration of 1 ml labeling reagent per 100 g body weight. After 2 hours, the mice were sacrificed and the distal growth plates of both the radius and ulna were harvested for embedding in paraffin according to established protocol. Serial sections were made at a suggested thickness of 5 microns using a Leica RM2155 microtome and collected on

Superfrost/Plus microscope slides (Fisher). Sections were then stained using a BrdU immunohistochemistry staining kit and protocol (Zymed Laboratories), which utilizes a biotinylated monoclonal anti-BrdU primary antibody, thus eliminating the need for a species-specific secondary antibody. This reduces the amount of background staining seen in conventional BrdU staining techniques.

A ratio indicative of proliferation in the growth plate was obtained by counting the BrdU positive cells in a set field and dividing by the total number of BrdU negative cells in the field. Counting was done manually on printouts of BrdU stained sections using a cell counter and pen.

3.5. DIG *In Situ* Hybridization

A section *in situ* hybridization protocol using digoxigenin-labeled RNA probes was adapted from that used by Murtaugh *et al.* (Murtaugh, 1999).

Ribo probes for *Ihh, Ptc, Col2, ColX, Gli-1,* and *Hipp-1,* previously described by St-Jacques et al. (1999), were prepared using DIG labeling mix and protocol (Roche).

LiCl precipitation was then used for RNA purification. This method offers some advantages over sodium or ammonium precipitation methods in that it does not efficiently precipitate DNA, protein, or carbohydrate and is also very effective at

removing free nucleotides (Barlow et al, 1963). The following were added to DIG RNA labeling reaction (20 µl):1 µl Glycogen 20 mg/ml (Roche),10 µl 4M LiCl-DEPC, 100 µl TE-DEPC, pH 8.0, 300 µl EtOH abs (-20°C).

The RNA was then precipitated at -20°C overnight and spun down for 15 minutes at 13,500 rpm at 4°C. A wash of 70% EtOH (DEPC) was carried out followed by a second round of centrifugation. The riboprobes were then resuspended in 100 µl of nuclease free water and were stored at -80°C.

The slides were prepared for hybridization according to the Murtaugh *et al.* (1999) protocol with the following changes. The concentration of proteinase K was doubled to 20 mg/ml in 200 ml PBS-DEPC. Probes were suspended in hybridization solution (see below) at a dilution of 3:100 instead of 1:100. The probe-hybridization solution mix was then applied to the sections circled with a PAP-Pen and covered with paraffin. The paraffin melts and sticks to the PAP-Pen at 63°C resulting in a sealed bubble that prevents evaporation. Slides were then placed in a tape-sealed VWR slide box at 63°C with an SSC soaked towel to provide a moist environment and incubated for at least 16 hours.

Hybridization Solution: 50% formamide, 10 mM Tris (pH 7.6), 200 µg/ml yeast tRNA, 1× Denhardt's solution, 10% dextran sulphate, 600 mM NaCl , 0.25% SDS , 1

mM EDTA (pH 8.0). Subsequent washes and detection were carried out as in Murtaugh et al. (1999).

3.6. Real-time PCR

The ABI 7500 Real-time PCR system was used for quantitative analysis of *Ptch*, *Gli-1*, and *Ihh* expression in RNA collected from the hearts, kidneys, thymus, and growth plates of treated mice. Tissue RNA was extracted using a Tissuemizer (Tekmar) to homogenize the tissue in Trizol reagent (Invitrogen). Reverse transcription products were then synthesized using the High Capacity cDNA Archive Reagents (Applied Biosystems), 5µl of cDNA products were used for each PCR amplification. The Taqman Gene Expression Assays (Applied Biosystems) Mm00439613_m1, Mm99999915_g1, Mm00436026_m1, and Mm00494645_m1 were used for amplification of *Ihh*, *Gapdh*, *Ptch1*, and *Gli1* respectively. The conditions for Real-time PCR were programmed by the manufacturer. Saline injected mice were used as a calibrator and fold induction of the respective genes, normalized to GAPDH levels, were calculated using the comparative ΔCt method (Applied Biosystems).

3.7. Genotyping

Tail and liver DNA was obtained for the purposes of genotyping. Biopsies were placed in microfuge tubes containing 0.5 ml of lysis buffer and digested overnight with proteinase K at 55°C in a hybridization oven. After Phenol-Chloroform extraction, DNA was precipitated and resuspended in 100 µl of nuclease free water.

Lysis Buffer: 100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 µg/ml Proteinase K.

ACH mice

Genotypes were confirmed by PCR using the conditions described by Naski *et al.*, (1998). Primers for the PCR reaction were hGH-1 (in the human growth hormone tag of the transgene, 5'-AGGTGGCCTTTGACACCTACCAGG-3') and hGH-2 (in the human growth hormone tag of the transgene, 5'-TCTGTTGTGTTTCCTCCCTGTTGG-3'). Amplification was with 28 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1.5 min.

SADDAN mice

Originally, genotypes were confirmed by PCR using the conditions described by Iwata et al., (2000). Primers for the PCR reactions were TW13 (in the *neo* gene, 5'-CAGCTCATTCCTCCCACTCATGAT-3') and R3-36s (in the *Fgfr3* gene, 5'-CATACAACGTGGGGTGGGCT-3'). Amplification was with 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. Later, a more sensitive assay using primers *neo*-1F (in the *neo* gene, 5'-CCGGCCGCTTGGGTGGAGA-3') and *neo*-1R (in the *neo* gene, 5'-GCAGGTAGCCCGGATCAAGCGTATG-3') was developed. Amplification was with 35 cycles of 95°C for 1 min, 61°C for 1 min and 72°C for 3 min. This assay revealed the presence of the *neo* cassette in genomic DNA from pups of "intermediate" phenotype.

3.8. Faxitron X-Rays

Radiography of SADDAN mice was carried out at the Centre for Bone and Periodontal Research in Montreal, QC. Faxitron X-Rays were done on euthanized mice injected with either Hh-Ag 1.4 or saline control using the Faxitron MX-20 radiography cabinet for small animals.

3.9. Skeletal Preparations

In some instances the growth plates were not collected. The P15.5 SADDAN and wild-type were skinned and eviscerated in ice-cold PBS. Skeletal preparations for the P15 day old mice were made according to the following protocol (Wallin et al., 1994). As much loose tissue as possible was removed from the skeleton before they were placed in 95% ethanol for 24 hours to be fixed prior to skeletal preparations. This was followed by a 24 hour incubation period in acetone to break down lipids. Skeletons were stained overnight at RT° in a solution Alizarin Red and Alcian Blue.

Skeletal Prep Staining Solution: 1 vol 0.3% Alcian blue in 70% Ethanol, 1 vol 0.1% Alizarin red in 95% Ethanol, 1 vol acetic acid, 17 vol 70% ethanol.

Alcian blue stains the cartilage matrix blue while Alizarin red stains calcified tissues (both the calcified cartilage and the bone) red. Skeletons were then briefly rinsed in ddH_2O twice and then transferred into a 1% KOH/water solution and left for 24 hours without agitation. Skeletons were then transferred to a 1% KOH in 20% glycerol/water solution and left without agitation until cleared. They were subsequently transferred progressively to 50%, 80%, and 100% glycerol over 2 days and stored in 100% glycerol.

3.10. Imaging

All histological images were taken using a Leica DM LB2 slide microscope connected to an Infinity Capture 3 digital camera and Infinity software (Lumenera Scientific). For BrdU proliferation assays, growth plate images were printed at the same magnification using a HP Colour LaserJet 3600dn printer (Hewlett-Packard), and positive and negative nuclei were counted manually within a designated field.

4. Results

4.1. Characterisation of a "mosaic" Phenotype in the SADDAN Mouse Model

While analyzing SADDAN mice treated with Hh-Ag 1.4, it was noticed that there are actually two distinct mutant phenotypes based on the average body size of the mice (Figure-7, Figure-10). Although Iwata et al. (2001) described two groups of mutant mice; they failed to provide a clear explanation for this observation, only speculating on the contribution of different genetic backgrounds. Using an improved PCR assay for detection of the *neo* cassette (see Materials and Methods) we could show that the intermediate phenotype results from inefficient Cre mediated excision of the *neo* cassette inserted in the mutated *Fgfr3* allele (**Figure 7**). As long as the neo cassette is present, a cell is phenotypically normal but upon *neo* excision the cell expresses the dominant mutant allele (Iwata et al. 2001). If excision is very efficient, all (or almost all) cells in the growth plate will express the mutant allele resulting in a SADDAN phenotype. However, if excision is only partial a significant portion of the cell population will retain the *neo* cassette and a wild type phenotype. Because pups of the intermediate phenotype are positive for the presence of *neo*, we conclude that inefficient excision of the *neo* cassette results in tissues that are mosaics for the *Fgfr3* mutation and produces an intermediate phenotype. Hematoxylin and

eosin staining of the growth plates in the radius and ulna of post natal day 18 mice supports this interpretation (**Figure 8**). There are areas of chondrocytes in the growth plate in the mosaic mice that have a very different phenotype than the normal adjacent cells (**Figure 9**). In WT, chondrocyte maturation progresses normally and the growth plate displays clear zones of resting, proliferating, pre-hypertrophic, and hypertrophic chondrocytes and a clear chondro-osseus junction with a zone of cell death and mineralization (**Figure 8A**). The resting chondrocytes in the mosaic growth plates seem to be phenotypically normal. However, there seems to be pools of "normal" and "abnormal" proliferating chondrocytes in the next stage of maturation (**Figure 8B**). These pools persist through to the chondro-osseus junction. These chondrocytes are rarely BrdU positive (**Figure 9**), indicating a much reduced rate of proliferation and suggesting that they express the mutant allele of *Fgfr3*. These areas of reduced proliferation lead to an eventual deficit in hypertrophic chondrocytes (**Figure 9**). At first glance, the growth plates of the mosaic mice appear even more disorganized than the SADDAN, even though there are normal cells present within a larger abnormal population. Whereas there is only a partial deficit in the mosaic mice of columnar proliferating chondrocytes, SADDAN mice never seem to develop proper columnar cells. There is also a much larger population of resting chondrocytes in the SADDAN growth plates, possibly because the cells never mature into proliferating columns. There is also a reduction in the overall size of the

hypertrophic zone, the cells of which eventually will die and form the scaffold that new mineral will be deposited into (**Figure 8C**).

For the above reasons, "mosaic" mice were not used in most experiments. They were identified using both phenotype and genotype analysis. For example, a smaller mouse that is positive for the *neo* cassette would be taken as a mosaic individual. Both WT and SADDAN mice do not genotype for *neo*. Mice determined to be wild type, mosaic, or SADDAN by genotyping and weighed over the course of Hh-Ag 1.4 treatment show three different weight gain trajectories over the course of 6 day treatment with Hh-Ag 1.4 starting at postnatal day 10 (**Figure 10**).

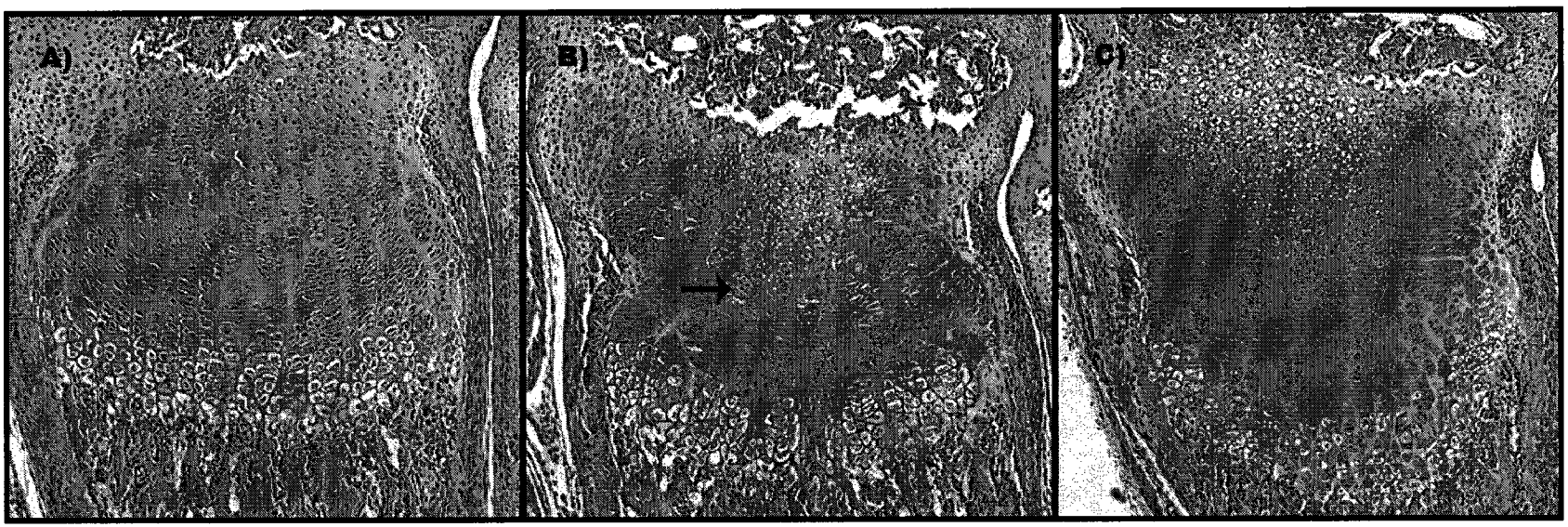

Figure 8: Hematoxylin and Eosin stained growth plates of wild-type, mosaic, and SADDAN mice. **A)** *Wild-Type C57BL6*: These mice have normal chondrocyte populations in the growth plate. **B)** *Mosaic*: Mosaic individuals tend to have groups of chondrocytes that behave normally in an abnormal SADDAN growth plate. **C)** *SADDAN*: Chondrocytes in the SADDAN growth plate do not mature normally into columnar chondrocytes or into hypertrophic chondrocytes as a result of reduced *Ihh* signalling.

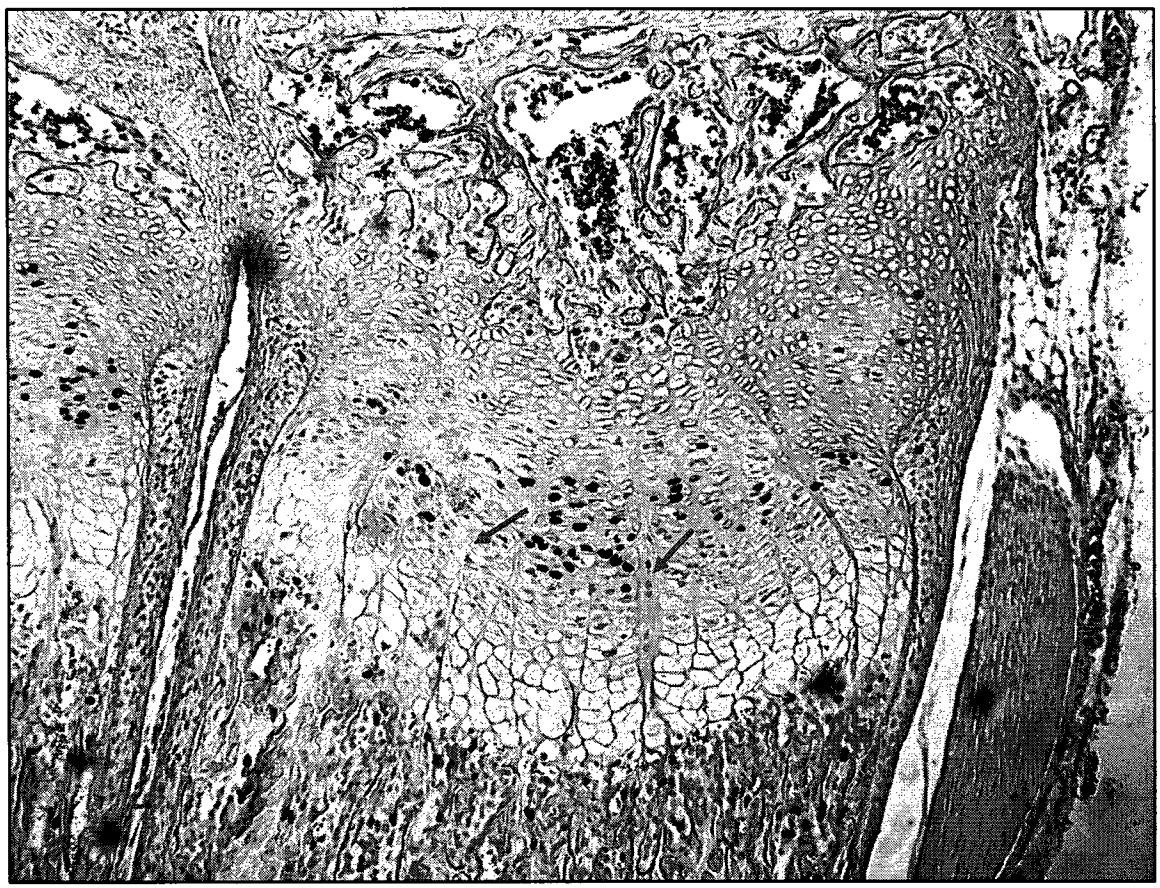

Figure 9: BrdU staining of the radial growth plate in a mosaic mouse. There are areas of chondrocytes in the growthplate in the mosaic mice that have a very different phenotype (*red arrows*) than the normal adjacent cells. WT cells seem to show more BrdU staining when compared to abnormal "mosaic" chondrocytes. These areas of reduced proliferation lead to an eventual deficit in hypertrophic chondrocytes.

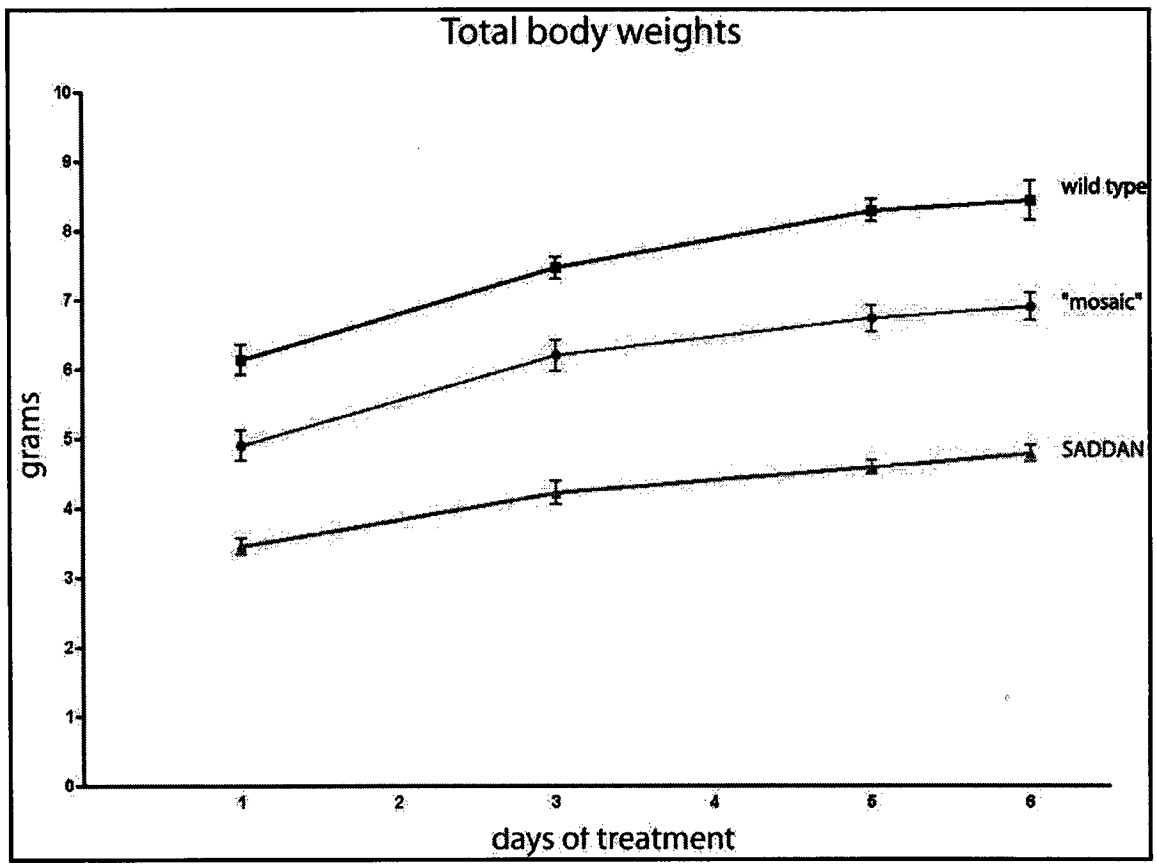

Figure 10: Change in total body weight over the course of Hedgehog Agonist 1.4 treatment. Mice determined to be wild type, mosaic, or SADDAN by genotyping show three different weight gain trajectories over the course of 6 day treatment with Hh-Ag 1.4 starting at postnatal day 10.

4.2. Induction of the Hh Signalling Pathway by Hh-Ag 1.4

To determine whether or not the hedgehog agonist 1.4 (Curis) had an effect on growth plate expression of genes in the hedgehog signalling pathway we performed real time PCR on cDNA obtained by reverse transcription of RNA recovered from growth plates in the long bones of treated mice. *Gapdh* was used as a control in this process.

Because Ptch is not only a receptor for Ihh, but also a downstream target for Hh signalling, the expression of *Ptch* and one of its downstream targets *Gli-1* are good indicators of Hh-Ag 1.4 activity in the growth plate (**Figure 5**). Indeed the genes immediately downstream of *Ihh, Ptch* and *Gli-1* are both upregulated more than 2-fold as a result of a single dose of Hh-Ag 1.4 at 5 mg/kg versus saline (Statistically significant by one-way ANOVA with $P<0.05$) (**Figure 11**). However, at the higher dose of 10 mg/kg there is no statistical difference from saline injection. According to our quantitative PCR data, *Ihh* expression is unchanged as the agonist binds to Ptch (**Figure 11**). However, *in situ* hybridization of ACH mice injected 5X with 2.5 mg/kg of Hh-Ag 1.4 reveals that *Ihh* expression may even be reduced in the pre-hypertrophic chondrocytes. This would make sense as there are negative feedback loops associated with the hedgehog pathway and the agonist mimics endogenous Ihh to increase downstream targets (**Figure 12**).

4.3. Changes in Growth Plate Length in Mice Treated with Hh-Ag 1.4

Histology of the growth plates in the radius and ulna of wild type and mutant mice revealed a significant lengthening of the growth plates and an overall increase in cell number within growth plates treated with Hh-Ag 1.4 (**Figure 13**; **Figure 14**; **Figure 15**). Change in growth plate length was measured by taking the length of the growth plate and dividing it by length of the hypertrophic zone resulting in the ratio GP/Hyp used in **Figure 13** (**Figure 14**; **Figure 15**). This larger ratio is the result of an expanded proliferative zone as well as a reduced hypertrophic region (**Figure 14**; **Figure 15**). A similar effect was seen in SADDAN mice treated with Hh-Ag 1.4, but the number of mice (*n*) analyzed was too small to assess the statistical significance of this difference. It is likely that by changing the level of signalling of a gene like *Ihh*, which is known to be responsible for both chondrocyte proliferation and maturation, we are causing this effect.

Further measurements of the humerus in a small number of mice were made using Faxitron X-Rays and a ruler. This demonstrated that despite a longer overall growth plate the bones were in fact significantly shorter (**Figure 16**) (See discussion for detailed analysis).

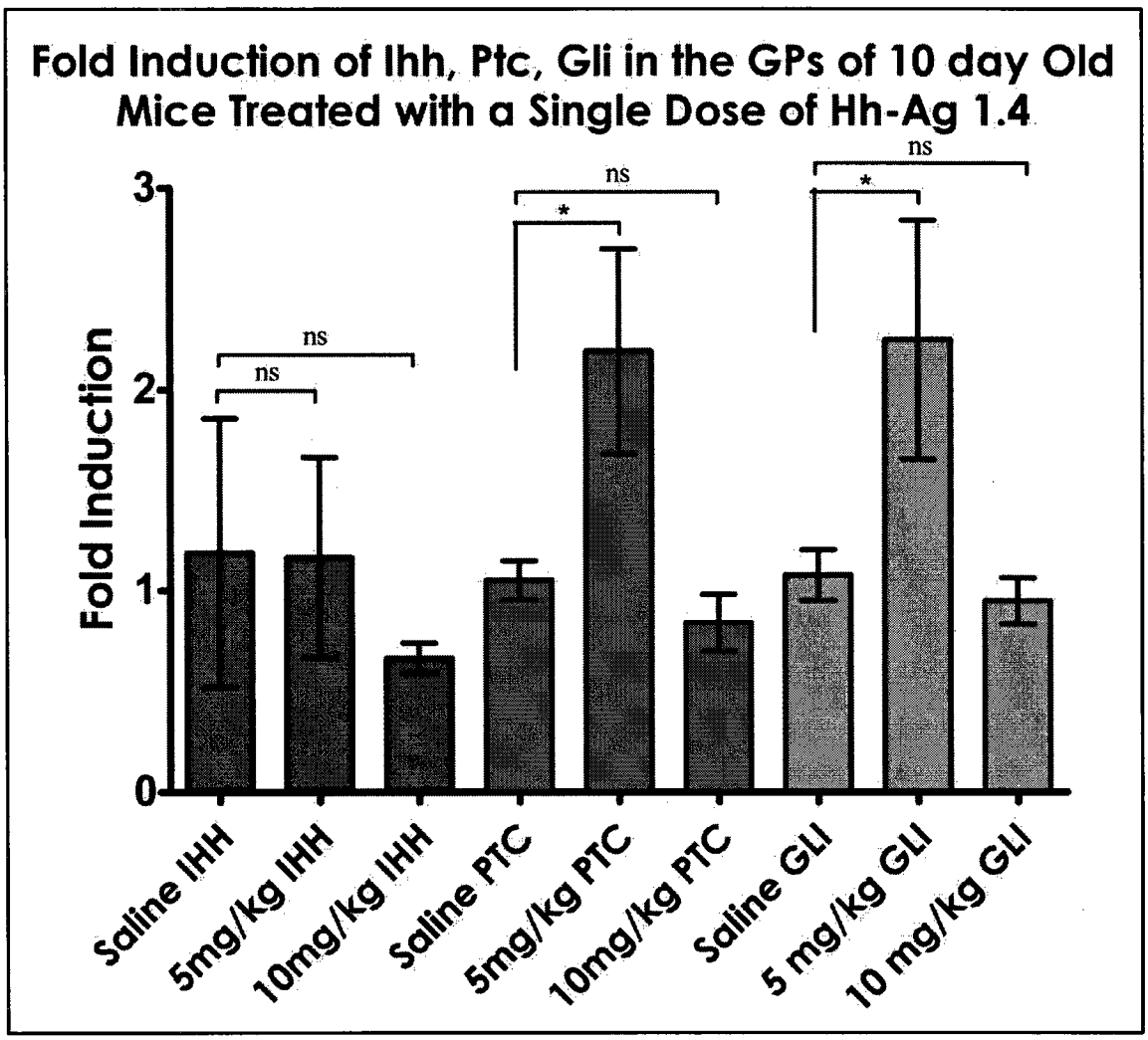

Figure 11: Real-Time PCR fold induction of *Ihh*, *Ptc*, and *Gli-1* in the growth plates of 10 day old mice treated with a single dose of, saline, 5 mg/kg Hh-Ag 1.4, or 10 mg/kg Hh-Ag 1.4 (Curis). Mice injected with single dose of 5 mg/kg show higher induction of both *Ptc* and *Gli-1*. Similar results were obtained by RT-PCR in the thymus, kidney, and the heart (Data not shown). N = for saline, 5 mg/kg, and 10 mg/kg were (12), (6), and (5) respectively. (**ns**) Not significant. (*****) Statistically significant by one-way ANOVA with $P<0.05$.

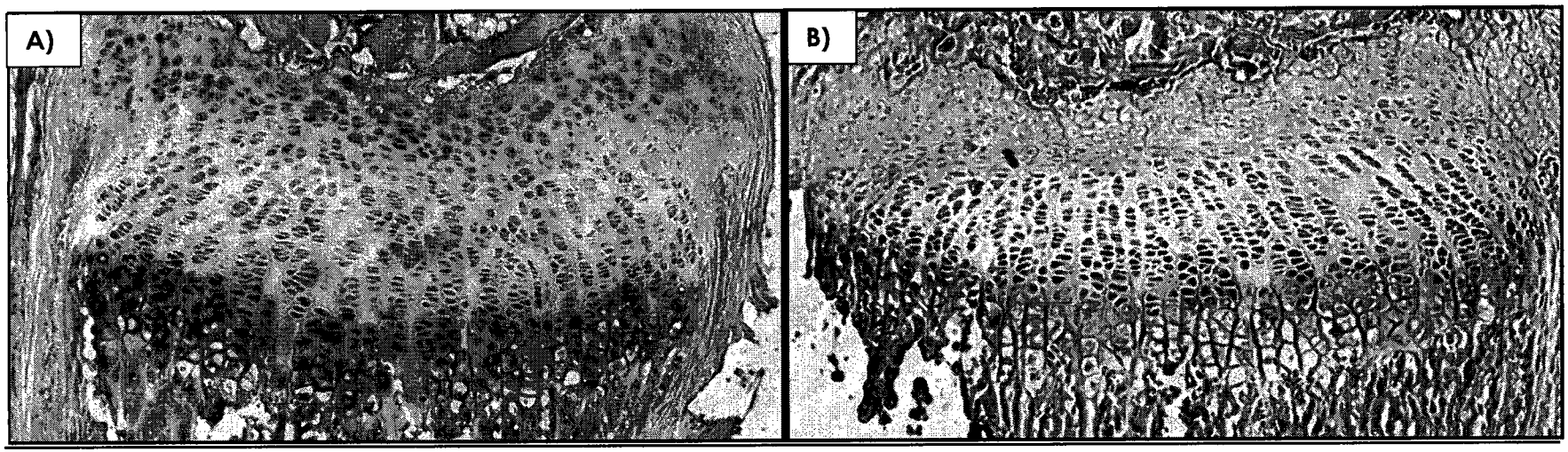

Figure 12: *In situ* hybridization for expression of *Ihh* in the growth plate of ACH mice injected 5X with 2.5 mg/kg of Hh-Ag 1.4 over a 6 day period. **A)** ACH mouse injected with saline. There is expression of Ihh in the pre-hypertrophic region of chondrocytes. **B)** ACH mouse injected with Hh-Ag 1.4. While the location of expression is the same in both the control and injected, there is a marked reduction of staining in mice injected with Hh-Ag 1.4.

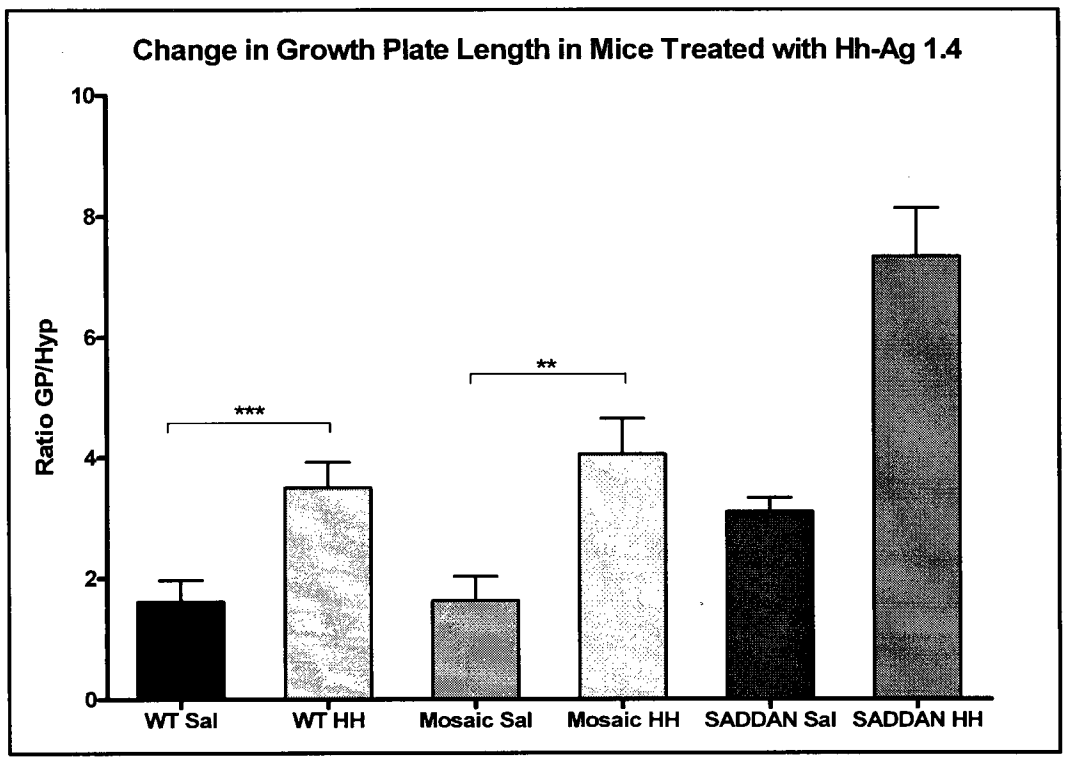

Figure 13: The ratio of the growth plate length over the length of the hypertrophic zone. Mice were injected 3 times over a period of 6 days with 10 mg/kg Hh-Ag 1.4 (Curis). Mice injected with Hh-Ag 1.4 show a longer growth plate and a smaller hypertrophic zone. (**), (***) Statistically significant by two-tailed t-test with $P<0.001$ and $P<0.0001$ respectively.

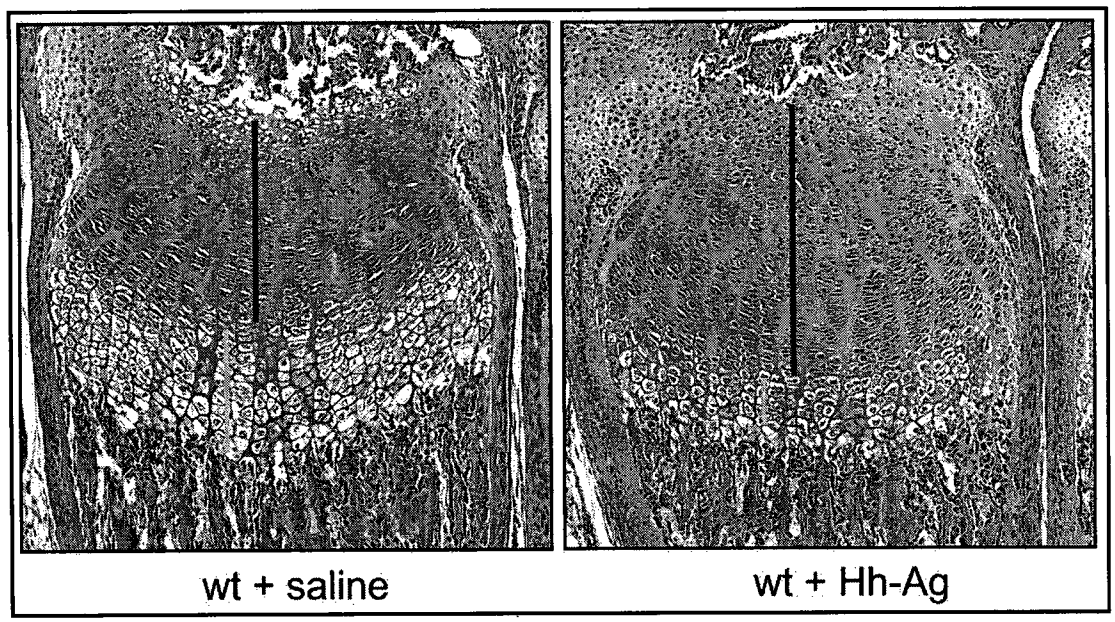

Figure 14: Wt growth plates of mice treated with saline or Hh-Ag-1.4. There is an increased proliferative zone (Black Bar) (see Fig 2.) and a decreased hypertrophic zone (Yellow Bar) in mice treated with Hh-Ag.

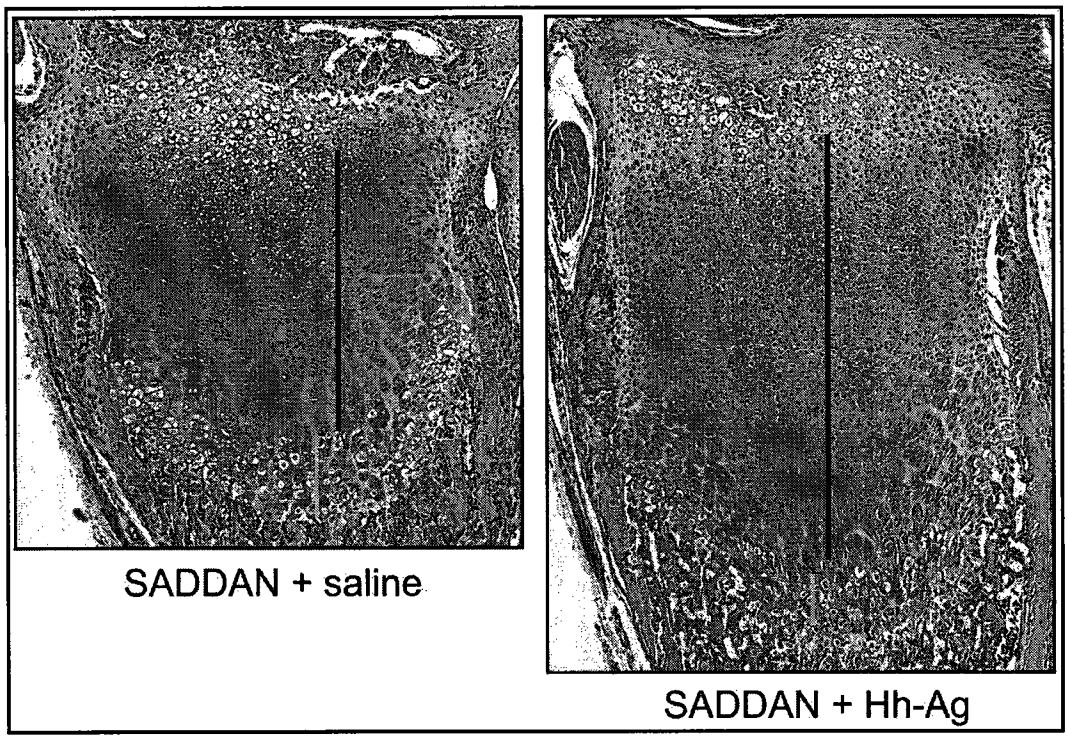

Figure 15: SADDAN growth plates of mice treated with saline or Hh-Ag-1.4. There is obvious enlargement of the proliferative zone (Black Bar) and a decrease in hypertrophy (Yellow Bar).

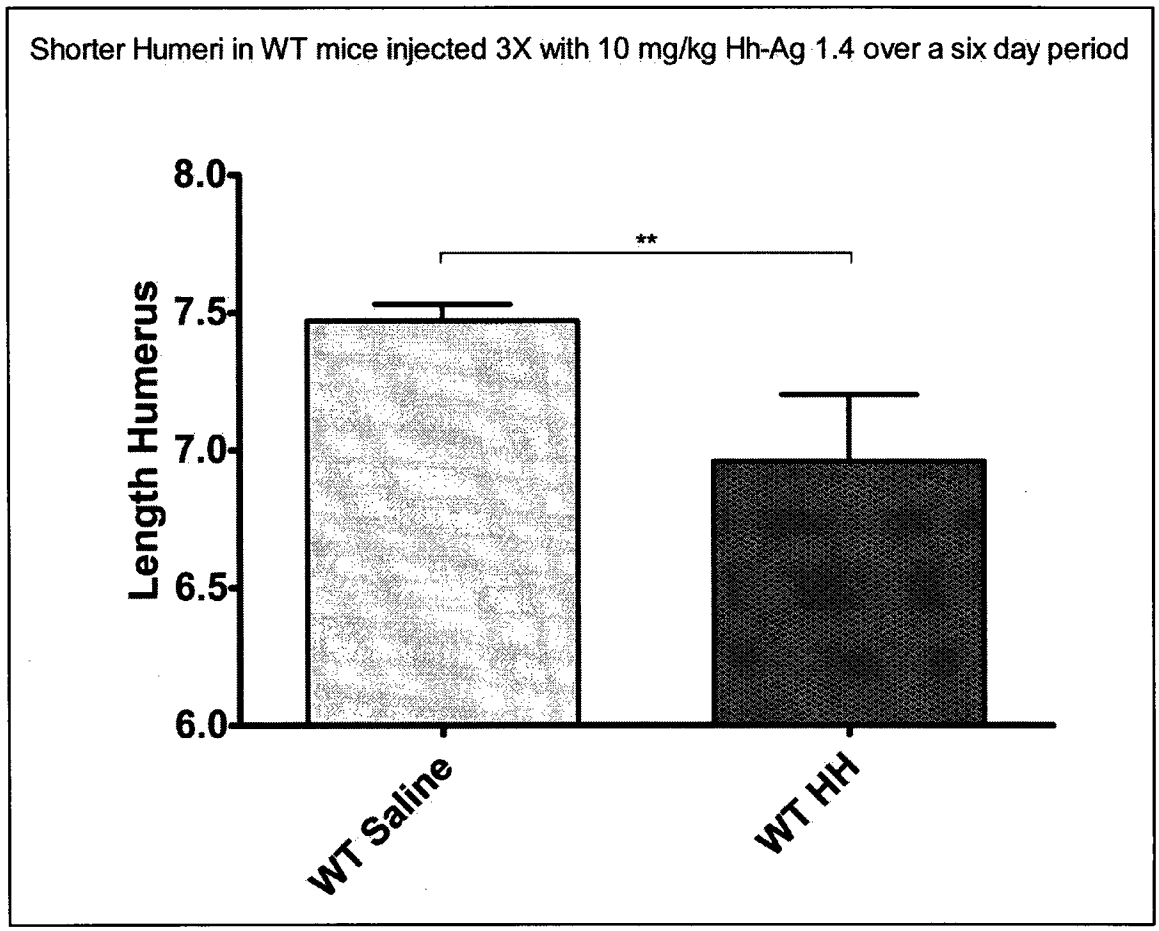

Figure 16: WT mice injected with Hh-Ag 1.4 show significantly shorter humeri than those treated with saline ($p < 0.01$). Measurements were made using a ruler on printouts of Faxitron X-Rays at same magnification (See discussion for detailed analysis).

4.4. Changes in Cortical Bone Thickness in Mice Injected with Hh-Ag 1.4

Histology of the radius growth plate also demonstrated a significant effect of Hh-Agonist 1.4 treatment on cortical bone. Hh-Ag 1.4 significantly induced bone formation as indicated by thickening of the cortical bone or osteoid surrounding the growth plates of the radius and ulna (**Figure 17**; **Figure 18**).

The thickness of the cortical bone (OR osteoid) was measured adjacent to the hypertrophic zone in three places (Top, middle, and bottom). An average of these three measurements was then taken for each mouse. This result is compatible with a number of observations showing that *Ihh* is a positive regulator of osteoblastogenesis (St-Jacques et al. 1999; Long *et al.* 2004; Hu *et al.* 2005).

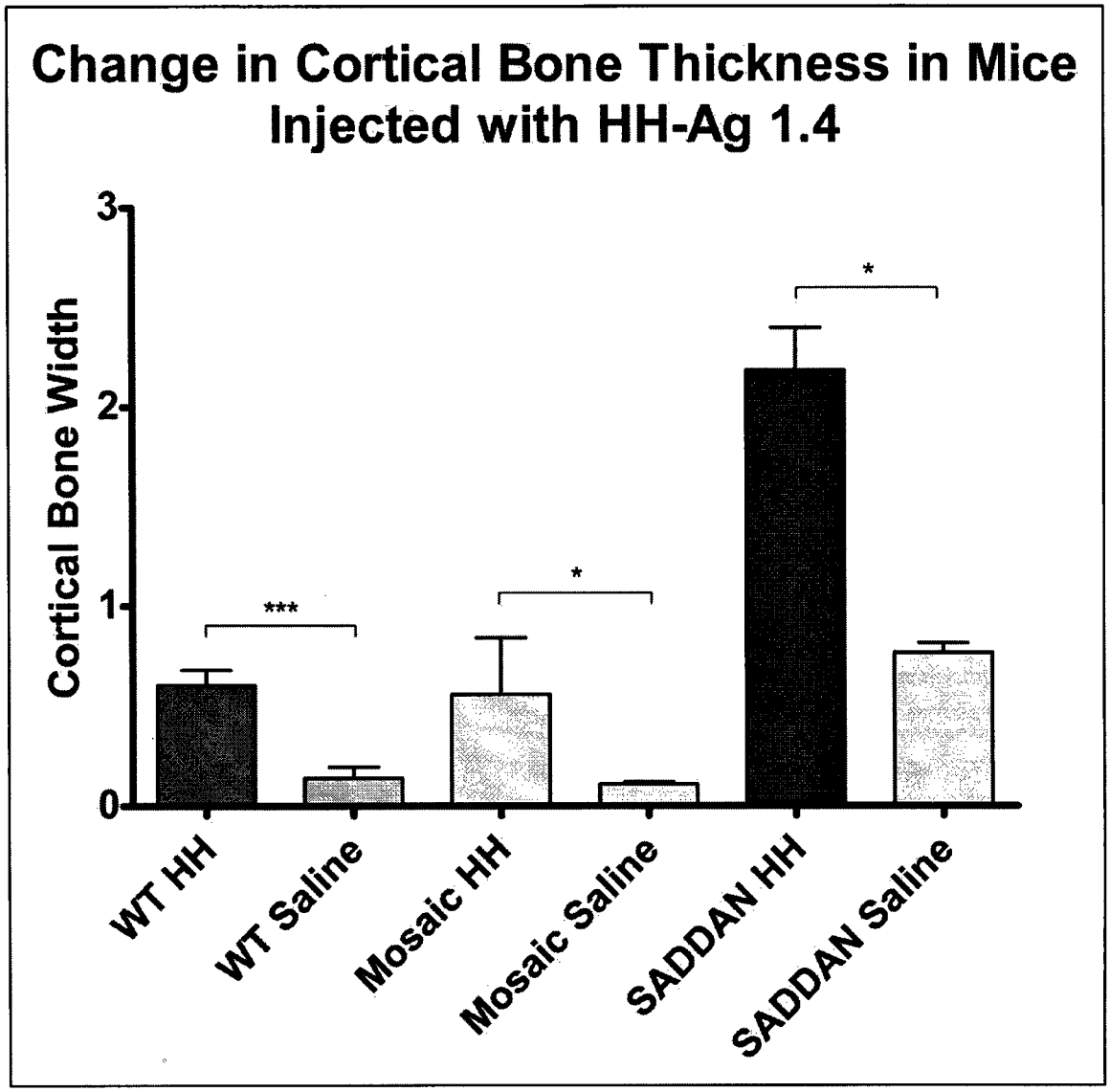

Figure 17: Change in cortical bone thickness (Or osteoid) in mice injected with Hh-Ag 1.4. (*), (***) Statistically significant by two-tailed t-test with $P<0.05$ and $P<0.001$ respectively.

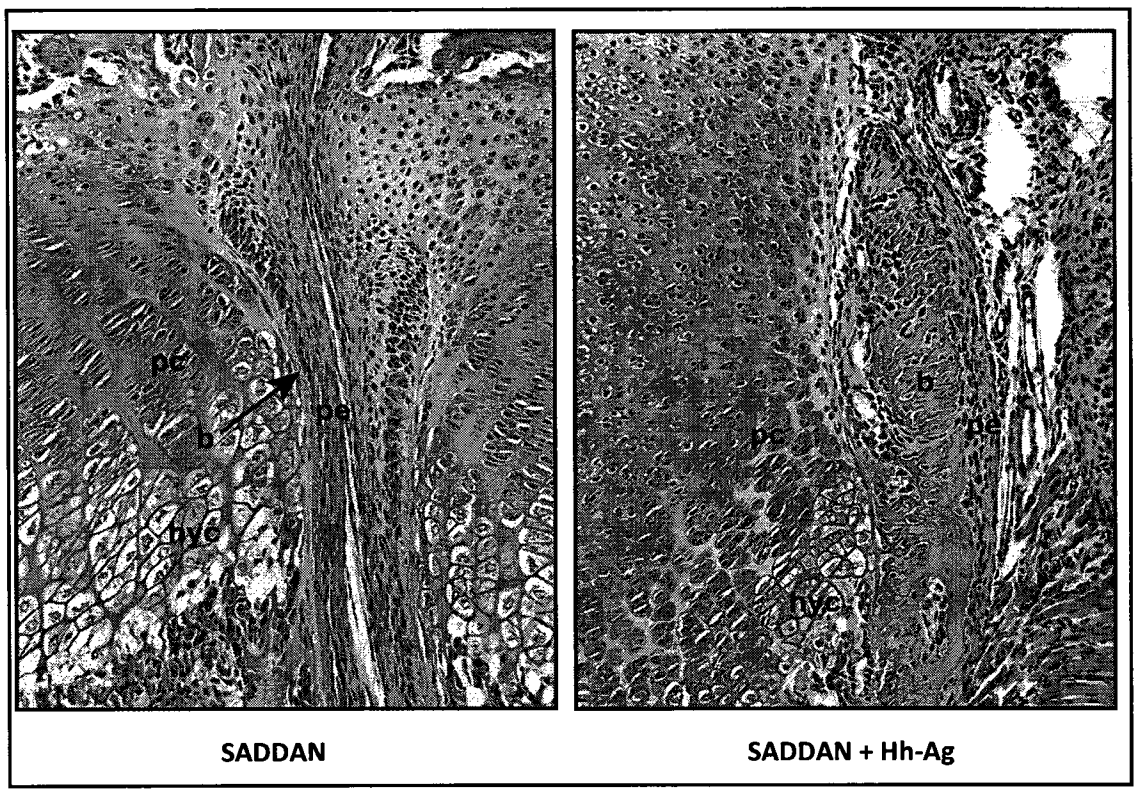

Figure 18: Stimulation of cortical bone (Or osteoid) formation by Hh-Ag-1.4 injections. Left panel shows the appearance of the cortical bone in SADDAN pups that have not been treated (arrow). The right panel shows the dramatically increased thickness of the cortical bone flanking the growth plate in a pup injected with Hh-Ag. (**b**, bone; **hyc**, hypertrophic chondrocytes; **pc**, proliferating chondrocytes; **pe**, periosteum.)

4.5. Reduction in Chondrocyte Proliferation in Mice Injected with Hh-Ag 1.4

Ihh and its downstream effectors are known to mediate cellular proliferation by targeting *Patched* and *Gli-1* (St-Jacques *et al.* 1999). In our study there was a marked reduction in chondrocyte proliferation within the growth plate when mice were treated with Hh-Ag 1.4 (**Figure 19**; **Figure 20**). A qualitative look at BrdU staining in the growth plates of Hh-Ag 1.4 injected versus saline injected WT mice reveals significantly less positive cells throughout the proliferative zone of chondrocytes in those mice treated with Hh-Ag 1.4 (**Figure 19**).

These data were quantified by taking the ratio of BrdU+/BrdU – chondrocytes within the growth plate. In all cases (WT, mosaic, SADDAN), mice injected 3 times with 10 mg/kg of Hh-Ag 1.4 over a period of six days, show a reduction in proliferation by measure of the BrdU+/BrdU- ratio (**Figure 20**). The reduction is significant in both the WT and the mosaic mice by two-tailed t-test ($P< 0.05$). We are confident that had the "*n*" of the SADDAN test group been larger, significance would have been attained here as well.

The reduction in proliferation was of obvious concern, as one of the stated goals of this project was to restore the deficit in cellular proliferation reported in the $Ihh^{-/-}$ null, ACH, and SADDAN mouse lines.

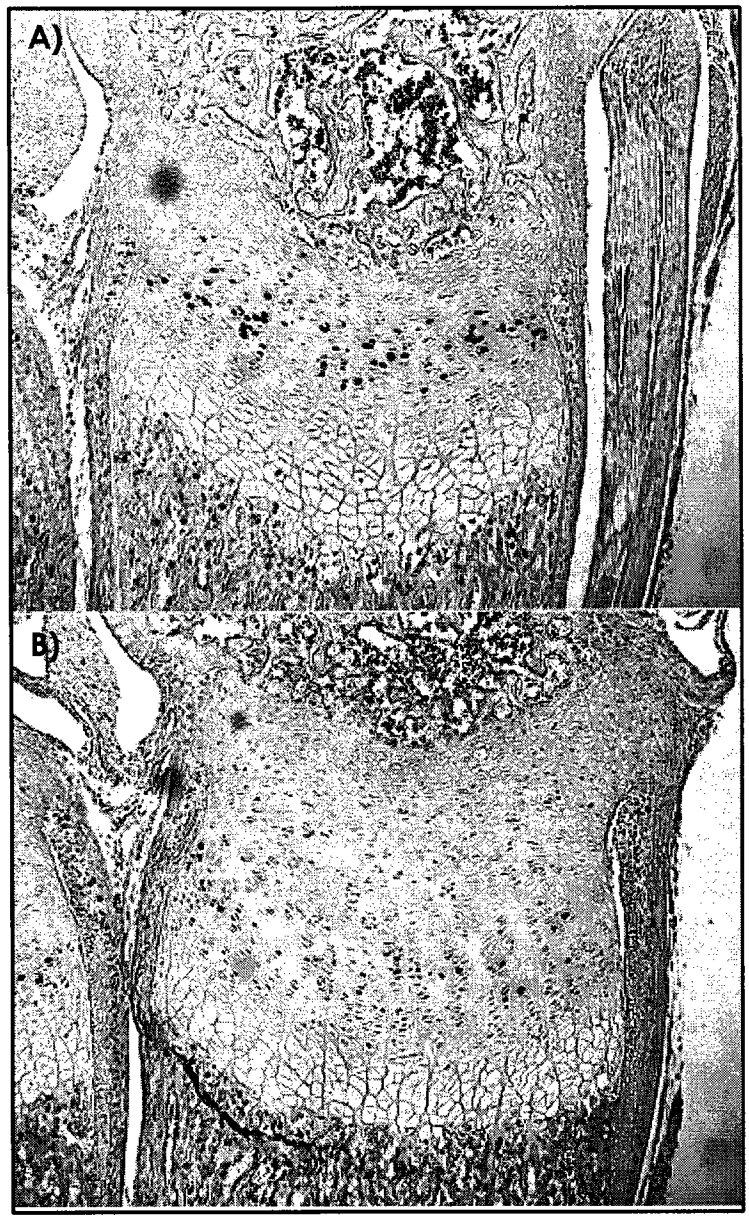

Figure 19: BrdU staining of the growth plate in WT mice treated with Hh-Ag 1.4 or Saline. **A)** BrdU staining of the growth plate of a WT mouse injected with saline three times over a period of six days. **B)** BrdU staining of the growth plate of a WT mouse injected 3 times over 6 days with 10 mg/kg of Hh-Ag 1.4. There is an overall decrease in BrdU staining in the Hh-Ag 1.4 injected mice.

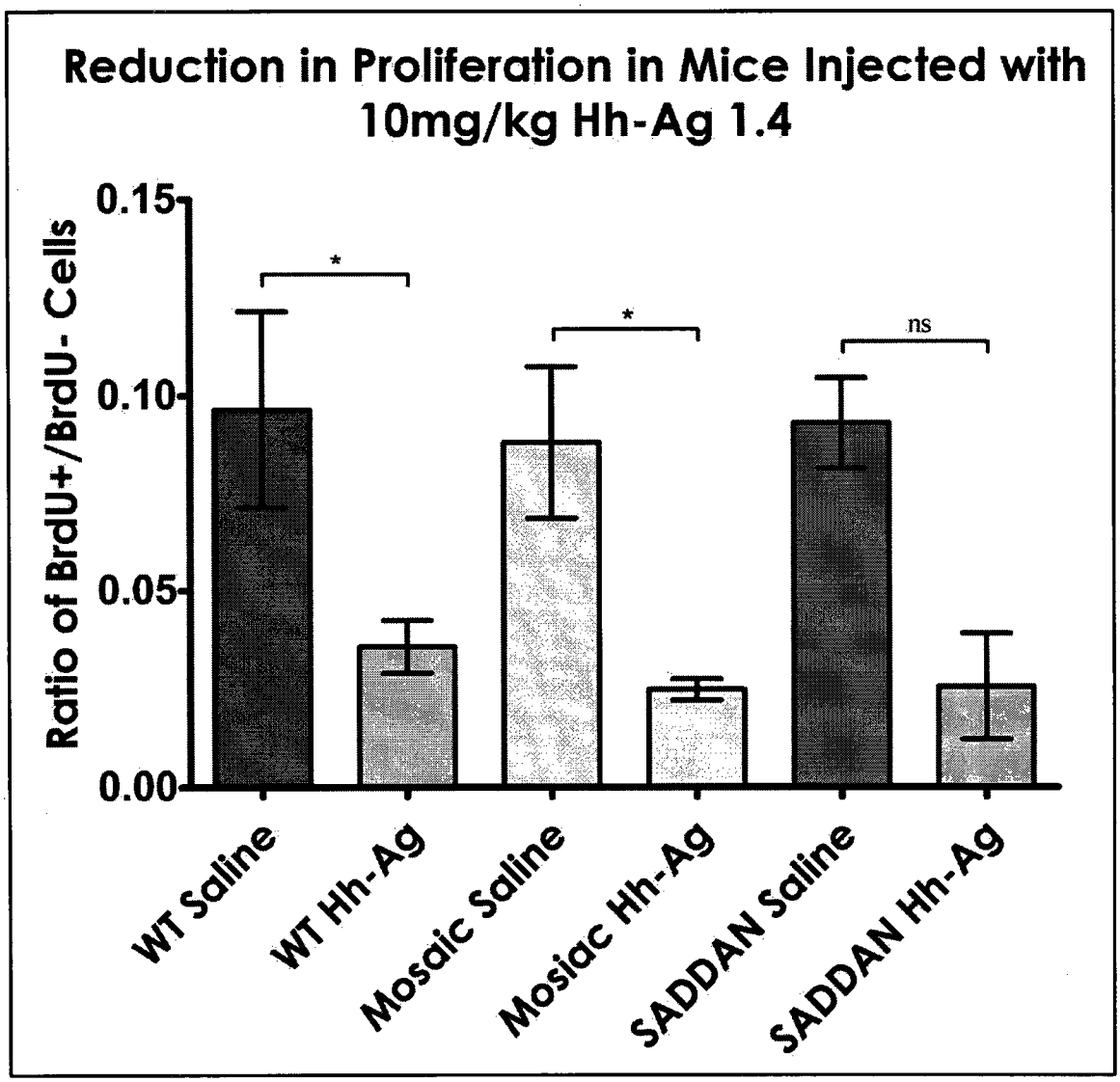

Figure 20: Reduction in proliferation in mice injected with 10 mg/kg Hh-Ag 1.4. Measured as a ratio of BrdU positive to BrdU negative cells located within the growth plate. (*) Statistically significant by two-tailed t-test with $P<0.05$.

5. Discussion

The goal of this research project was to investigate the use of a synthetic hedgehog agonist to determine whether or not it could rescue or lead to an improvement in the condition of SADDAN and ACH mouse models of chondrodysplasia. In both SADDAN and ACH there is above normal activation of the Fgfr3 receptor leading to down regulation of Ihh signalling. Ihh is a well-established regulator of endochondral ossification, and as such, is a good potential target for a pharmacological intervention. This was attempted using the hedgehog agonist Hh-Ag 1.4 described by Frank-Kamenetsky *et al.* (2002).

To prove whether or not Hh-Ag 1.4 did indeed have a positive impact on the chondrodysplasia condition we monitored changes in growth plate length, cortical bone thickness, BrdU incorporation, and gene expression (Real Time PCR and *in situ* hybridization) in both SADDAN and ACH mice injected with Hh-Ag 1.4.

5.1. Discovery of the "Mosaic" Phenotype in the SADDAN Mouse Model

In order to prove that the growth plate of intermediate phenotype animals ("mosaics") is indeed constituted of a mix of wild-type and mutant cells, one could use antibodies specific for the phosphorylation of Fgfr3 to stain growth plates of mosaic mice using immunohistochemistry. It has been shown that phosphorylated

ERK 1/2 and STATs are indicators of elevated Fgfr3 signalling. Antibodies for both phosphorylated ERK 1/2 and STATs exist (Murakami *et al.* 2004; Li *et al.* 1999). There should be a difference in staining between the normal cells and those with activated Fgfr3. Normal cells that display lower levels of staining for phosphorylated ERK 1/2 (wild-type) should overlap with cells that are positive for BrdU staining (**Figure 9**) (Murakami *et al.* 2004).

5.2. Induction of the Hh Signalling Pathway by Hh-Ag 1.4

In our study there was a two-fold induction of both *Ptch* and *Gli1* after injection with a single dose of 5 mg/kg Hh-Ag 1.4. This was shown by quantitative PCR for these markers after RNA extraction in dissected growth plates. Interestingly, a higher dose of 10 mg/kg Hh-Ag 1.4 resulted in a slight decrease in induction in *Ptch* and *Gli1*. One possible explanation for such an effect is that Ihh functions as a morphogen. For instance, hedgehog proteins have the ability to direct neural progenitors to acquire specific cell identities (Fuccillo *et al.* 2006). In particular, members of the GLI family of transcription factors act as effectors of hedgehog signalling in the spinal cord, allowing an extracellular concentration gradient of hedgehog to be translated into different cell identities (Fuccillo *et al.*

2006). Perhaps by administering such a high dose of agonist we are changing the way the downstream targets respond to Hh.

It was also shown by quantitative PCR that there was minimal change in expression of native *Ihh*. *In situ* hybridization using probes for *Ihh* seem to show some down-regulation in *Ihh* after injection of Hh-Ag 1.4. This is most likely the result of negative feedback through PTHrP, which has been shown to control the expression of *Ihh*. This could easily be tested using either immunohistochemistry or *in situ* hybridization for PTHrP.

5.3. Changes in Growth Plate Length, Cortical Bone Thickness, and reduction in proliferation in Mice Treated with Hh-Ag 1.4

The reduction in chondrocyte cellular proliferation in our study of Hh-Ag 1.4 was completely unanticipated. In fact, we hypothesized that induction of the hedgehog pathway would lead to an overall increase in chondrocyte proliferation thus leading to an increase in bone length and rescuing, at least in part, the chondrodysplasia phenotype seen in the ACH and SADDAN mouse models. At the same time there was a massive increase in cortical bone flanking the pre-hypertrophic region of the growth plate. As increased *Ihh* has been reported to lead

to osteoblastogenesis via *Runx2*, this is not completely unexpected, but again conflicts with the discovery that there is a reduction in proliferation.

How can the finding of reduced proliferation be reconciled with that of the induction of *Ptch* and *Gli-1*, indicators of increased Ihh signalling, seen in the real-time PCR results?

A tissue known as the perichondrium surrounds the growth plate cartilage. It consists of undifferentiated mesenchymal cells and functions to regulate chondrocyte maturation through poorly understood mechanisms (Hinoi *et al.* 2006). The changes in GP length, cortical bone thickness, and proliferation reduction seen in mice treated with Hh-Ag 1.4 may be due to increased *Fgf18* expression in perichondrium via increased Runx2/Wnt signalling pathway (Hinoi *et al.* 2006). There are several reasons for this hypothesis. SADDAN mice have only one allele with an activated Fgfr3 receptor. It is likely that a significant increase in the level of FGF ligand, would up-regulate signalling through the wild type allele and this could result in a more severe phenotype. In the *FGFR3* allelic series in human chondrodysplasia, it is well known that the severity of the phenotype correlates with the level of activation of the receptor (up-regulation of signalling). Indeed, severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN), and thanatophoric dysplasias type I (TDI) and type II (TDII), are caused by higher levels

of constitutive activation of the receptor than HCH or ACH (Ornitz and Marie, 2002).

What are the indications that Fgf18 signalling in the perichondrium is increased via the hedgehog signalling pathway in mice treated with Hh-Ag 1.4? It was recently documented by Hinoi *et al.* (2006) that *Twist-1* regulates the function of *Runx2* which inhibits chondrocyte proliferation and hypertrophy through its expression in the perichondrium and that this reduction on proliferation was the result of increased Fgf18. *Ihh* null mice completely lack osteoblasts in the endochondral skeleton, which is at least due in part to a deficiency in Runx2 (St-Jacques *et al.* 1999). Long *et al.* (2004) demonstrate that cells lacking smoothened, thus Hh signalling, fail to undergo osteoblast differentiation. This signalling cascade most likely normally functions through the canonical *Wnt* pathway eventually leading to an activation of *Runx2*, *Osx*, and *Fgf18* (Hu *et al.* 2005; Hinoi *et al.* 2006).

Also, removal of the perichondrium in chicken tibia organ cultures leads to an increase in proliferation in growth plate chondrocytes and to a larger hypertrophic zone (Long and Linsenmyer, 1998; Di Nino *et al.* 2001). The exact opposite of this phenotype is seen in our SADDAN and ACH mice injected with Hh-Ag 1.4. We show a two-fold reduction in chondrocyte proliferation and a smaller hypertrophic zone when injected with agonist (**Figure 14**; **Figure 15**; **Figure 20**).

An analysis of mice harboring an activating *Fgfr3* mutation revealed that in early development, *Fgfr3* functions to promote chondrocyte proliferation and decrease differentiation (Iwata *et al.* 2000). This suggests that *Fgfr3* signalling has a biphasic role during chondrocyte development. At early embryonic stages it promotes chondrocyte proliferation and postnatally it acts to suppress chondrocyte proliferation. Consistent with this hypothesis, cultured limb mesenchymal cells from *Fgfr3* null mice fail to differentiate into chondrocytes and do not proliferate in the presence of *Fgf18* (Davidson *et al.* 2005).

Fgf18–/– mice, have expanded hypertrophic zones at late developmental stages, with histology similar to that seen in mice lacking *Fgfr3*. This is consistent with the proposed model that *Fgf18* signals through *Fgfr3* to negatively regulate chondrocyte proliferation and differentiation during late embryonic and early postnatal development. Liu et al. (2007) studied the *Fgf18*–/– phenotype at early embryonic stages and found that the size of the early hypertrophic chondrocyte zone was significantly decreased at E14.5, and BrdU analysis for proliferating cells showed significantly decreased chondrocyte proliferation in E14.5 *Fgf18*–/– growth plates. This contradictory finding supports a model in which chondrocytes at different stages of development may change their response to *Fgf18*/*Fgfr3* signalling from a mitogenic/differentiation response to a non-mitogenic/reduced differentiation response (Liu *et al.* 2007). Liu *et al.* (2007) also note that the shortened hypertrophic

chondrocyte zone could also be explained by a more general delay in endochondral bone development.

In previous studies *Fgf18* and *Fgfr3* were shown to inhibit *Ihh* expression in pre-hypertrophic chondrocytes at late embryonic and postnatal stages (Liu *et al.* 2002; Naski *et al.* 1998. The recent work by Liu *et al.* (2007) suggests that Fgf18/Fgfr3 may regulate chondrocyte proliferation and differentiation indirectly by regulating Ihh signaling. In support of this theory, Iwata *et al.* (2000) observed increased chondrocyte proliferation and increased *Patched* expression in E15.5 mice harboring an *Fgfr3* gain of function mutation.

The best way to test the validity of this hypothesis would be to compare levels of *Runx2* and *Fgf18* expression in perichondrium of Hh-Ag 1.4 treated and untreated wild type mice (and/or ACH-SADDAN mice). This could be done by *in situ* hybridization for *Fgf18, Wnt, Runx2,* and *Twist-1.* Another possible method would be by quantitative PCR on dissected perichondrium from chicken tibia as done in Long and Linsenmayer (1998), and by Ducy *et al.* (2001) or on rib tissue as in Hinoi *et al.* (2006). As well, in the chondrocytes of the growth plate, Hh-Ag 1.4 treatment should result in increased phosphorylation of ERK 1/2 and cellular localization of STATs. One could potentially stain for phosphorylated ERK using antibodies described by Murakami *et al.* (2004). In Li *et al.* (1999) they demonstrate higher levels of STAT nuclear localization in the activated *Fgfr3* mutant. One could

stain using antibodies against Stat1, Stat5a and Stat5b to determine if hedgehog agonist Hh-Ag 1.4 leads to a similar activation of STATs and their nuclear localization. Finally, if Fgf18 is indeed up-regulated by hedgehog signalling in the perichondrium there should also be increased α1-integrin expression which appears to be a specific target of Fgf18 signalling and may be required for chondrocyte differentiation and cartilage matrix production (Davidson *et al.* 2005; Ekholm *et al.* 2002).

In addition, comparing all the same parameters between treated and non-treated *Fgfr3* -/- described by Colvin *et al.* (1996) mice would be informative . In this case, treatment should still lead to increased Runx2/Fgf18 levels but this should not lead to reduced proliferation since this effect is presumably a result of signalling through the Fgfr3 receptor. There should also be no increase in ERK 1/2 phosphorylation, STATs nuclear localization, or α1 integrin expression as a result of Hh-Ag 1.4 administration.

Ideally, the same tests should be performed on *Runx2* -/- or *Fgf18* -/- mice and give similar results. Unfortunately, both Runx2 -/- and *Fgf18* -/- mice die at birth (Liu *et al.* 2002). The experiment could be done on *Fgf18* +/- or Runx2 +/- mice or better yet on *Runx2* +/-;*Fgf18* +/- compound heterozygote's which show a level of α1

integrin expression in chondrocytes as low as in *Runx2* -/- or *Fgf18* -/- (Hinoi *et al.* 2006).

5.4. Future Directions

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is a method used to detect DNA fragmentation by labelling the terminal end of nucleic acids, thus determining whether DNA fragmentation from apoptotic signalling cascades has occurred (Labat-Moleur *et al.* 1998). One possible future experiment would be to stain the growth plates of treated mice with TUNEL in order to determine whether an increased rate of apoptosis may also contribute to the smaller hypertrophic zone.

A different approach to up-regulate Ihh signalling in growth plates of ACH and SADDAN pups could be to manipulate gene expression *in vivo*. However, expression of *Ihh* under the control of the chondrocyte specific collagen type II promoter/enhancer (*Col2a1*) leads to perinatal lethality and prevents the establishment of transgenic line of mice (Tavella *et al.* 2004) In order to circumvent this complication one could create a transgenic line of mice in which the expression of *Ihh* is inducible and therefore can be activated *in utero* or in young pups (**Figure 20**) The inducible "Tet-on" system uses a modified form of the tet repressor called reverse tet trans-activator (rtTA), that can activate transcription from the target

promoter containing tetracycline responsive elements (TREs), only in the presence of doxycycline (Backman *et al.* 2004)). By further placing expression of the rtTA under control of the *Col2a1* promoter, one would obtain a cell-type specific inducible system.

A vector containing an improved *TRE* promoter (*TRE-tight*) and an rtTA optimized for translation in mammalian cells (**Figure 21**) (Backman *et al.* 2004) will be used in this case. Furthermore, in this construct it would be advisable to use the chick *Ihh* cDNA sequence (*cIhh*) to allow distinguishing between transgene (*cIhh*) and endogenous (*Ihh*) expression in mice.

Once the transgenic mice are generated using standard protocol for pronuclear injection, carriers of the *TetOn-Col2-cIhh* transgene will be crossed with mutants carrying the ACH and SADDAN mutations (Auerbach *et al.* 2003).

In fact, part of the vector to be used in this transgenic mouse has already been cloned. Once a transgenic line is produced mice could be crossed with various other strains to test many of the hypotheses outlined here. *Fgfr3* -/- mice and *Fgf18* -/- mice could be crossed with the tetracycline inducible *Col2a1-Ihh* to test whether or not Fgf18 signalling is biphasic in nature and also to determine if the concomitant changes in proliferation and maturation seen in our study mice is due to Fgf18 signalling in the perichondrium.

It will also be necessary to test different dosing levels and regiments in future experiments. A dose of 10 mg/kg was used in this study as a proof of principle that subcutaneous injections of Hh-Ag 1.4 could indeed reach the growth plate and induce Hh signalling. It is also possible that many other cell types in the body are responding to increased Hh signalling producing a systemic or endocrine effect. Therefore, blood and urine analysis for various hormonal changes should be monitored.

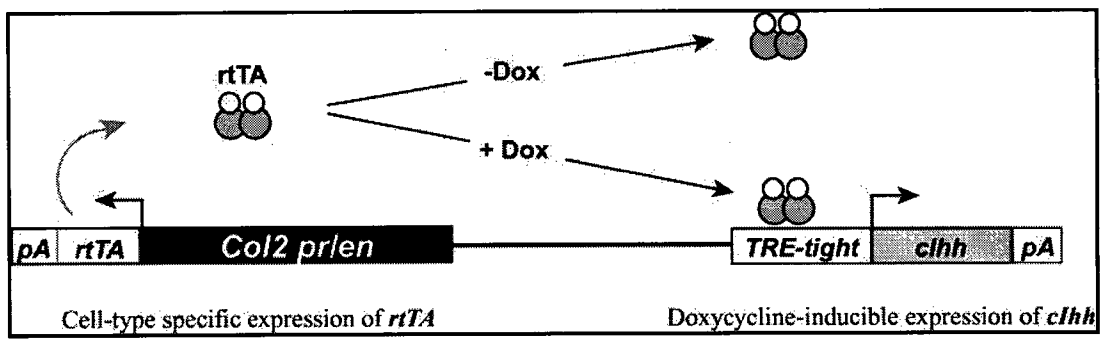

Figure 21: Tetracycline inducible *clhh* transgene under control of the Col2a1 promoter.

6. Conclusion

The role of hedgehog signalling in controlling the rate of growth plate proliferation and maturation has been extensively studied. It is likely that decreased *Ihh* expression is one of the main factors resulting in the chondrodysplasia phenotypes. However, there are still no effective treatments for this group of diseases.

After completing various experiments on this project it may be concluded that further work needs to be done to verify whether or not hedgehog agonists can rescue the defects seen in chondrodysplasia. We may have discovered a previously unknown effect that was not predicted. Based on work recently published by another group, we hypothesize that postnatally increased Ihh signalling may lead to an increase in Fgf18 signalling via Wnt and Runx2 leading to a reduction in proliferation and chondrocyte hypertrophy. This hypothesis may be tested using the various methods outlined in the discussion.

At present it does not seem that the hedgehog agonist Hh-Ag 1.4 will be useful in the treatment of chondrodysplasia. However, their use in models of low bone density in adults, such as osteoporosis, may be of clinical interest as Hh-Ag 1.4 administration does lead to a significant increase in cortical bone thickness. It was recently shown *Patched1* haploinsufficiency increases adult bone mass and modulates Gli3 repressor activity (Ohba *et al.*2008).

Appendix

Animal Use Protocol

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

McGill University Animal Care Committee
RENEWAL of Animal Use Protocol

For: Research ☒ Teaching ☐ project

Principal Investigator: Benoit St-Jacques **Protocol #** 4629

Protocol Title: Role of Indian hedgehog signaling in skeletal development-treatment of achondroplasia **Category:** C

Unit, Dept. & Address: Genetic Unit, Shriners Hospital, 1529 Cedar avenue, Montreal, QC, Canada H3G 1A6

Email: bst-jacques@shriners.mcgill.ca **Phone:** 514-282-7153 **Fax:** 514-842-5581

Funding source: The Shriners of North America

Start of Funding: January 2005 **End of Funding:** December 2007

Emergency contact #1 + work AND home phone #s: Benoit St-Jacques, work = 514-282-7153, home = 514-483-6015

Emergency contact #2 + work AND home phone #s: Marguerite Desbarat, work = 514-842-5964, home = 514-932-5149

1. Personnel and Qualifications

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

Name	Classification	Animal Related Training Information		Occupational Health Program *	Signature *"Has read the original full protocol"*
		UACC on-line Theory course	Workshops + others		
Benoit St-Jacques	PI	Completed "advanced level" theory course on animal ethics and McGill ARC "Mouse workshop" , Dec 2004			
David Morisson	Grad student	Completed "advanced level" theory course on animal ethics and McGill ARC "Mouse workshop", Sept 2005			
Judy Cowan	Technician	Certified Animal Health Technician			
Mia Esser	Technician	Certified Animal Health Technician			

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

Approved by:

2. Approval Signatures

Principal Investigator/ Course Director		**Date:** Oct 30, 2006
Chair, Facility Animal Care Committee		**Date:**
UACC Veterinarian		**Date:** Nov 1 2006

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