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In Vitro and In Vivo Characterization of Mutations in the Human Indian Hedgehog Gene Associated
with Brachydactyly Type A1 and Acrocapitofemoral Dysplasia

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***In Vitro* and *In Vivo* Characterization of Mutations
In the Human Indian Hedgehog Gene Associated with
Brachydactyly Type A1 and Acrocapitofemoral Dysplasia**

By

Graeme Cunningham

THESIS

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Abstract

Brachydactyly type A1 (BDA1) is a congenital disorder that affects normal bone development and patterning. Affected individuals have short fingers, broad hands and are generally short in stature. Acrocapitofemoral dysplasia (ACFD) is a chondrodysplasia characterized by variable short stature of the affected individual with short limbs, brachydactyly in the hands and feet, a relatively large head, and a narrow thorax with pectus deformities. Missense mutations in the Indian hedgehog (*IHH*) gene have been shown to be responsible for both disorders. We were the first group to successfully characterize the functional consequences of four BDA1-associated mutations (E95K, D100N, R128Q and E131K), and one ACFD-associated mutation (V190A). In stably transfected HEK293 cells, production and secretion of each mutant was significantly compared in comparison to the wild-type. When a LIGHT2 and a differentiating ATDC5 reporter cells were treated separately with normalized amounts of BDA1-associated mutant *IHH*-N, significantly reduced reporter activity in both cell lines was detected. Little increase in activity was noted when the reporter cells were treated with an equal combination of mutant and WT *IHH*-N, consistent with a dominant-negative effect.

Furthermore, morpholino-induced 'knock-down' of zebrafish *ihha* revealed a crucial role for *ihha* in early zebrafish angiogenesis. Co-injection of human *Ihh* mRNA, although improving the phenotype, did not completely rescue it. Injection of mutant *Ihh* mRNA alone resulted in approximately 25% of the embryos displaying an altered phenotype – further evidence of a dominant negative effect. Ultimately, our *in vitro* and *in vivo* analysis led to the development of a likely model for the pathogenesis of both brachydactyly type A1 and acrocapitofemoral dysplasia.

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Abbreviations

°C	Degrees celcius
μL	Microlitre
μM	Micro-Molar
2q	q-arm of chromosome 2
3D	Three-dimensional
A	Adenine
A	Alanine
ab/am	Anti-biotic/anti-mycotic
ACFD	Acrocapitofemoral dysplasia
AF	Anal fin
Ala	Alanine
Arg	Arginine
Arrb2	β-Arrestin
Asp	Aspartate
BDA1	Brachydactyly Type A1
bh	Basihyal
BMP	Bone morphogenic protein
bp	Base pairs
C	Cytosine
C	Cysteine
c.	Notation for cDNA indicating 'clone'
CA	California
Ca(NO ₃) ₂	Calcium nitrate
cb	ceratobranchial cartilage
cc	Cardiac chamber
ch	Ceratohyal cartilage
cM	centi-Morgan
cm	Centimetre
CMV	Cytomegalovirus
co	Coracoid of pectoral girdle
CO ₂	Carbon dioxide
ColII	Collagen Type II
ColX	Collagen Type X
CV	Caudal vein
Cys	Cysteine
D	Aspartate
DA	Dorsal aorta
DAPI	4',6-diamidino-2-phenylindole
ddH ₂ O	Double-distilled Water

DF	Dorsal fin
Dhh	Desert hedgehog
Disp	Dispatched
DLAV	Dorsal longitudinal anastomotic vessel
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
dpf	days post-fertilization
E	Glutamate
E. coli	Escherichia coli
ECL	Enzymatic chemiluminescence
EDTA	Ethylene diamine tetracetic acid
EGFP	Enhanced green fluorescent protein
ehh/ihhb	echidna hedgehog
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FGF	Fibroblast Growth Factor
FP	Floor plate
Fu	Fused
G	Guanine
G	Glycine
GAG	glycosaminoglycan
GE	General Electric
Gln	Glutamine
Glu	Glutamate
H	Histidine
H ₂ O ₂	Hydrogen peroxide
Hsa	Homo sapiens
HCl	Hydrochloric acid
HEK	Human embryonic kidney
Hh	Hedgehog
HHIP	Hedgehog inhibiting protein
Hh-N	Hedgehog amino-terminal, signaling peptide
His	Histidine
HPE	holoprosencephaly
hpf	hours post-fertilization
hr(s)	hour(s)
hrp	Horseradish peroxidase
hs	Hyosymplectic
HSPG	Heparan sulphate proteoglycan

I	Isoleucine
ICM	Intermediate cell mass
IFT	Intraflagellar transport
Ihh	Indian hedgehog
Ihha	Danio rerio indian hedgehog a
IHH-N	Ihh Amino-terminal, signaling peptide
Ihh-Np	Palmitoylated Indian hedgehog N-terminal signaling peptide
ISH	In situ hybridization
K	Lysine
KCl	Potassium chloride
kDa	KiloDaltons
KOH	Potassium hydroxide
L	Leucine
LARII	Luciferase assay reagent II
LB	Luria-Bertani
Leu	Leucine
Lys	Lysine
M	Molar
m	Meckel's cartilage
MA	Massachusetts
MD	Maryland
MEM	Minimum essential medium
mg	Micrograms
MgCl ₂	Magnesium chloride
MgSO ₄	Magnesium sulphate
min(s)	Minutes
mL	Millilitres
mM	Milli-molar
mm	Millimetre
MO	morpholino
MO	Missouri
MOPS	3-(N-Morpholino)-propanesulfonic acid
mRNA	Messenger ribonucleic acid
My	Myoseptum
N	Asparagine
NaCl	Sodium chloride (salt)
NC	Notochord
NCBI	National Center for Biotechnology
NEB	New England Biolabs
ng	Nanograms
NH ₂	Amido
nm	Nanometre

NY	New York
OD	Optical density
OHRI	Ottawa Health Research Institute
OMIM	Online Mendelian Inheritance in Man
ON	Ontario
OR	Oregon
p.	Notation for protein sequence
P	Proline
PAGE	Polyacrylamide gel electrophoresis
PAV	Parachordal vessel
pBS	pBluescript II KS+
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
Pfu	Pyrococcus furiosus
pg	Picograms
pH	Potential of Hydrogen
PKA	Protein Kinase A
PLB	Passive Lysis Buffer
POC	Primary ossification centres
polyA	Poly-adenine
PPR	Parathyroid-related Peptide/Parathyroid Hormone Peptide
pq	Palatoquadrate
Pro	Proline
Ptch1/Ptch	Patched
PTHrP	Parathyroid hormone-related Peptide
PTU	phenylthiourea
PVDF	polyvinylidene difluoride
Q	Glutamine
R	Arginine
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
S	Somite
SDS	Sodium dodecyl sulfate
sec	Seconds
SeV	Intersegmental vessels
Shh	Sonic Hedgehog
Shh-C	Sonic hedgehog carboxy-terminus
Shh-N	Shh Amino-terminal, signaling peptide
Shh-N	Sonic hedgehog amino-terminal, signaling peptide

s-Ihh-Np	Soluble, palmitoylated indian hedgehog amino-terminal, signaling peptide
Skn	Skinny hedgehog
Smo	Smoothened
SOC	Secondary ossification centres
SSD	Sterol-sensing domain
s-Shh-Np	Soluble, palmitoylated sonic hedgehog amino-terminal, signaling peptide
SuFu	Supressor of Fused
SV40	Simian virus 40
syu	sonic-you
T	Thymidine
T	Threonine
TBS-T	Tris-Borate Saline Tween-20
TM	Transmembrane
twhh	tiggy-winkle hedgehog
TX	Texas
U/ μ L	Units per microlitre
UK	United Kingdom
UTR	Un-translated region
UV	Ultraviolet
V	Valine
V	Volts
VA	Virginia
Val	Valine
vegf	Vascular endothelial growth factor
W	Tryptophan
WT	Wild-type
X	Denotes 'times' as a measure of concentration
YS	Yolk sac
Zn ²⁺	Zinc ion

Chapter 1

Introduction

1.1 Bone Development

1.1.1 *Endochondral Ossification and the Growth Plate*

Bones. Rigid. Strong. Scaffolding of life. They give us blood, regenerate when they break, and ache while we age. The human skeleton is a magnanimous entity. It consists of 206 bones and is divided into an axial skeleton, which includes the skull, vertebral column, and ribs, and an appendicular skeleton, which includes the pectoral girdle, the pelvic girdle, and the bones of the arms, legs, hands, and feet.

The human skeleton arises from the mesoderm during the gastrulation stage of development. There are two methods in which bones are formed; intramembranous ossification and endochondral ossification. The former is a very rare process, occurring primarily in the flat bones of the skull. In both processes, mesenchymal cells of the mesoderm will condense into pre-cartilaginous clusters of cells that will adhere [1]. Intramembranous ossification will see these mesenchymal cells differentiate directly into osteoblasts, which will secrete a type 1 collagen-rich matrix. Contrary to this, clusters of mesenchymal cells destined for endochondral ossification will differentiate into chondrocytes, while cells at the border of this pre-cartilaginous region will form a perichondrium. These chondrocytes will produce a matrix rich in type II collagen and the proteoglycan, aggrecan [1].

The condensed cartilage will then expand through proliferation and matrix deposition, until a point where chondrocytes at the centre of the condensation will undergo hypertrophic differentiation [1]. These hypertrophic chondrocytes will then control the mineralization of the surrounding matrix and, through the expression of

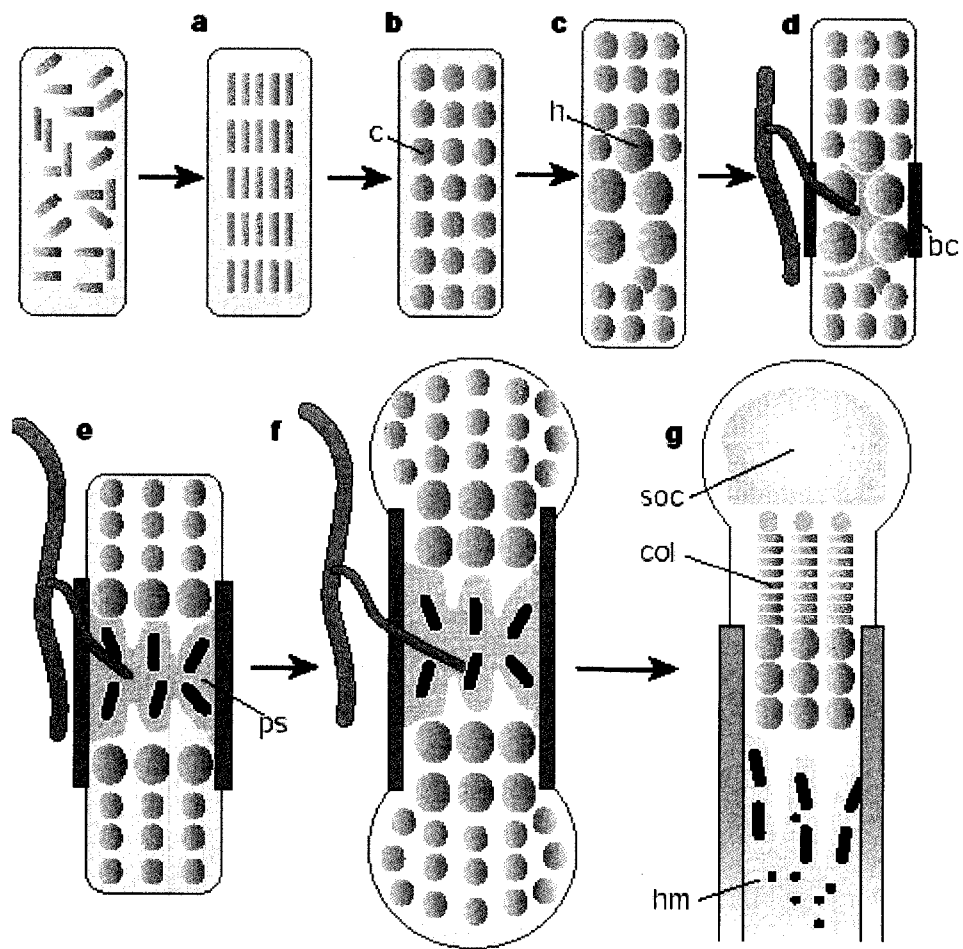
vascular endothelial growth factor (vegf) will cause the invasion of the developing bone. These hypertrophic chondrocytes will also signal perichondrial cells to become osteoblasts, thus forming the bone collar. In the final step, the hypertrophic chondrocytes will then undergo apoptosis, leaving behind a scaffold, through which osteoblasts and blood vessels can invade and create the final bone matrix structure.

As this phase of terminal differentiation, and eventual apoptosis, of these initial hypertrophic chondrocytes is occurring, the developing bone is extending through continued proliferation of chondrocytes. In the case of a long bone, two separate secondary ossification centres (SOC) are created at opposite ends of the bone, and between the primary ossification centre (POC) and these SOC's is a region of chondrocytes in varying stages of proliferation and differentiation – this region is known as the growth plate. At the terminal end of the bone, encapsulated by the periarticular perichondrium, are the round, non-proliferating reserve chondrocytes, which serve as precursors for the adjacent group of cells more proximal to the POC, the proliferating columnar chondrocytes. Through a series of molecular signalling pathways, these will differentiate into pre-hypertrophic chondrocytes, then onto hypertrophic chondrocytes and finally into bone. This process is illustrated in figure 1.1 [1].

1.1.2 *Bone development disorders*

Not surprisingly, when the process of endochondral ossification goes awry, the potential consequences for the development of the axial and appendicular skeleton can be variable, occasionally severe. A chondrodysplasia presents itself as abnormally short and/or deformed limbs, and often results in general short stature (dwarfism). Some cause

Figure 1.1. Illustration of endochondral ossification. Mesenchymal cells condense (**a**) and become chondrocytes (labelled **c**) (**b**). These chondrocytes differentiate into hypertrophic (labelled **h**) chondrocytes at the centre of the developing bone (**c**). Perichondrial cells surrounding the developing bone form the bone collar (labelled **bc**) while the hypertrophic chondrocytes attract blood vessels, secrete a mineralized matrix and undergo apoptosis (**d**). Perichondrial cells will differentiate into osteoblasts and form the primary spongiosa (labelled **ps**) (**e**). Proliferation perpetuates lengthening of the bone (**f**), until finally a secondary ossification centre (labelled **SOC**) is formed at the ends of the developing bone forming the growth plate between it and the primary ossification centre (**g**). The growth plate is comprised largely of flat proliferating columnar chondrocytes (labelled **col**) which differentiated into pre-hypertrophic and subsequently hypertrophic chondrocytes, which will ultimately form the haematopoietic marrow (labelled **hm**) in the marrow space. *Modified from Kronenberg, 2003.*



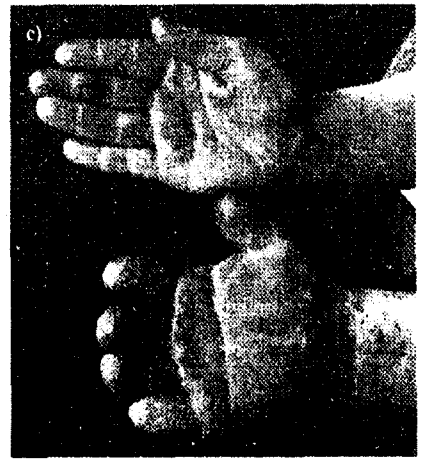
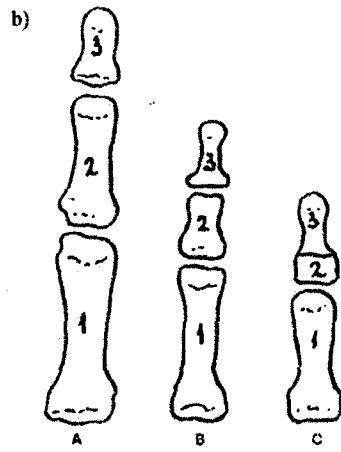
more shortening of the limbs than the trunk (short-limbed dwarfism). Still others can cause more shortening of the trunk, as opposed to the limbs. Some symptoms in both children and adults can include short limbs, bowlegs, a bulky forehead, and an arched back. On occasion, joints do not develop the capacities for their full range of motion. In this study we will be focusing on two disorders of bone development, brachydactyly type A-1 (BDA1), a congenital disorder affecting the development of the hands and feet, and Acrocapitofemoral dysplasia (ACFD), a chondrodysplasia.

1.1.3 *Brachydactyly Type A1* (OMIM 112500)

William C. Farabee was the first to describe this human hand malformation in terms of an autosomal dominant Mendelian trait. Farabee's work initiated much of the early work in the field of human genetics [2]. Brachydactyly can be characterized by generally shortened digits in the hands and feet due to malformation of the phalanges and metacarpals [3]. In 1951, Julia Bell classified brachydactyly into five different categories; A, B, C, D and E, on the basis of malformation of the five digits. Category A was further subdivided into A1, A2 and A3 subtypes [3, 4]. This thesis will focus on BDA1.

BDA1 is characterized by shortening of the middle phalange of all digits in the hands and feet, shorting of the proximal phalange of the first digit, and occasional fusion of the middle and terminal phalanges (figure 1.2) [5]. In fact, all the hand bones may be shorter than those in the normal hands but the middle phalanx and the proximal 1st phalange are the most severely shortened as they are the last to ossify [6] (figure 1.2). In some individuals, the metacarpals are also short. Affected members are generally shorter

Figure 1.2. Pictures of normal hands and hands with Brachydactyly type A1. (A) Top hand, unaffected individual; bottom hand, related affected individual with mild BDA1. (B) A drawing depicting approximate size differences in the proximal (1), middle (2), and distal (3) skeletal phalanges of A – an unaffected individual, B – an individual with mild BDA1, and C- an individual with severe BDA1. (C) Top hand, unaffected individual; bottom hand, related affected individual with severe BDA1. *Modified from Drinkwater, 1915.*



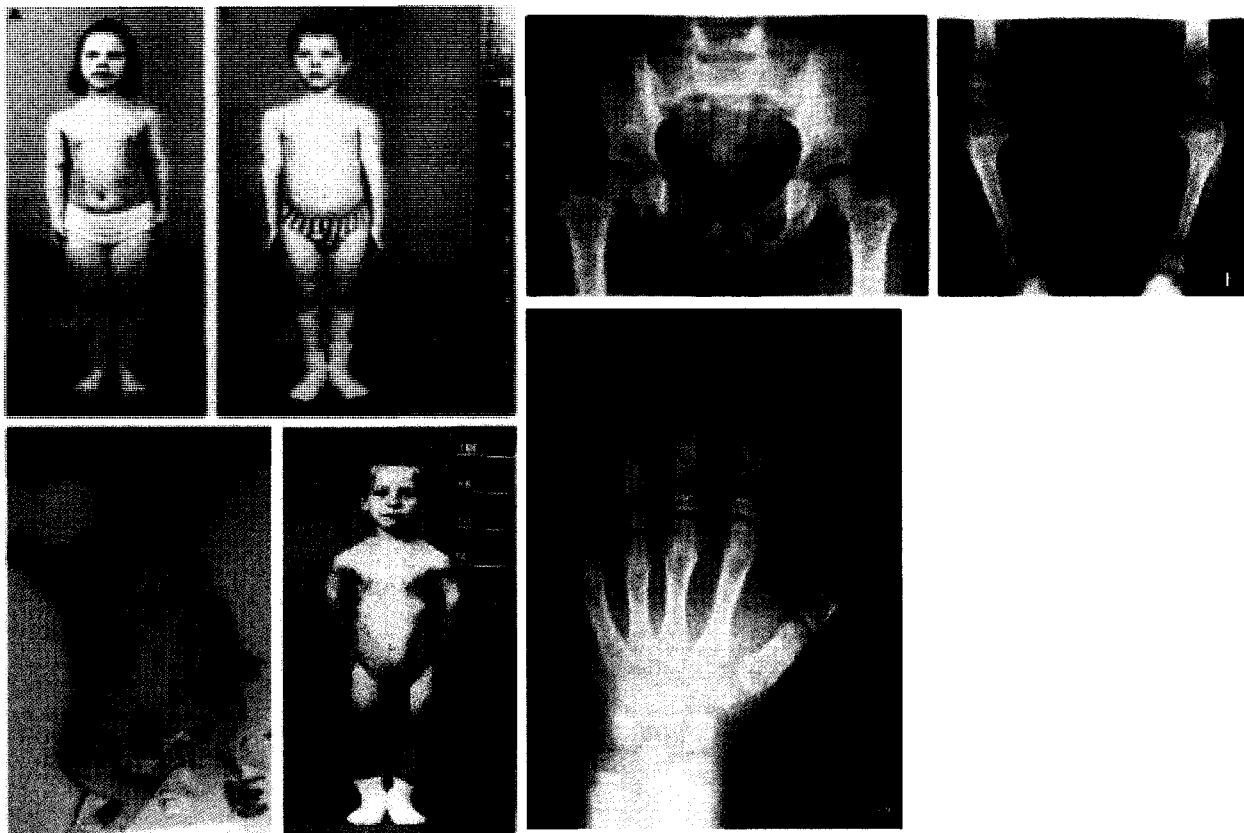
in stature, when compared to their unaffected family members. Often BDA1 can also been described as part of complex syndromes, including nystagmus [7, 8], mental retardation [7-10], scoliosis [7, 11, 12], and club feet [12].

In 2000, BDA1 was mapped to a narrowly defined region of 2q35-q36 [13]. Subsequently, three mutations were identified in the *Indian hedgehog* gene (*IHH*) [4]. Locus heterogeneity was implied when a second locus for BDA1 was reported on chromosome 5p13.3-p13.2 (designated BDA1B – OMIM 607004) in a Canadian family [14]. In addition to this, a third locus associated with BDA1 was discovered in at least one other family, when *IHH* and the chromosome 5p13.3-p13.2 region were excluded [15].

1.1.4 *Acrocapitofemoral Dysplasia* (OMIM 607778)

ACFD is characterized by postnatal onset of variable short stature – measured at various ages, ranging from 2.3 to 8.6 standard deviations from the mean [16]. The affected individual generally possesses short limbs, brachydactyly in the hands and feet, a relatively large head, and a narrow thorax with pectus deformities. Patients exhibit cone-shaped epiphyses when examined radiographically in the hands, the proximal part of the femur and, to a variable degree, the shoulders, knees, and ankles [17] (figure 1.3). Chiefly, these cone-shaped epiphyses result from the premature epimetaphyseal fusion resulting in growth arrest and shortening of the bone involved [16], with variable severity. The spine is only mildly affected, presenting a slightly ovoid appearance of the vertebral bodies. Affected individuals do not exhibit associated congenital anomalies and are of normal intelligence. Individuals with ACFD should not be confused with

Figure 1.3. Photographs of four of the five Patients with ACFD aged 10.5(A), 10(B), 4 months (C), and 9 years (D), each showing varying degrees of short-limb dwarfism. Also shown are radiographs of the pelvis (E), lower limbs (F), and hand (G) of different patients, each with ACFD. Note the egg-shaped femoral heads and very short femoral neck in panel E. In panel F, the distal epiphyses are voluminous, there is notable deformity in the tibia with proximal and premature epimetaphyseal fusion. In the hands, note the cone-shaped epiphyses in the distal phalynx of all digits, as well as shortening of the middle phalynx in digits II-V. *Modified from Mortier et al., 2003.*



those with hypochondroplasia and asphyxiating thoracic dysplasia. In each case, ACFD can be differentiated from these conditions by the presence of the premature closure of the growth plate and the development of cone shaped epiphyses.

In 2003, ACFD was linked to the q-arm of chromosome 2, which subsequently led to the discovery of two different homozygous mutations in *IHH* as the causative agent [16, 17]. The parents carrying one mutant allele show no signs of altered skeletal development [17].

1.2 Indian Hedgehog

1.2.1 Gene and Protein

Indian hedgehog (IHH) belongs to the family of mammalian hedgehog (Hh) genes, which includes *Sonic hedgehog (SHH)* and *Desert hedgehog (DHH)*. Highly conserved Hh genes encode for secreted intercellular signalling peptides that are crucial for growth and patterning in a number of tissues and organs in the developing embryo (amino acid sequence alignment; table 1.1) [18]. Specifically, *IHH* has been shown to play a significant role in the development of the axial and appendicular skeletons, angiogenesis and hematopoiesis [1, 61, 63]. *IHH* is also thought to play a role in the pancreas and gut based on its high level of expression in these tissues [18].

IHH is located on the q arm of chromosome 2 (2q34). The gene spans 5762 base pairs of chromosome 2, and is comprised of 3 exons. Expression of the gene results in a transcript approximately 1500 nucleotides in length, which in turn codes for the full-length precursor protein of approximately 51 kDa. The 5' end of the transcript is

Table 1.1. Sequence alignment of amino acids of hedgehog signaling peptide across and within species. The chart illustrates the alignment of amino acid sequence of human IHH-N with IHH-N homologs from mouse (*Mus musculus*), the chicken (*Gallus gallus*), African clawed frog (*Xenopus laevis*), and the zebrafish (*Danio rerio*). Human IHH-N sequence was also aligned with the N-terminal signaling peptide of human sonic hedgehog (SHH) and desert hedgehog (DHH). Residues highlighted in red indicate the conserved residues of the the BDA1- and ACFD-associated mutations - each mutation is indicated.

		P46L(137C>T)			
<i>Homo sapiens</i> IHH 28	CGPGRVVGSRRRPPRK-LVPLAYKQFSPNVPEKTLGASGRYEGKIARSSERFKELTPNY				
<i>Mus musculus</i>	CGPGRVVGSRRRPPRK-LVPLAYKQFSPNVPEKTLGASGRYEGKIARSSERFKELTPNY				
<i>Gallus gallus</i>	CGPGRVVGSRRRPPRK-LIPLAYKQFSPNVPEKTLGASGRYEGKIARNSEFRKELTPNY				
<i>Xenopus laevis</i>	CGPGRVVGRRRR-PTK-LSPLSYKQFSPNVPEKTLGASGRYEGKISRNSERFKELTPNY				
<i>Danio rerio</i>	CGPGRGYGKRRT-PRK-LTPLAYKQFSPNVAEKTGASGRYEGKVTPSSERFKELTPNY				
<i>Homo sapiens</i> SHH	CGPGRGFGKRHH-PKK-LTPLAYKQFIPNVAEKTGASGRYEGKISRNSERFKELTPNY				
<i>Homo sapiens</i> DHH	CGPGRGPVGRRRYARKQLVPLLYKQFVPGVPERTLGASGPAEGRVARGSERFRDLVPNY				
		E95E(283G>A) E95G(284A>G)	D100E(300C>A) D100N(G298>A)*	R128Q(383G>A)*	T130N(389C>A)* E131K(391G>A)*
<i>Homo sapiens</i> IHH 87	NPDIIFKDEENTGADRLMTQRCKDRLNSLAISVMNQMPGVKLRVTEGNDGEDGHHSEESL				
<i>Mus musculus</i>	NPDIIFKDEENTGADRLMTQRCKDRLNSLAISVMNQMPGVKLRVTEGNDGEDGHHSEESL				
<i>Gallus gallus</i>	NPDIIFKDEENTGADRLMTQRCKDRLNSLAISVMNQMPGVKLRVTEGNDGEDGHHSEESL				
<i>Xenopus laevis</i>	NPDIIFKDEEITGADRLMTQRCKDRLNSLAISVMNQMPGVKLRVTEGNDGEDGHHFEESL				
<i>Danio rerio</i>	NPDIIFKDEENTGADRLMTQRCKDKLNSLAISVMNLMPGVRLRVTEGNDGEDGLHSEESL				
<i>Homo sapiens</i> SHH	NPDIIFKDEENTGADRLMTQRCKDKLNALAISVMNQMPGVKLRVTEGNDGEDGHHSEESL				
<i>Homo sapiens</i> DHH	NPDIIFKDEENSGADRLMTQRCKERVNALAIAMNMMPGVRLRVTEGNDGEDGHHAAQDSL				
		T154E(461C>T)			V190A(569T>C)
<i>Homo sapiens</i> IHH 145-202	HYEGRVDITTSORDRNKYGLLARLAVEAGFDWVYYESKAHVHCSVKSEHSAAAKTGG				
<i>Mus musculus</i>	HYEGRVDITTSORDRNKYGLLARLAVEAGFDWVYYESKAHVHCSVKSEHSAAAKTGG				
<i>Gallus gallus</i>	HYEGRVDITTSORDRNKYGLLARLAVEAGFDWVYYESKAHIHCSVKSEHSAAAKTGG				
<i>Xenopus laevis</i>	HYEGRVDITTSORDRNKYGLLARLAVEAGFDWVYYESKAHIHCSVKSEHSAAAKTGG				
<i>Danio rerio</i>	HYEGRVDITTSORDRNKYRLMARLAVEAGFDWVYYESKGVHCSVKSEHSVAAKTGG				
<i>Homo sapiens</i> SHH	HYEGRVDITTSORDRSKYGLMARLAVEAGFDWVYYESKAHIHCSVKAENSVAAKSGG				
<i>Homo sapiens</i> DHH	HYEGRALDITTSORDRNKYGLLARLAVEAGFDWVYYESRNHVHVSVKADNSLAVRAGG				

comprised of a short signalling peptide which localizes the precursor to the nuclear membrane and facilitates its transport to the endoplasmic reticulum [19].

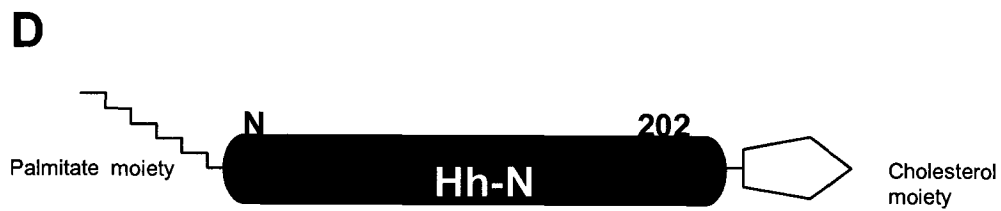
Biologically active amino-terminal IHH signalling peptides (IHH-N) are generated by auto-catalytic cleavage catalyzed by amino acid residues located in the carboxy-terminus of the Hh precursor protein [20]. It is known that SHH-N protein produced *in vivo* contains additional modifications; palmitoylation of an NH₂-terminal cysteine residue and covalent attachment of cholesterol at the C-terminus, both of which are required for activity and long range patterning of the molecule [21, 22](figure 1.4). And by strong similarity of IHH-N with SHH-N, it is believed that IHH-N also undergoes the same post-translational modifications. The addition of this palmitate is facilitated by a protein called Skinny Hedgehog (Skn) [23]. Goetz *et al.* [24], demonstrated that amino acids 27-34, 35-48 and the palmitate acceptor site, Cys-25 are essential to facilitate protein-protein and protein-lipid interactions in SHH-N, and that these interactions are necessary for multimerisation of SHH into the palmitoylated, cholesterol modified, diffusible multimer of SHH-N, denoted s-SHH-Np [24].

1.2.2 Signalling Pathway

As we will be referring primarily to IHH signalling during the process of endochondral ossification we will examine here IHH signalling in the developing long bone. During embryogenesis, *IHH* is expressed throughout the primordial cartilage, but eventually becomes restricted to the pre-hypertrophic chondrocytes in the growth plate after birth [25].

In vitro studies have indicated that IHH-N becomes membrane anchored as a

Figure 1.4. Cleavage and processing of the Indian hedgehog protein. (*A*) The mature hedgehog mRNA codes for an approximately 45 kDa precursor protein, which is composed of a signal peptide (27 amino acids), an N-terminal fragment (175 amino acids) responsible for all signaling functions, and a C-terminal fragment (209 amino acids) with no apparent function beyond catalysing the cleavage reaction. During hedgehog processing to the mature peptide, (*B*) the signal sequence is cleaved; (*C*) autocleavage of the mature N-terminal portion from the C-terminal portion is catalysed by residues in the C-terminal fragment; and (*D*) palmitate and cholesterol moieties are added to the N-terminus and C-terminus of the N-terminal fragment, respectively.



result of lipid modification [23, 24]. Although unconfirmed, it is likely that this is a process essential to localize the protein to the membrane and interact with the protein Dispatched (Disp), a twelve-pass transmembrane protein that has been shown to be essential for Hedgehog signalling [26]. Disp is part of an emerging family of multi-pass membrane proteins, which contain a characteristic sterol-sensing domain (SSD) [27].

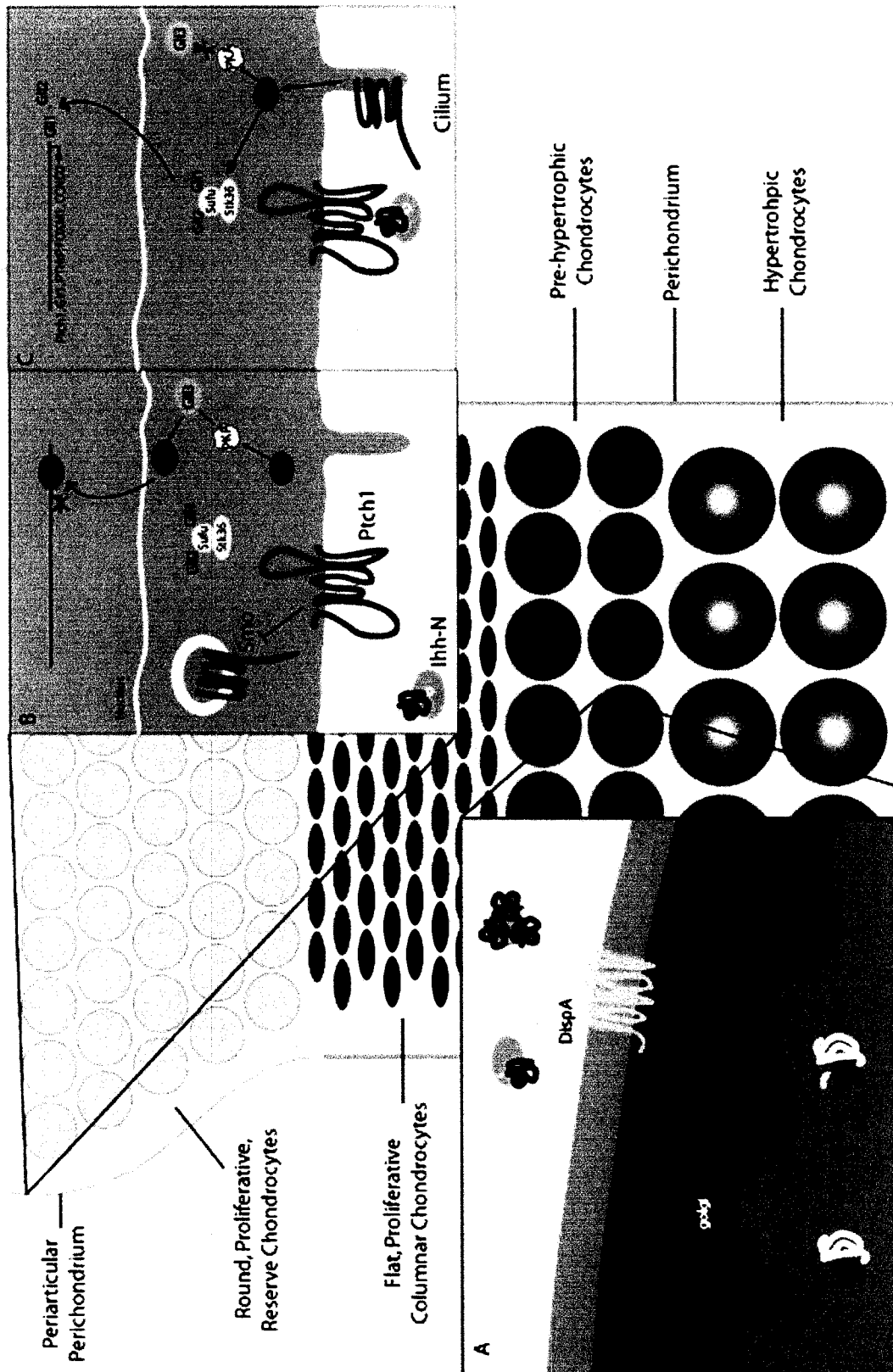
Once multimerized, IHH-N can act as both a short-range (over 10-15 cell diameters, $\sim 20\ \mu\text{m}$) and long-range morphogen (numerous cell diameters, $\sim 200\ \mu\text{m}$) [28]. IHH-N will typically target cells in both the perichondrium surrounding the developing bone collar, and the periarticular perichondrium [29]. Both of these cell types express the Hedgehog-ligand-binding protein, Patched (Ptch1/Ptch or Ptch2) [19, 30, 31]. Although there are two genes of the 12-transmembrane protein, in embryonic development, evidence suggests that Ptch1 is the primary target of IHH [32]. In the absence of a binding ligand, it is known that Ptch1 inhibits the activity of the seven-TM domain structure of Smoothened (Smo) [33]. However, recently it has been discovered that cilia play a much more important role in this process. Recent evidence suggests that Ptch localizes to the cilia of Hedgehog-responsive cell lines, and inhibits Smo activity by preventing its accumulation within the cilia [34, 35]. Furthermore, Ptch inhibits Smo in a catalytic manner, as a single molecule of Ptch can inhibit the activity of approximately 50 Smo molecules [36]. This type of result would suggest that there are likely intermediate components, such as a small molecule, that mediate the signals between Ptch and multiple Smo molecules.

Again, in the absence of Hedgehog, unknown interactions between the Hedgehog receptor Ptch and Smo prevent Smo from initiating the Hedgehog signalling cascade. At

this point, the 155 kDa zinc-finger transcription factor, Gli3, is phosphorylated by protein kinase A (PKA) and subsequently proteolytically processed into a 75 kDa product, still containing its zinc-finger DNA-binding domain, which localizes to the nucleus, binds to Hedgehog target genes, thus preventing their transcription [37, 38]. This process is facilitated by intraflagellar transport (IFT) proteins, which are important in the trafficking of protein cargo along the microtubule cytoskeleton, and are also involved in the formation of cilia and flagella [Reviewed in 35]. In particular, the IFT anterograde motor subunit, Kif3a, and the IFT retrograde motor subunit, Dnchc2, have been shown to be specifically involved in Hh signalling between the Ptch and Gli transcription factors [34, 39]. At the same time, a complex of Stk36 (the vertebrate homolog of the *Drosophila* Hedgehog serine/threonine kinase, Fused; Fu), Suppressor of Fused (SuFu) as well as several kinesin-related proteins (possibly Kif7 and Kif27; homologous to the *Drosophila* *Cos2* gene), bind to activator forms of Gli1 and Gli2 and prevent their activation of Hh-target genes by inhibiting Gli translocation to the nucleus [34, 40-42]. This process is illustrated in detail in figure 1.5.

Upon binding of Hedgehog ligand to Ptch, mammalian Smo is phosphorylated by Grk2, a process that also involves β -arrestin (Arrb2), which internalizes the transmembrane (TM) protein [43, 44]. In cell culture, Smo is localized in clathrin-coated pits upon Hedgehog stimulation [44]. Sub-cellular localization of Smo terminates the phosphorylation and processing of Gli3, likely through the activity of IFTs, thus preventing Gli3 from localizing to the nucleus and inhibiting transcription of Hedgehog target genes. Furthermore, upon induction of Smo activity, again, through the mediation of IFTs, the repression of Gli1 and Gli2 activity by the Stk36/Sufu macromolecular

Figure 1.5. The Indian Hedgehog signalling pathway. IHH is produced in pre hypertrophic chondrocytes (**A**) and undergoes autocatalytic cleavage in the endoplasmic reticulum (ER), and subsequently addition of a palmitate and a cholesterol moiety at the N- and C-terminus, respectively. It is these post- translational modifications that localize the peptide to the sterol-sensing domain of Dispatched (DispA), which facilitates the diffusion of IHH through the membrane and possibly its multimerization. In the absence of Hedgehog (**B**), the hedgehog receptor Patched, Ptch1, inhibits the activity of Smoothened, Smo. Through the activity of intraflagellar transport proteins (IFTs), Protein Kinase A (PKA) phosphorylates Gli3 into its active repressor form, which then localizes to the nucleus and prevents the transcription of IHH target genes. When Hedgehog binds (**C**), it relieves the inhibition of Smo, which, through the activity of IFTs, terminates the phosphorylation of Gli3 into its repressor form and also relieves the inhibition of Gli1 and Gli2 by the Sufu/Stk36 macromolecular complex, which allows Gli1 and Gli2 to localize to the nucleus and initiate the transcription of Hedgehog target genes, such as *Pthrp*, *Gli*, *Hip*, *Foxm1*, *Ccnd2*.



complex is relieved. These transcription factors then localize to the nucleus and bind to, and initiate the transcription of Hedgehog target genes, including; *Ptch*, *Gli1*, *Parathyroid Hormone-related Peptide (PTHrP)*, *Foxm1* and *Ccnd2* [41, 45, 46] (figure 1.5).

Each of the individual genes targeted by IHH play a different role in endochondral ossification. *Foxm1* and *Ccnd2* are transcription factors essential for cycle progression and mitosis [47, 48]. These transcription factors are up-regulated by *IHH* expression in gastric cancers, and it is likely that these regulators of cell cycle play a role in the proliferation of chondrocytes in the developing long bone, independent of PTHrP signalling [47]. The expression of *Ptch* and *Gli1* are indicative of a self-amplification of the signal within the Hedgehog-signalled cell, theoretically in order to maximize the efficiency of the Hedgehog signal initially transduced in the cell. Coincidentally, *Ptch* and *Gli1* expression also provide a reliable readout for the status of IHH activity *in vitro* and *in vivo*. PTHrP is an essential regulator of development in the growth plate that participates in a negative feedback loop with IHH, which is essential to maintain the balance between proliferation and differentiation of chondrocytes in the growth plate [19, 25, 49-51].

PTHrP is an auto/paracrine factor with a role in a wide variety of adult and fetal tissues, including heart, kidney, hair follicles, placenta, breasts, lungs, and of course cartilage [52]. IHH-N signal reaches the periarticular perichondrium and induces the expression of *PTHrP*. The mRNA for its receptor; *Parathyroid hormone/Parathyroid hormone-related peptide receptor (PPR)* is expressed in a region of the growth plate that spans the proliferating columnar chondrocytes into, primarily, the pre-hypertrophic zone

[53, 54]. Studies examining *PTHrP*-null (*PTHrP*^{-/-}) mice have elucidated that activation of PPR maintains the cell in a proliferative state, while activation of PPR in chicken chondrocytes is sufficient to down-regulate *IHH* expression [51]. This suggests that *IHH* up-regulates *PTHrP* in order to delay chondrocyte hypertrophy. In this manner, the rate of cells leaving the proliferating zone and therefore the rate of long bone growth is carefully controlled [50, 54, 55]. It is believed that *PTHrP* regulates more than just *IHH* expression in chondrocyte hypertrophy [51]. However, the mechanism by which it does this remains relatively unknown.

1.2.3 *BDA1* and *ACFD*-associated mutations in *IHH*

It has been over 100 years since William C. Farabee first characterized brachydactyly type A1 as a disease transmitted as an autosomal dominant condition [2]. After Gao *et al.*, [13] mapped the locus for *BDA1* in three large, unrelated families, a subsequent focus on the *IHH* gene led to the discovery of three mis-sense mutations in *IHH* [56]. These mutations were a c.G283A transition in exon 1, a c.G391A transition in exon 2, and a c.C300A transversion in exon 1, resulting in p.Glu95Lys; p.Glu131Lys and p.Asp100Glu, respectively [56].

Our group examined DNA from the two families initially studied by Drinkwater in 1908 [57] and 1915 [5], and found that, although reported to be unrelated, both families shared the same c.G298A transition, resulting in a p.Asp100Asn substitution [58]. Our group also discovered an additional mutation in a separate Pennsylvanian family; a c.G383T transversion, resulting in a p.Arg128 Gln [unpublished].

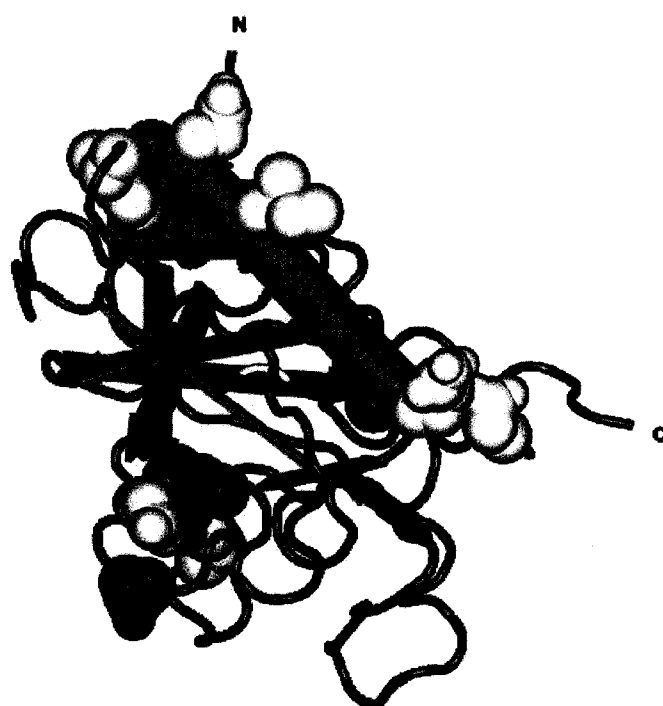
Although other mutations have since been discovered; E95G, T130N and T154I,

this project will focus on the following BDA1-causing mutations: E95K, D100N, R128Q, E131K.

In 2003, Dr. Geert R. Mortier *et al.*, used a genome-wide homozygosity mapping approach in two separate consanguineous families with a total of five individuals affected with ACFD, and linked the disease to a marker on chromosome 2q35-36 [17]. This subsequently led to a focus on *IHH* as a likely candidate gene. Two separate mutations in *IHH* in each family were discovered. Family one contained a homozygous c.C137T transition, which resulted in a Pro to Leu substitution at position 46, while family 2 contained a homozygous c.T569C transition, resulting in a Val to Ala substitution at amino acid position 190 [17]. ACFD is transmitted as an autosomal recessive disorder. The parent carriers of affected individuals are, although somewhat smaller in stature, relatively unaffected [16].

As no crystal structure of the amino terminus of human Indian hedgehog has been deduced, and considering the high similarity between human IHH-N and mouse Shh-N, 3-dimensional position of these mutated IHH-N residues has been inferred by comparison to the crystal structure of amino terminal of mouse Sonic hedgehog (Shh) (figure 1.6) [59]. A comparison of the properties of the original and mutated residues, are summarised in table 1.2. All of the BDA1 mutations appear to be clustered together on the surface of the protein, while the ACFD mutations, P46L and V190A, are located at the amino- and carboxy-termini of the signalling peptide, respectively. The P46L mutation has been purported to reside in the region important for binding of Shh-N protein to Ptch [60]. However, recent evidence suggests that the P46L mutation resides in one of three regions of amino acids at the amino-terminal end of the signalling peptide

Figure 1.6. 3D model of mouse Shh-N illustrating BDA1, ACFD and HPE3 mutations. Illustrated is the 3-dimensional structure of the mouse Shh-N. Illustrated in yellow are the residues of Shh which when mutated cause holoprosencephaly (HPE3). Illustrated in red are the two ACFD-causing mutations in *IHH*, and in green, the three previously published BDA1-causing mutated residues (E95, D100, and E131). The R128 residue resides in this region as well. *Modified from Hellemans et. al., 2003.*



ACE2 mutations (HH)	red
ESV2 mutations (HH)	green
HIP2 mutations (SH)	yellow

Table 1.2. Summary of amino acid changes for each mutation. Listed in the table are each of the ACFD mutations (P46L, V190A) and BDA1 mutations (E95K, D100N, R128Q, E131K). For each mutation the original and the new amino acid is listed and for each, the general properties; polarity, acidity, and hydrophobicity (more negative values denote more hydrophilic residues). Note the significant change in both the residues which were originally Glutamic acid (a proton donating group) to the more basic residue, lysine.

Mutation	Original residue	New residue
P46L	Proline (Pro) Non-polar - neutral -1.6	Leucine (Leu) Non-polar - neutral 3.8
V190A	Valine (Val) Non-polar - neutral 4.2	Alanine (Ala) Non-polar - neutral 1.8
E95K	Glutamate (Glu) Polar - acidic -3.5	Lysine (Lys) Polar - basic -3.9
D100N	Aspartate (Asp) Polar - acidic -3.5	Asparagine (Asn) Polar - neutral -3.5
R128Q	Arginine (Arg) Polar - basic -4.5	Glutamine (Gln) Polar - neutral -3.5
E131K	Glutamate (Glu) Polar - acidic -3.5	Lysine (Lys) Polar - basic -3.9

crucial for palmitoylation modification [24].

1.2.4 Other Roles of *IHH*

In addition to its role in moderating proliferation and differentiation in the developing long bone, *IHH* has also been shown to coordinate angiogenesis in the growth plate as well [61]. It has been established that *IHH* is expressed in the visceral endoderm of ES embryoid bodies and mouse embryonic yolk sacs [62]. In the yolk sacs of mice lacking *Ihh*, normal endothelial cell-containing blood islands and a primary capillary plexus develop, and yet branching blood vessels from these blood islands appear smaller, less organized and also appear flattened or collapsed [61]. It has been shown that *SHH*, produced in the endoderm of the developing chick embryo, acts upstream of VEGF and Notch signalling to promote *de novo* neovascularization through the assembly of angioblasts and endothelial progenitors into vessel tubes [63, 64]. And yet, both *Ihh* and *vegf* are expressed in the visceral endoderm layer, and it appears that this source of *vegf* is required for proper angiogenesis, suggesting that *Ihh* and *vegf* may act in concert rather than in tandem, as is the case with *Shh*, to promote vascular remodelling [65]. Furthermore, analysis of the *Ihh*^{-/-} mouse chondrocytes, normally kept in a state of proliferation and delayed differentiation, undergo hypertrophy and terminal differentiation. These chondrocytes express *vegf* and are invaded aberrantly by blood vessels [66]. Subsequently, vessel expansion in the developing long bone is impaired, resulting in a degraded cartilage matrix that is devoid of blood vessels [66]. While one report refutes that *Ihh* acts directly on endothelial cells to confer identity [63], others suggest that *Ihh* directly induces endothelial cells to form capillaries [64, 67]. In either case, it can be concluded that *Ihh* plays a role in determining endothelial cell fate in

mammals.

Hedgehogs (*SHH*, *IHH* and *DHH*), BMP/TGF β , WNT and Notch signalling pathways are essential to the stem cell signalling network. This network plays a vital role in a variety of processes, including embryogenesis, maintenance of adult tissue homeostasis, tissue repair during chronic persistent inflammation, and carcinogenesis [41]. BMP-RUNX3 signalling induces *IHH* expression in differentiated epithelial cells around the surface of the stomach and intestine [1, 68]. Hedgehog signalling is frequently activated in oesophageal cancer, gastric cancer and pancreatic cancer due to transcriptional up-regulation of Hedgehog ligands and epigenetic silencing of *HHIP1/HHIP* gene, encoding the Hedgehog inhibitor. *SHH* and *IHH* are expressed in all of the six esophageal cancer cell lines tested, all of the six human gastric cell lines tested, and five of six tested human pancreatic cancer cell lines [69]. Up-regulation of genes, such as *Hedgehog*, encoding proponents of onco-developmental pathways occurs during chronic inflammation, in order to repair damaged tissues. Epigenetic silencing of negative regulators of these onco-developmental pathways, such as PTCH1 and HHIP, can occur during chronic persistent inflammation and/or ageing. Finally, mutation, amplification and/or loss of genes encoding these onco-developmental signalling molecules then occurs to advance the multi-stage carcinogenesis [47].

In addition to neoplasms associated with altered *IHH* signalling in the gastrointestinal tract, aberrations in Hedgehog signalling in its more common place of endochondral ossification can result in the development of cartilaginous tumours. These tumours are the most common neoplasms affecting bone. They can range from benign lesions, such as enchondromas and osteochondromas, to malignant chondrosarcomas.

For example, patients with multiple osteochondromas [70], an autosomal dominant disorder, develop cartilage-capped, benign, bony neoplasms on the outer surface of bones [70]. These benign lesions can cause disability because of pain, limb deformity, and pathological fracture. Furthermore, these lesions have a high potential (50% in some cases of multiple osteochondromas) for malignant change [71]. Chondrosarcomas can be very difficult to treat, as neither chemotherapy nor radiotherapy is typically effective.

Chondrosarcomas arise typically as a result of disruption of Hedgehog signalling. Examples include a constitutively active PTHrP receptor (PTHR1), as a result of a mutation (R150C PTHR1)[49]. Constitutive activation of PTHR1 would maintain chondrocytes in a state of uninhibited proliferation, resulting in the consequent neoplasm. Another example is mutations in *EXT* genes, which regulate the biosynthesis of heparan sulphate proteoglycans (HSPGs); multifunctional macromolecules involved in facilitating the diffusion of IHH to PTCH [38]. Recently, some successful reports have been generated suggesting that blockage of Hh signalling, through the use of small molecule inhibitors, such as triparanol, in chondrosarcoma explant cultures and xenografts can attenuate the neoplastic phenotype by decreasing tumour cell proliferation, tumour cellularity, and tumour volume [72]. These results suggest that targeting Hh signalling may be an effective chemotherapeutic strategy for patients with chondrosarcomas.

1.3 Zebrafish: A unique model

1.3.1 Zebrafish

The zebrafish, *Danio rerio*, is a tropical fish, belonging to the family *Cyprinidae*.

Originating from Eastern India and Southeast Asia, it now inhabits numerous tropic regions of the world as a consequence of its popularity both as an easily domesticated and traded aquarium fish and its usefulness in scientific research. It is so named for its five uniformly pigmented, horizontal blue stripes on its sides that extend from the head to the caudal fin. The zebrafish grows to approximately 3.8 cm in length, has a three year lifespan and an individual fish can produce between 100 and 300 eggs each spawning. It is these large, robust, transparent embryos that develop from egg to larvae in three days, external to the mother, that make the zebrafish embryo such an excellent model for experimental manipulation and examination.

Furthermore, zebrafish reach maturation and begin to reproduce between three and four months after fertilization [73]. This feature, along with the large progeny size, makes the zebrafish an excellent vertebrate model for the study of forward genetic screening in a Mendelian fashion. In addition to this, several gene knockdown and/or over-expression technologies, and small-molecule-induced phenotypes can be employed using this model; an excellent tool with which to dissect vertebrate gene function and genetic networks. One such technique employs the use of morpholino (MO) technology. A morpholino is a synthetic oligonucleotide, typically between 25 and 35 bases. It is so named for the backbone of the molecule, which consists of a six-member morpholine ring in place of the ribose-based sugar group, and a phosphorodiamidate group in place of the phosphate group. A morpholino can be designed antisense to any portion of a transcript of interest, and depending on where the MO is designed to bind, it can serve a variety of purposes. For example, if designed to complement the 5' UTR and start site of a transcript, binding of the MO to the message will sterically hinder the binding of the

ribosomal complex and prevent translation of the message, effectively “knocking-down” expression of the target gene. We have employed this technology in this study, and have designed a MO to inhibit the expression of the *IHH* homolog in zebrafish, *ihha*.

1.3.2 Zebrafish hedgehog family

There are five different genes in the zebrafish *hedgehog* family that play a wide variety of roles not only in the developing organism but also in the adult. The five genes include, *shh*, *ihha*, *dhh*, the floor-plate specific *tiggy-winkle hedgehog (twhh)* [74], and the notochord specific *echidna hedgehog (ehh/ihhb)* [75]. Hedgehog signalling in the developing zebrafish embryo serves several functions, ranging from Shh and Twhh-mediated patterning of the left-right axis [76], to regulation of chondrocyte hypertrophy in the developing axial and appendicular skeleton by Ihha [77] to patterning of regenerating fin rays after injury [78]. More specifically, transcripts of *ihha* are first evident in 2-3 cells in the parachordal cartilage in an embryo 4 days-post-fertilization (dpf) [77]. By 6 dpf, a larval cartilaginous skeleton has formed and *ihha* expression can be detected in hypertrophic chondrocytes in several cartilaginous elements of the craniofacial and fin endoskeleton [77]. Thus far, no other evidence exists to suggest that *ihha* is expressed any earlier in embryogenesis. It is interesting to note that *twhh* and *ehh* expression can partially compensate for lost *shh* expression in the *sonic you (syu)* mutants [79], and it is believed that there may be a greater functional redundancy of hedgehog genes in zebrafish than in other vertebrates [80]. In this study, we will analyze Ihha signalling pathways in the developing zebrafish embryo, and we will examine the effects of over-expression of human *IHH* in this model.

Chapter 2. Project Goals

2.1 Rationale

Extensive study has been conducted analyzing the genetics of brachydactyly type A1 and acrocapitofemoral dysplasia, however little has been done to analyze how the various mutations to the human Indian hedgehog gene lead to the pathogenesis of these disorders. We strongly believe that the study of these mutations to *IHH* will yield novel information regarding how the characteristics of the mutant peptide result in BDA1 and ACFD, and will also provide insight into the molecular signalling pathway of *IHH*. There is great need to better understand the molecular pathways inherent in bone development in order to generate therapeutic treatments for such degenerative bone disorders as arthritis and osteoporosis.

2.2 Hypothesis

Previous studies examining mutations to other Hedgehog proteins, most notably Sonic Hedgehog, have provided significant insight into the properties of Hedgehog proteins. Based on previous reports, I hypothesize that there will be distinct similarities, in terms of processing and activity, between each BDA1 mutation and each ACFD mutation, but there will be a great deal of difference between the biochemical properties of each of the separate groups of mutations from each other, and from the wild-type variant. Furthermore, we believe that the BDA1 and ACFD-associated mutations to IHH will lead to mis-folding of the protein, altered IHH auto-proteolysis, and ultimately to a reduction in the amount of mutant IHH-N that is secreted by the IHH-producing cell.

2.3 Project Objectives

1. To construct full-length *IHH* cDNA
2. To analyze IHH production, secretion and activity *in vitro*
 - a. To create six different point mutated variants of the *IHH* cDNA by site-specific mutagenesis
 - b. To develop lines of HEK293 cells stably expressing one of *IHH* variant constructs (mutants and wild-type).
 - c. To examine IHH-N production and secretion by western blot of stable cell lysates and media
 - d. To examine IHH-N activity in a non-chondrogenic hedgehog-responsive reporter cell line
 - e. To examine IHH-N activity in a chondrogenic hedgehog-responsive cell line
3. To analyze expression of *IHH* and *ihha* in a zebrafish model
 - a. To assess the ability of human *IHH* overexpression to rescue an *ihha*-morphant zebrafish phenotype
 - b. To assess the impact of over-expression of human *IHH* alone in a wild-type phenotype

Chapter 3

Production of full-length *Indian Hedgehog* cDNA, and synthesis of BDA1 and ACFD-associated mutations

3.1 Introduction

As previously stated, it was our goal to analyze the production, processing, secretion, and activity of both wildtype and mutant recombinant *IHH*. However, to do this we required cDNA of *IHH* in an expression vector in order to generate this recombinant protein. To date, no group has produced a full-length cDNA clone of human *Indian hedgehog*. However, I utilized a commercially available clone, IMAGE clone 5182642, which consisted of a small portion of the 3' end of the first exon, followed by the complete sequence of exons 2 and 3 of *IHH*. My plan was to genomically amplify a exon 1, digest this amplified product, along with the IMAGE clone, and the vector pBluescript II KS+, and create the first ever full-length cDNA clone of the human *Indian hedgehog* gene. From this product, constructs containing each of the BDA1- and ACFD-causing mutations previously cited were produced via site-directed mutagenesis employing reverse complementing primers flanking the region of the single nucleotide polymorphism. The success of this step of the project enabled us to move forward with our functional characterization of the BDA1- and ACFD-causing mutations in human *Ihh*.

3.2 Materials and Methods

3.2.1 PCR amplification of *IHH* exon1

IHH exon 1 was amplified using *IHH*cDNAx1-F forward primer (5'-ATCTGGATCCATGTCTCCCGCCCGGCTCCG-3') and the reverse primer, *IHH*x1-R (5'-GAGCGTGCCAGCCAGTCG-3'). All primers were synthesized by Sigma Genosys

(Sigma-Aldrich, Oakville, ON). PCR conditions included 0.3 mM dNTPs, 1.5 mM MgCl₂, 100 nM KCl, and 1 µL of 1U/µL of Native *Pfu* DNA polymerase (Stratagene, La Jolla, CA). Amplification conditions were; (1) 98°C for 45 sec; (2) 98°C for 45 sec; [81] 57°C for 45 sec (4) 72°C for 2 min (5) 72°C for 10 min with steps 2, 3 and 4 repeated for a total of 40 cycles.

3.2.2 Production of full-length IHH cDNA

As mentioned, the IMAGE clone contained *IHH* cDNA coding for exons 2 and 3, but it also contained a small portion of exon 1, which possessed a *Bsp*HI restriction site. Approximately 75 ng of amplified exon1 and 1 µg of the IMAGE clone were digested in 20 µL volumes, each, in 2.0 µL of 10X New England Biolabs Buffer #4. The amplified section of exon 1 was digested with 1 µL of 1U/µL *Bam*HI and 1 µL of 1U/µL *Bsp*HI and the reaction was incubated in a PTC-225 thermal cycler (MJ Research, Waltham, MA) at 37°C for 1 hour. The IMAGE clone was digested with 1 µL of 1U/µL *Apa*I, incubating in the same PTC-225 thermal cycler at 25°C for one hr, at which point, 1 µL of 1U/µL *Bsp*I was added and the reaction incubated at 37°C for 1 hr. The restriction digest of the vector, pBluescript KS+ was performed in a 20 µL reaction, containing 100 ng of pBluescript KS+, 2.0 µL of 10X New England Biolabs Buffer #4, and 1.0 µL of 1U/µL *Apa*I and incubated at 25°C for 1 hour. At which point, 1.0 µL of 1U/µL *Bam*HI was added and the reaction mixture was incubated at 37°C for another hour. The digested products from all reactions were separated by size on a 1.0% agarose gel for 25 minutes at 120V.

3.2.3 *Purification of digested IHH cDNA*

The appropriate bands were excised and purified using the GE Healthcare illustra GFX PCR DNA and Gel band purification kit (GE Healthcare, Buckinghamshire, UK). The purified products were quantitated via agarose gel electrophoresis. Five μL of each purified sample were electrophoresed on a 1.0% agarose gel for 25 minutes at 100V. This gel was subsequently photographed under UV light and the concentration of each sample was calculated based on comparison of band intensity with bands of the HighRanger Plus 100bp DNA ladder (Norgen Biotech, Thorold, ON). These products were ligated in a 30 μL reaction with 50 ng of pBluescript and the amplified exon 1 and the IMAGE clone insert, along with 3.0 μL of the 10X ligase buffer, and 1.0 μL of 1U/ μL T4 DNA ligase (both from Invitrogen, Carlsbad, CA). This reaction mixture was incubated at 16°C in the MBS Satellite 0.2G thermal cycler (Thermo Electron Corporation, Milford, MA) for 16 hours.

3.2.4 *Preparation of pBSIhhWT*

The ligated products were subsequently transformed in competent *E. coli* DH5 α (Invitrogen, Carlsbad, CA) by heat-shock at 42°C. An inoculated suspension of the transformants was plated on an Luria-Bertani (LB)-agar plate, containing ampicillin (100 $\mu\text{g}/\text{mL}$), and incubated overnight at 37°C. Colonies were selected and used to inoculate a 5 mL seeding culture, which was incubated overnight at 37°C until the optical density (OD) at 600 nm (OD₆₀₀) reached 1.0. The plasmid was isolated using the GenElute™ Plasmid Miniprep kit (Sigma, St. Louis, MO), according to the manufacturer's

instructions. Confirmation of construction of the full-length *IHH* cDNA in pBluescript was done by restriction digest with *Bsp*HI, according to manufacturer's recommended reaction conditions.

3.2.5 *Mutagenic primer design*

All site-directed mutagenesis primers were designed using the software, DS Gene (Accelrys, San Diego, CA). All primers were synthesized by Sigma Genosys (Sigma-Aldrich, Oakville, ON). Concentrated primer stocks were re-suspended in 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA, and diluted in ddH₂O to a 10 μ M working concentration. The mutagenic primer sequences are summarized in Table 3.1.

3.2.6 *Site-directed mutagenesis of each IHH variant*

Each mutation was introduced to the pBSIhhWT construct using specific mutagenesis primers detailed in section 2.2.3. Site-directed mutagenesis was employed using the Quikchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), following explicitly the manufacturer's recommended protocol.

3.2.7 *Confirmation of mutations by ABI sequencing*

Successful introduction of the following mutations; E95K, D100N, E131K and V190A, was confirmed via sequencing of the corresponding pcDNAIhh construct. Each construct was sequenced using the T7 promoter primer (5'-TAATACGACTCACTATAGGG-3') from the forward direction and the Sp6 promoter

Table 3.1. Primers for site-directed mutagenesis. The table of primers listed in ascending order of the position of the mutated residue. All primers are listed as 5' to 3' as read left to right. The mutated nucleotide is highlighted in red.

Primer Name	Primer Sequence
P46L	P46L - For P46L - Rev
E95K	E95K - For E95K - Rev
D100N	D100N - For D100N - Rev
R128Q	R128Q - For R128Q - Rev
E131K	E131K - For E131K - Rev
V190A	V190A - For V190A - Rev

primer (5'-TCGACATTTAGGTGACACTATAGAA-3') from the reverse direction, using the BigDye™ v3.1 terminator cycle sequencing kit (Applied Biosystems, Foster City, California) according to the manufacturer's suggested protocol. Sequencing products were evaluated on an Applied Biosystems 3130x1 genetic analyzer.

3.2.8 *Confirmation of mutations by restriction digest*

Successful introduction of the P46L and R128Q mutations was confirmed via restriction digest. Restriction digest of the pBSIhhP46L construct was performed in 20 µL volumes, containing 0.5 µg of the pcDNAIhhP46L construct, 2.0 µL of 10X NEB #2 Buffer, and 1.0 µL of *Bsr*BI. The reaction was incubated at 37°C in a MBS Satellite 0.2G thermal cycler (Thermo Electron Corporation, Milford, MA) for 1.5 hrs. The digested products were loaded on a 1.0% agarose gel containing ethidium bromide, and electrophoresed for 25 min at 120V and photographed under UV light. Similarly, to confirm introduction of the R128Q mutation, restriction digestion of the pBSIhhR128Q construct was performed in 20 µL volumes, containing 0.5 µg of the pBSIhhR128Q construct, 2.0 µL of 10X NEB #3 Buffer, and 1.0 µL of *Pst*I. The reaction was incubated at 37°C in a MBS Satellite 0.2G thermal cycler (Thermo Electron Corporation, Milford, MA) for 1 hr. The digested products were loaded on a 1.0% agarose gel containing ethidium bromide, and electrophoresed for 25 mins at 120V and photographed under UV light.

3.2.9 Sub-cloning *IHH* variants in *pcDNA3.1(+)*

Initially, the full length *IHH* cDNA was excised from the pBSIhhWT construct by a restriction digest performed in 20 μ L volumes containing 500 ng of either pBSIhhWT or pcDNA3.1(+), 2.0 μ L of 10X NEB #4 Buffer, 1 μ L of *Apa*I, 1 μ L of *Bam*HI, in ddH₂O. The reaction was incubated at 25°C for 1.5 hrs, then at 37°C for 1.5 hrs, in an MBS Satellite 0.2G thermal cycler (Thermo Electron Corporation, Milford, MA). The digested products were purified using the GE Healthcare illustra GFX PCR DNA and Gel band purification kit (GE Healthcare, Buckinghamshire, UK) according to manufacturer's specifications. The concentration of the purified digested products was determined by running a sample of each on a 1.0% agarose gel for 25 minutes at 100V. The gel was photographed under UV light and the concentration of each sample was calculated based on comparison of band intensity with bands of the HighRanger Plus 100bp DNA ladder (Norgen Biotech, Thorold, ON). A four-fold stoichiometric excess of insert (*IHH* cDNA) in comparison to the vector (pcDNA3.1) were ligated in a 30 μ L reaction containing 3.0 μ L of the 10X ligase buffer, and 1.0 μ L of 1U/ μ L T4 DNA ligase (both from Invitrogen, Carlsbad, CA). The reaction was incubated at 16°C in the MBS Satellite 0.2G thermal cycler (Thermo Electron Corporation, Milford, MA) for 16 hours.

With the exception of the pcDNAIhhR128Q construct, which was prepared in a manner exactly the same as that listed above for the pcDNAIhhWT construct, the remaining mutant pcDNA3.1 constructs were prepared by digesting both the respective mutant pBSIhh construct and pcDNAIhhWT with both *Bam*HI and *Pst*I. The reactions were prepared in 30 μ L volumes, containing 500 ng of either of pcDNAIhhWT or one of the mutated pBSIhh constructs, 3 μ L of 10X Amersham One-Phor-All buffer (GE

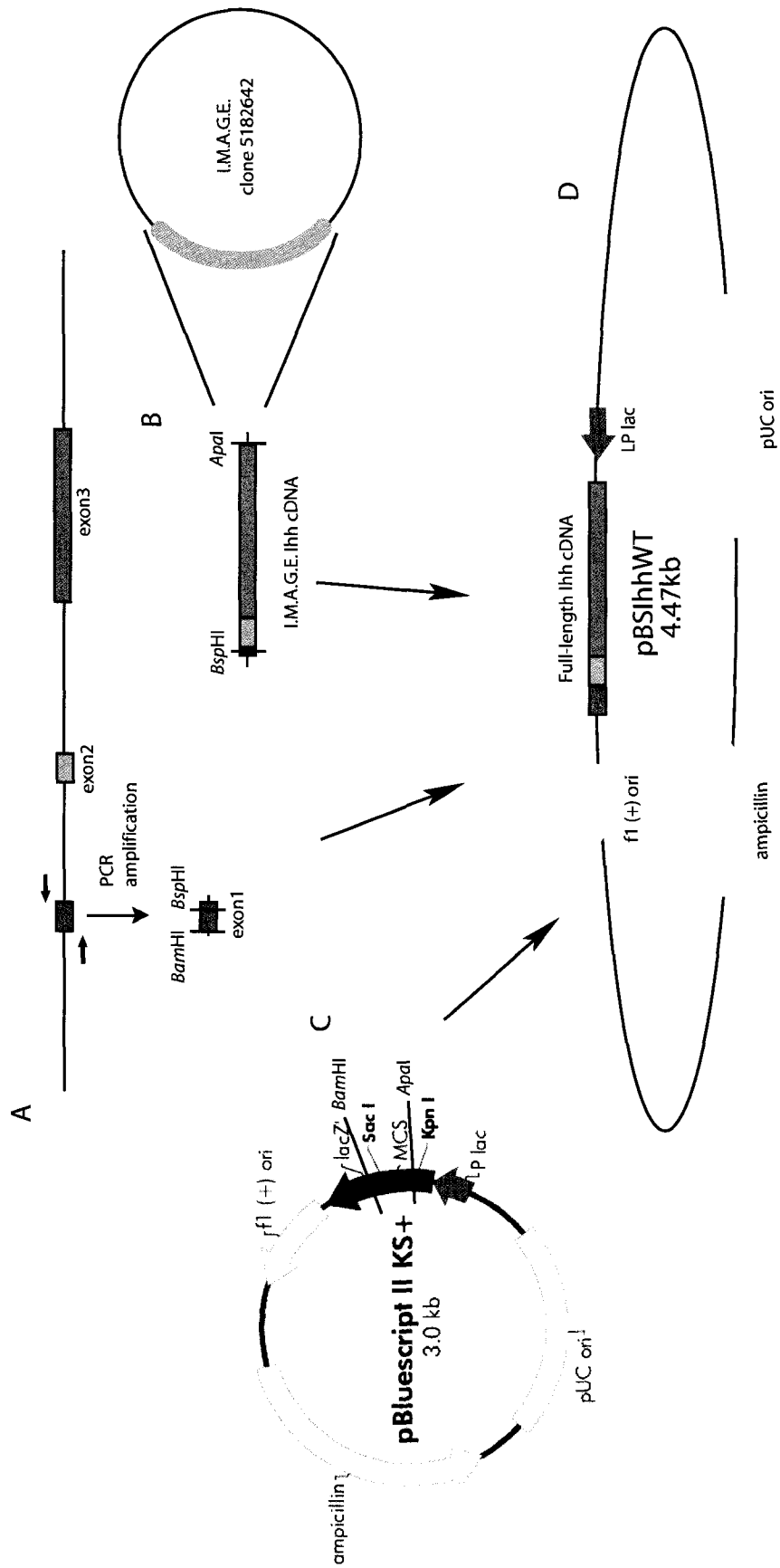
Healthcare, Buckinghamshire, UK), 1 μ L *Bam*HI, and 1 μ L *Pst*I, all in ddH₂O. The reactions were incubated at 37°C for 1.5 hrs in an MBS Satellite 0.2G thermal cycler (Thermo Electron Corporation, Milford, MA). The digested products were purified and ligated according to previously detailed protocols.

3.3 Results

3.3.1 Production of *pBSIhhWT*

The IMAGE clone, obtained from Open Biosystems (Open Biosystems, Huntsville, AL), contained a portion of the 3' end of the first exon, followed by exons two and three. Therefore, the first exon as well as a portion of the 5' UTR had to be amplified from a genomic sample of human DNA. The complete sequence of *Ihh* and flanking regions was downloaded from the NCBI database and all primers and sequence alignments were based on this genomic sequence. A product of 400 bp, stretching from just upstream of the ATG start site of *IHH* to exon 2 was amplified by PCR. The IMAGE clone was digested, with both *Bsp*HI and *Apa*I, while the amplified region spanning exon1 was digested with both *Bsp*HI and *Bam*HI. The digested products, in accordance with the plan illustrated in figure 3.1, were purified by gel purification and subcloned into pBluescriptII KS+, digested with *Bam*HI and *Apa*I, in a three-part ligation. The resultant construct was named *pBSIhhWT*. Successful production of the full-length *IHH* cDNA and its subsequent sub-cloning in pBluescriptII KS+, was confirmed via restriction digest with *Bsp*HI. When digested with *Bsp*HI, pBluescriptII produces two bands, one of 1969 bp and one of 1008 bp, while the digestion of *pBSIhhWT* produces

Figure 3.1. Schematic representation of pBSIhhWT cloning plan. A portion of exon1 was amplified by PCR and digested with *Bam*HI and *Bsp*HI (A) . The *IHH* cDNA from the IMAGE clone (clone #5182642) was excised by restriction digest with *Bsp*HI and *Apa*I (B). At the same time, pBluescript II KS+ was digested with *Bam*HI and *Apa*I (C). The three pieces were ligated in a single reaction to produce the pBSIhhWT construct (D).



three bands of 2383 bp, 1079 bp, and 1008 bp. An agarose gel was used to visualize the digested products and to screen for positive clones (figure 3.2).

3.3.2 *Successful introduction of BDA1- and ACFD-causing mutations into pBSIhhWT*

Site-directed mutagenesis was employed to introduce the BDA1-causing mutations in *IHH*, E95K, D100N, R128Q, E131K, and the ACFD-associated mutations, P46L and V190A. The resultant constructs were named, pBSIhhE95K, pBSIhhD100N, pBSIhhR128Q, pBSIhhE131K, pBSIhhP46L and pBSIhhV190A, respectively. For each mutation, a set of reverse complementing mutagenic primers, each containing the corresponding single-nucleotide polymorphism were developed and the reactions were carried out using the Stratagene Quikchange Site-Directed Mutagenesis Kit.

Confirmation of the introduced mutations was conducted via different mechanisms. The E95K, D100N, and E131K mutations were confirmed via ABI sequencing of the corresponding pcDNAIhh construct using the T7 forward primer. We opted to sequence the pcDNA-based construct, as we were having a significant amount of difficulty sequencing the pBluescript-based constructs. The V190A mutation was confirmed via sequencing of the pcDNAIhhV190A construct with the reverse Sp6 primer. Figure 3.3 illustrates the chromatograms covering the region immediately surrounding each mutated site in comparison to the chromatograms of the same region from ABI sequencing of the pcDNAIhhWT construct using either the T7 or Sp6 primer. The full sequence of the *IHH* cDNA was also analyzed by ABI sequencing of the pcDNAIhhWT construct using both the T7 and Sp6 primers, and was compared to the

Figure 3.2. Confirmation of pBSIhhWT production by restriction digest. When digested by *Bsp*HI, pBluescript produces two bands of 1969 bp and 1008 bp (lane 2). The successful introduction of the full *IHH* cDNA introduces a new *Bsp*HI cut site and results in the production of three bands of 2383 bp, 1079 bp, and 1008 bp (lane 3). These digested products were compared against the HighRanger plus 100bp DNA Ladder (lane 1). The sizes of each band in the DNA ladder are indicated.

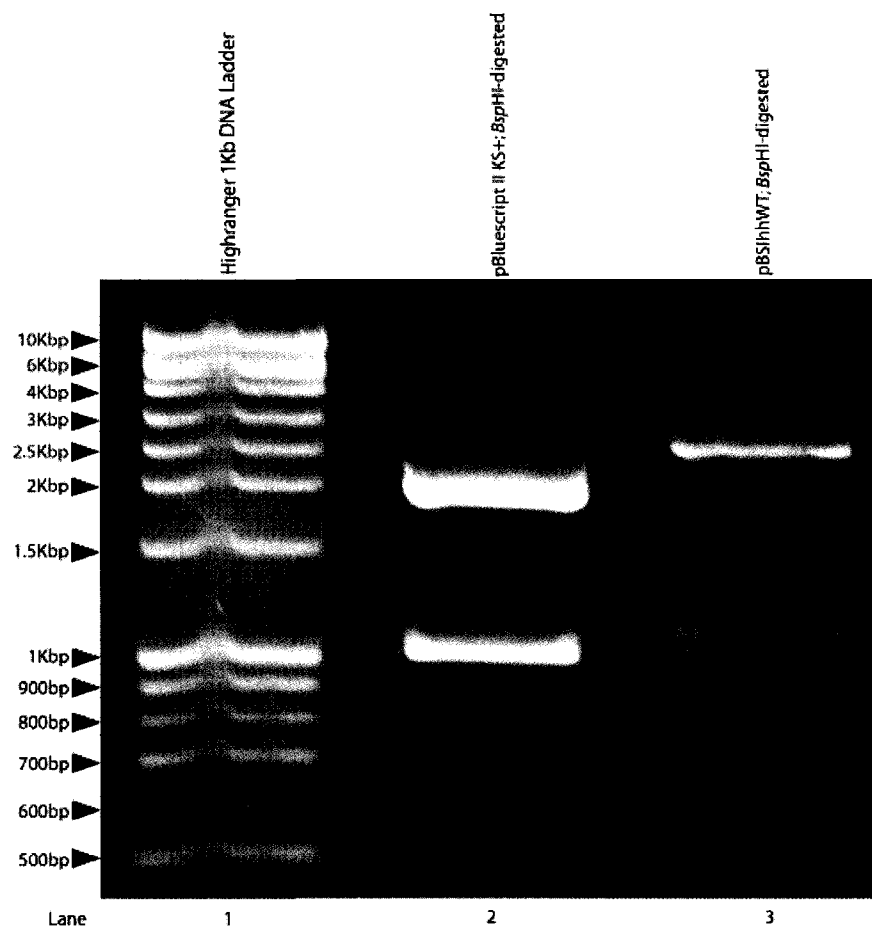
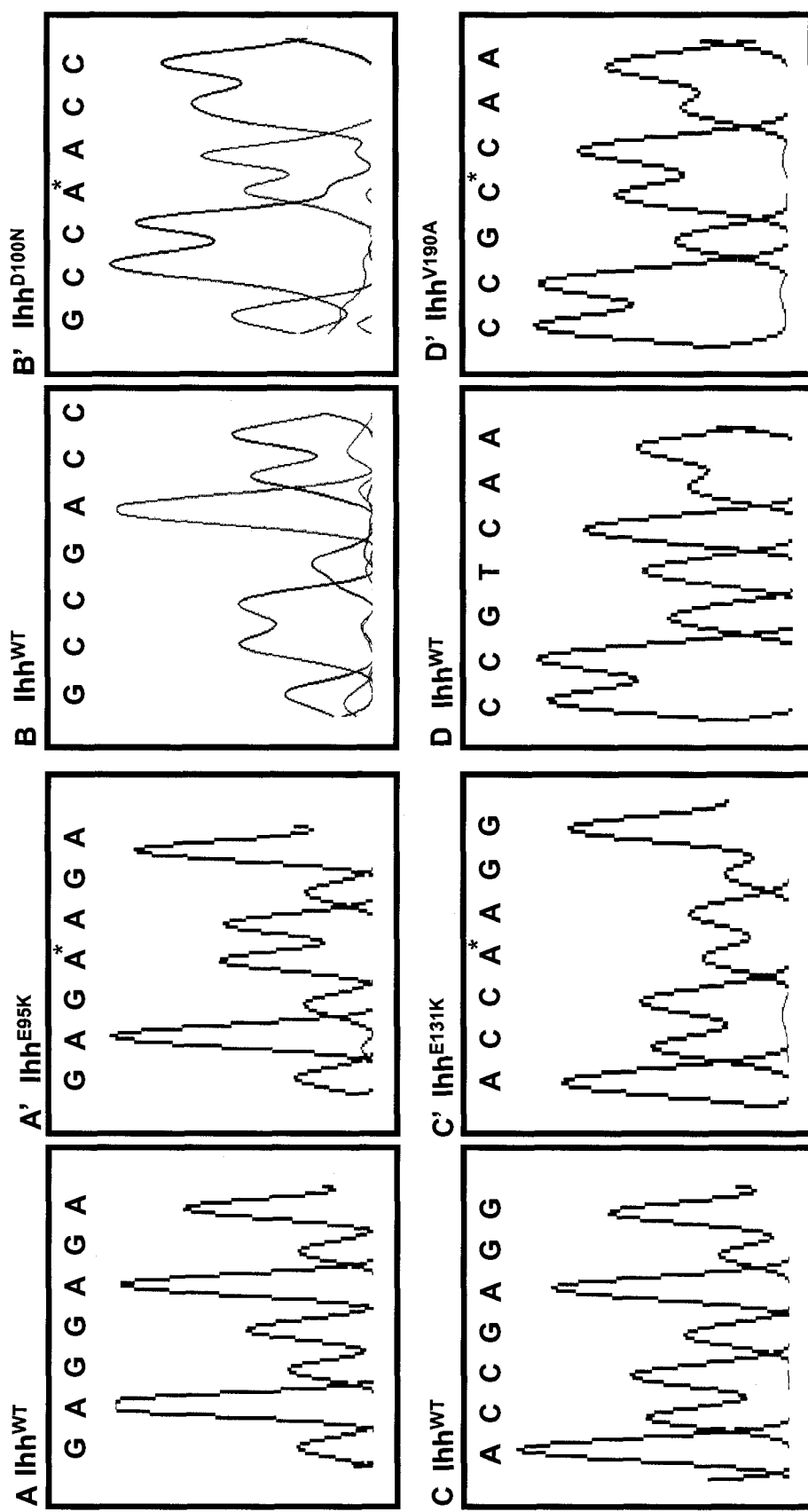


Figure 3.3. Confirmation of production of E95K, D100N, E131K and V190A mutants by ABI sequencing. Illustrated are excerpts from the chromatograms obtained by ABI sequencing of the indicated mutant construct. The location of each wild-type nucleotide sequence (A, B, C, D) is compared to the same locations in each of mutant pBSIhhWT construct, confirming the successful introduction of the E95K (A'), D100N (B'), E131K (C'), and V190A (D') mutations. The mutated nucleotide in each case is indicated by a *.



GenBank sequence (Accession number NM_002181) of the *IHH* message, to ensure that there were no additional mutations introduced to the sequence.

The introduction of the P46L and R128Q mutations were confirmed via restriction digest of pBSIhhP46L and pBSIhhR128Q with *Bsr*BI and *Pst*I, respectively. The digestion of pBSIhhWT produces bands of 1801 bp, 682 bp, 667 bp, 545 bp, 241 bp, 173 bp and 157 bp. The introduction of the P46L mutation eliminates a *Bsr*BI cut site, producing products of 1801 bp, 839 bp, 667 bp, 545 bp, 241 bp and 173 bp. Figure 3.4 illustrates a 1.5% agarose gel, on which were run the digested products of both pBSIhhWT and a selected clone of pBSIhhP46L. Our pBSIhhWT construct contains only one cut site for *Pst*I at position 1632 of the *IHH* cDNA, therefore when cut with *Pst*I, pBSIhhWT produces one 4470 bp band. The introduction of the R128Q mutation introduces an additional *Pst*I cut site, henceforth, when cut with *Pst*I, pBSIhhR128Q produces one band of 3916 bp and one of 554 bp. The digested products of both constructs were run on a 1.5% agarose gel, which is illustrated in figure 3.5.

3.4 Discussion

Prior to this study, we were unable to uncover any study, which functionally characterized the properties of Indian hedgehog nor any of its respective, naturally occurring mutations in humans. However, at least one group had characterized various HSPE-causing mutations in *Sonic hedgehog* [82]. These studies analyzed the processing of the *SHH* mutations *in vitro*, as well as the activity of the protein employing a hedgehog responsive reporter cell line. It was our intention to follow a similar type of

Figure 3.4. Confirmation of successful introduction of P46L mutation by restriction digest. When digested by *Bsr*BI, pBSIhhWT produces the banding pattern visible in lane 3. The successful introduction of the P46L mutation removes a *Bsr*BI site and the 157 bp band and the 682 bp band combine to form a 839 bp band visible in lane 4. Lane 5 illustrates a colony from which a clone was obtained that did not contain the mutation. Lane 2 illustrates the banding pattern of pBluescript when it is digested by *Bsr*BI. The banding pattern of each was compared to the HighRanger 100bp DNA ladder (lane 1).

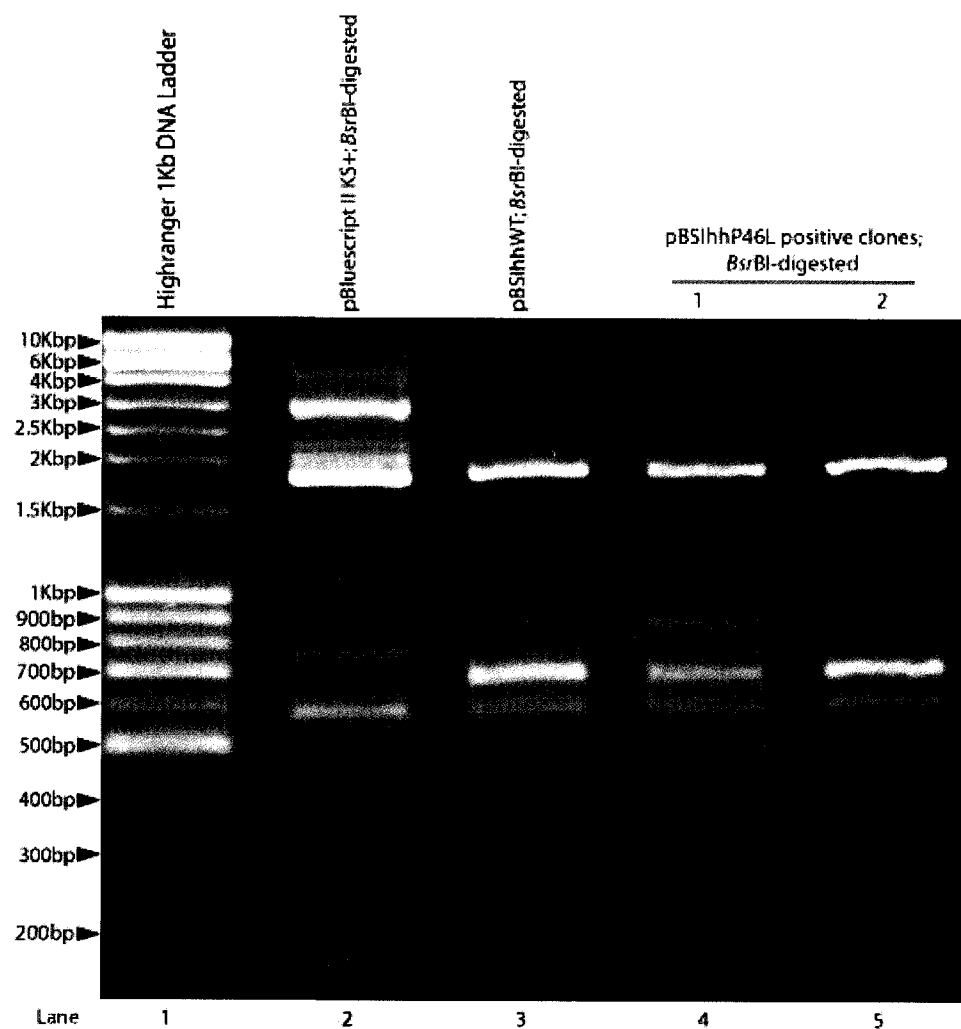
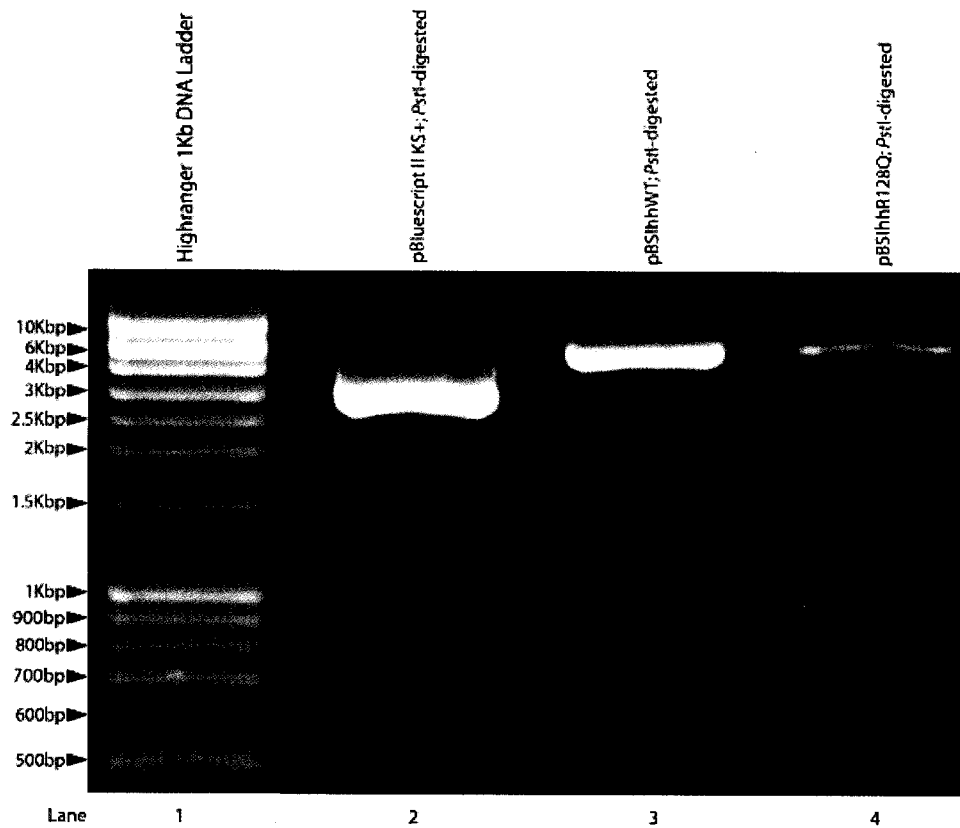


Figure 3.5. Confirmation of successful introduction of R128Q mutation by restriction digest. The pBluescript II KS+ plasmid vector does not contain a *Bsr*BI cut site and thus is not cut by the restriction enzyme (lane 1). When the pBSIhhWT construct is digested by *Bsr*BI, it produces one band of 4470 bp (lane 3). The successful production of the R128Q mutation introduces a new *Bsr*BI site. The banding pattern of the resultant pBSIhhR128Q construct produces two bands of 3916 bp and 554 bp. This banding pattern visible in lane 4 confirms the successful introduction of the mutation. Each was compared to the HighRanger 100bp DNA ladder (lane 1).



approach to characterize the BDA1- and ACFD-causing mutations in *IHH*, both *in vitro* and *in vivo*. However, in order to conduct this analysis, we first had to develop a full-length copy of *IHH* cDNA.

At the outset of this project, no full-length cDNA of *IHH* was in existence. Therefore, it was our primary goal to design a full-length cDNA of *IHH* that could be used to generate constructs that would express each of the various BDA1- and ACFD-causing mutations.

We obtained the IMAGE clone of *IHH*, which spanned a small portion of exon 1, and all of exons 2 and 3. This clone existed largely to serve as a means to generate short-sequence oligonucleotides that could be used to probe for *IHH* message in an expression library. However, without exon 1, which contained the transcriptional start site, the IMAGE construct was completely ineffective as an expression vector. The full-length cDNA that was subsequently developed and sub-cloned into pBluescript II KS+ contained no introns. All of the BDA1- and ACFD-associated mutations are exonic missense mutations.

We chose to use pcDNA3.1+ as our expression vector because of the high level of expression of our gene, inserted downstream of the human cytomegalovirus (CMV) immediate-early promoter. Also, pcDNA3.1+ contains the SV40 early promoter and origin of replication, which facilitates efficient episomal recombination in cells expressing the SV40 large T antigen, such as our chosen line of HEK293 cells [83]. This approach ensured high levels of the episomal plasmid in the cell and the highest level of *IHH* expression in our stably-transfected cell lines, and thus, theoretically, the greatest amount of recombinant protein recovered. Furthermore, HEK293 cells have been used

previously to analyze the processing and activity of hedgehogs [82], as well as immunohistochemistry of various other sub-cellular molecular pathways [84]. As it was our purpose to analyze the processing and secretion of IHH-N, and collect IHH-N from an *in vitro* model in order to test its activity, we concluded that it was in our best interest to use this previously tested model. Having successfully created the first full-length cDNA clone of *IHH*, developed efficient expression vectors to produce the recombinant protein, and successfully developed additional constructs possessing one of the BDA1 or ACFD-causing mutations, we were perfectly placed to move forward with our *in vitro* and *in vivo* characterization of both mutant and wild-type *Indian Hedgehog*.

However, as is often the case, in scientific research, it can be difficult to account for unforeseen human error. It was clear that site-directed mutagenesis had produced a pBSIhhP46L clone that contained the P46L mutation (figure 3.4). And yet, when Kelly Patterson began her research she started first with re-sequencing all of the constructs I had generated. It was discovered that our stock of pcDNAIhhP46L contained no variations in sequence from the pcDNAIhhWT construct. In the fourth lane in figure 3.4 the 839 bp band is, in fact, less intense than the 682 bp band that follows it. This would indicate that the sample that was digested and loaded was not a pure sample of pBSIhhP46L. A pure sample of pBSIhhP46L would yield equal stoichiometric amounts of the 839 and 682 bp bands, and the larger band should contain more EtBr and thus have a greater intensity. Therefore, it is likely that the sample is contaminated with pBSIhhWT – a result of faulty clone selection. Therefore, it is plausible that in the development of the stable cell lines a resistant cell that was stably transfected with pcDNAIhhWT was the one that was selected. A theory supported when I analyzed the

episomal plasmid isolated from the HEK(IHH^{P46L}) cell line by ABI sequencing. This sample did not contain the mutation. Therefore, throughout the remainder of this thesis, the data for the Ihh^{P46L} mutation will be included in the calculations as an additional IHH^{WT} sample.

Chapter 4

***In Vitro* chracterization of BDA1- and ACFD-associated mutations in the human *Indian hedgehog* gene**

4.1 Introduction

Very few studies have been conducted to analyze the properties of mutant Hedgehog proteins and how they compare to their wild-type counterparts. A great deal of information about the protein can be learned from analyzing the mutants – crucial conserved regions of the protein, important domains that confer a specific function, how these mutated regions eventually lead to the development of the disease state. Two such studies conducted by Goetz *et. al.*, and Traiffort *et. al.*, have shown how the cephalic disorder, holoprosencephaly (HPE) can arise from mutant SHH that undergoes altered autoproteolytic cleavage [24, 82]. These groups have conclusively revealed a series of residues that are essential in the post-translational addition of palmitate, and how that modification is crucial in the secretion of soluble SHH-multimers [24]. As of yet, no group has undertaken the task of characterizing mutations to the human *Indian Hedgehog* gene.

Various methods have been used to analyze the mechanisms of the Hedgehog signalling pathway. Hedgehog-responsive reporter cell lines such as C3H10T1/2 and Shh-LIGHT2 are commonly used, as each is a rather simple, consistent, and accurate system of measuring Hedgehog activity. For example, the Shh-LIGHT2 cell line is a line of NIH 3T3 cells (endogenously expressing key players in the Hedgehog pathway) that has been stably transfected with two constructs: one constitutively expressing *Renilla* luciferase at a moderate level, and the other with *Firefly* luciferase cloned under the control of the Gli-promoter [85]. Therefore when active hedgehog is added to the media, hedgehog binds to PTCH and induces, through the cascade, the conformational change in Gli1 and Gli2 to activator forms, which then drive the expression of *Firefly* luciferase, the

level of which, as measured by luminometer, can be measured against the level of *Renilla* luciferase as a method of controlling for cell number and viability. In this study we opted to use the Shh-LIGHT2 cell line as one part of our *in vitro* model.

In addition to looking at general activity levels of the mutant vs. wild-type proteins, we also wanted to look at how these proteins behave in a model that more accurately reflects endochondral ossification. For this, we chose the mouse embryonic carcinoma cell line, ATDC5. These cells represent a clone of chondroprogenitor cells that mimic undifferentiated mesenchymal tissue. However, differentiation of these cells to chondrocytes can be induced through an insulin-dependent pathway that very closely mimics that of endochondral ossification *in vivo* [86, 87]. Through this differentiation process various markers and key players of ossification networks are expressed: IHH, PTHrP, FGFs, and BMPs [88]. One simple way to gauge the degree of differentiation is by staining the cells with alcian blue, a stain that targets glycosaminoglycans (GAGs). The emergence of GAGs, long unbranched polysaccharide groups, in differentiating ATDC5 cells typically indicates the expression of collagens type II and type X, to which, GAGs are covalently linked in large quantities [89, 90]. It is known that IHH plays a significant role in this process, as its expression spikes at 8 days into the 28 day differentiation process [88].

It was my intention to examine the processing, secretion and activity of mutant and wild-type IHH proteins. We developed several lines of stably transfected HEK293 cells expressing one of the *IHH* mutant variants or wild-type. Media from these cells was collected, concentrated and the amount of IHH protein in each sample was quantified by densitometry. We initially hypothesized that, similar to what was seen with the HPE-

associated mutations in Shh, some or several of the mutants would display a similar type of abrogation, or complete elimination, of the auto-catalytic cleavage of the C-terminal end of the IHH precursor protein [82]. With regards to both of the *in vitro* models we examined, LIGHT2 and differentiating ATDC5, we treated the cells with 500ng/ μ L of one of either the mutant variants, or the wild-type. We also wanted to analyze how signalling may differ between cells treated with IHH^{WT} only and those treated with a combination of IHH^{WT} and one of the BDA1 mutant proteins; as BDA1 is an autosomal dominant disorder [4, 15, 58]. Initially, based on evidence provided by Fuse *et. al.* [60] and Pepinsky *et. al.* [91] that suggests that the Glu95, Asp100, and Glu131 residues reside in a groove of the protein that is responsible for Ptch binding, we opted to treat these cells with equal molar quantities of IHH^{WT} and one of the BDA1-associated mutants to: 1) More accurately mimic the stoichiometric ratio of mutant:wild-type IHH that would be present in the growth plate, assuming normal levels of secretion of mutant IHH-N, and 2) more accurately assess the difference between the activity levels of those cells treated with either IHH^{WT} alone or a combination of IHH^{WT} and one BDA1 mutant.

4.2 Materials and Methods

4.2.1 Tissue culture reagents and conditions

A line of human embryonic kidney (HEK) 293 (referenced herein as HEK293) cells was generously donated by Dr. Robin Parks, Ottawa Health Research Institute (OHRI). These cells were maintained in minimal essential media (MEM) with 1% Gibco GlutaMAX (Glu)(Invitrogen, Carlsbad, CA), 1% antibiotic/antimycotic (ab/am)

(Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Carlsbad, CA). A line the chondroprogenitor cells, ATDC5 (ATCC, Manassas, VA) was maintained in Dulbecco's Modified Eagle Medium (DMEM)/F-12 HAM nutrient mixture (1:1) with 1% Gibco GlutaMAX (Invitrogen, Carlsbad, CA), 1% ab/am (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Carlsbad, CA). The Shh-LIGHT2 cell line was obtained from Dr. Valerie Wallace (OHRI). The LIGHT2 cells were maintained in DMEM containing 1% GlutaMAX, 1% ab/am, 17 mM sodium bicarbonate, 25 mM glucose, and 10% FBS, supplemented with 0.4 mg/mL G-418. All cell lines were maintained at a temperature of 37°C and 5% CO₂.

4.2.2 *Development of stably-transfected line of HEK293 cells expressing pcDNAIhh constructs*

A frozen stock of HEK293 cells was plated on a 10 cm plate (Corning Life Science, Corning, NY). 2.0×10^5 of these second generation cells were used to seed a 35 mm plate (Corning Life Science, Corning, NY) and allowed to reach approximately 60% confluency. Two µg of each of the pcDNAIhh constructs were added to separate 100 µL of Gibco Opti-Mem®I (Invitrogen, Carlsbad, CA), to which, 10 µL of Superfect® Transfection Reagent (Qiagen Corp., Valencia, CA) was added, mixed and incubated at room temperature for 10 minutes. Six hundred µL of growth media (MEM/1% Glu/1% ab/am/10% FBS) were added to this mixture, which was then transferred to a 35 mm dish containing washed HEK293 cells. The transfection reaction was incubated on the cells for 2-3 hrs, at which point the reaction mixture was removed, the cells washed once with PBS, and fresh growth media added. Twenty-four hours post-transfection, the cells were

passed to a 10 cm plate (Corning Life Science, Corning, NY) and maintained in growth media supplemented with 2 μ M Ganciclovir (Sigma-Aldrich, Oakville, ON) and 400 μ g/mL of G-418 (Sigma-Aldrich, Oakville, ON). The first four days in selection media saw significant death of non-resistant colonies. Between days 7 and 10, large colonies began to develop and become visible. These colonies were isolated and cultured in 24-well tissue culture plate (Falcon, BD Biosciences, San Jose, CA). As each individual cell line, expressing one of the *IHH* clones, reached 60% confluency, it was passaged in 10 cm tissue culture plates (Corning Life Science, Corning, NY). One plate was maintained and passed for additional assays, while the other was frozen for long term storage in liquid nitrogen in 10% DMSO and 20% FBS in MEM. Each cell line was given the name of HEK(IHH*) where '*' denotes the variant identity; ie. HEK(IHH^{WT}) or HEK(IHH^{E95K}).

4.2.3 *Western blotting techniques employed to confirm expression of IHH and analyze processing in vitro*

4.2.3.1 *Concentration of HEK(IHH) media*

The media from all plates of each stable cell line was collected and any particulate matter removed via low speed centrifugation using the Allegra X-15R Centrifuge (Beckman Coulter, Fullerton, CA). The media was then concentrated from a 45 mL total volume to approximately 500 μ L using Amicon Ultra Centrifugal Filter Devices with a 10kDa filtration limit (Milipore, Billerica, MA). Total protein was collected from the

stably-transfected HEK293 cell lysates using a RIPA buffer (50 mM TrisHCl pH7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS. All cell lysates were stored at -20°C until required.

4.2.3.2 *Polyacrylamide gel electrophoresis*

Concentration of total protein in the cell lysates was determined by Bradford assay, using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Hercules, CA). Protein samples to be analyzed were combined with 5X Laemmli Running Buffer to a final (1X) concentration of 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 0.005% bromphenol blue, 5% β -mercaptoethanol, and 10% glycerol. These samples were heated to 95°C for 10 minutes using the MBS Satellite 0.2G thermal cycler (Thermo Electron Corporation, Milford, MA). This sample was subsequently loaded into each well of a NuPAGE 4-12% Bis-Tris Gel (Invitrogen, Carlsbad, CA) and subjected to electrophoresis at 200V for 45 minutes in a X-Cell SureLock Mini-Cell (Invitrogen, Carlsbad, CA). The running buffer used contained 50 mM MOPS, 50 mM Tris-HCl, 0.8 mM EDTA, and 0.1% SDS. The protein was transferred to Immunoblot polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA) using a Mini Trans-Blot Cell (Bio-Rad Laboratories, Hercules, CA) in a buffer consisting of 48 mM Tris-HCl, 39 mM Glycine, 0.1% SDS, and 20% methanol, set at a constant voltage of 100V for one hour.

4.2.3.3 *Western Blot*

PVDF membranes containing transferred proteins were blocked in 5% milk in 1X Tris-Borate Saline Tween-20 (TBST) containing 10 mM Tris-HCl pH 8.0, 0.15 M NaCl, 0.05% Tween-20, at room temperature, shaking, for 1 hr. The membranes were probed with rabbit polyclonal α -SHH-N (H-160) (Santa Cruz, Santa Cruz, CA) primary antibody in 5% milk in 1X TBST (at varying concentrations ranging from 1:100 – 1:500). These membranes were left to incubate overnight, shaking, at 4°C. The membranes were then washed three consecutive times of 15 minutes at room temperature with TBST prior to and after being probed with a horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000)(Bethyl Laboratories, Montgomery, TX) in 5% milk in TBST for 1 hr at room temperature. Detection of the secondary antibody was performed with the Amersham ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK). The treated membranes, covered in saran wrap were exposed to Amersham HyperfilmTM MP (GE Healthcare, Buckinghamshire, UK) for a variety of times. The film was developed using the M35A X-OMAT Processor (Kodak, Rochester, NY).

4.2.3.4 *Quantification of IHH by densitometry*

The intensity of the 21 kDa band on an autoradiograph of western blot of concentrated media samples was normalized and an SDS-PAGE gel prepared to run 1 μ L, 10 μ L, 10 μ L, 15 μ L, 5 μ L, and 10 μ L of media (concentrated 60X) from stable HEK293 cells expressing IHH^{WT}, IHH^{E95K}, IHH^{D100N}, IHH^{R128Q}, IHH^{E131K}, and IHHV^{190A}, respectively. These samples were electrophoresed alongside a 15 ng aliquot of recombinant mouse SHH-N, donated generously by Dr. Valerie Wallace (OHRI). A photograph of the resulting autoradiograph was taken and the concentration of IHH-N in

each sample was calculated by densitometry in comparison to the intensity of the band of known quantity of Shh, using the program Alpha Imager (Alpha Innotech Corp., San Leandro, CA).

4.2.4 *Transient transfection of HEK293 cells with pcDNAIhh constructs*

A frozen stock of HEK293 cells was plated on a 10 cm plate (Corning Life Science, Corning, NY) and 1.0×10^4 of these second generation cells were used to seed each well of a Culture Slide (Falcon, BD Biosciences, San Jose, CA), where they were permitted to reach approximately 60% confluency. Two hundred and fifty ng of each of the pcDNAIhh constructs were added to separate 66 μ L of Gibco Opti-Mem®I (Invitrogen, Carlsbad, CA), to which, 5 μ L of Superfect® Transfection Reagent (Qiagen Corp., Valencia, CA) was added, mixed and incubated at room temperature for 10 minutes. 300 μ L of growth media (MEM/1% Glu/1% ab/am/10% FBS) was transferred to each well of the culture slide containing the HEK293 cells, and to this, the transfection reaction mixture was added. The transfection reaction was incubated on the cells for 24 hrs.

4.2.5 *Immunohistochemistry of transiently-transfected HEK293 cells*

After the 24 hr, the transfection reaction mixture was removed, the cells washed once with PBS and 200 μ L of 2% paraformaldehyde (PFA) was added to each well, and incubated at room temperature for 10 mins. The cells were then washed twice with 1X phosphate-buffered saline (PBS). At this point, 200 μ L of blocking solution (10% horse serum, 0.4% Triton X-100 in 1X PBS) was added to each well and incubated at RT for 1

hr. The blocking solution was removed and the cells treated with a combination of mouse monoclonal α -KDEL (Abcam, Cambridge, MA) and rabbit polyclonal α -SHH-N (H-160) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at concentrations varying from 1:100 – 1:300, in 10% horse serum in 1X PBS. The culture slides were then placed, uncovered, inside a humidified container and incubated for 16 hrs at 4°C. The primary antibody solution was removed and the cells were incubated in 1X PBS at RT for 10 mins, three times. The cells were then incubated in solution of 2% horse serum in 1X PBS, containing a mixture of Alexa Fluor 594 donkey α -mouse IgG (Invitrogen, Carlsbad, CA) and Alexa Fluor 488 donkey α -rabbit IgG (Invitrogen, Carlsbad, CA) at concentrations varying from 1:1000 – 1:2000, in a humidified container, shielded from light, at RT for 1.5 hr. The cells were then washed three times, in the dark, with 1X PBS, 75 μ L of 4',6-diamidino-2-phenylindole (DAPI) was then administered to each of the wells, and the cells were incubated in the dark for 2.5 min. The cells were then washed twice with 1X PBS, the chamber was removed, the wells were left to the air to dry (in the dark) and then 1-2 drops of Fluorescent mounting medium (Dako Cytomation, Glostrup, Denmark) was added to each well, and a 24mmX50mm coverslip was used to seal the slide. The cells were visualized using Zeiss AxioImager.M1 (Carl Zeiss MicroImaging, Inc., Thornwood, NY), using the program AxioVision – AxioVs40V4.6.1:0. (Carl Zeiss MicroImaging, Inc., Thornwood, NY).

4.2.6 *Shh-LIGHT2 luciferase reporter assay*

3.0×10^4 Shh-LIGHT2 cells were used to seed each well of a 96-well plate (Corning Life Science, Corning, NY) and permitted to grow to confluency in the medium

and conditions described in 3.2.1. Once confluent, the media was aspirated, the cells washed with sterile PBS, and 100 μ L of fresh growth media conditioned with 0.5 μ g/mL of one IHH-N variant (suspended in concentrated media collected from one of the stably transfected lines of HEK293) were added to each well. After 28 hours, the media was aspirated and the cells were lysed by the addition of 30 μ L of Passive Lysis Buffer (PLB) supplied with the Dual-Luciferase® Reporter 1000 Assay System (Promega Corp., Madison, WI). The PLB was incubated on the cells at 4°C, shaking, for 45 mins. The lysed cells, suspended in PLB, from each well were transferred to separate 5 mL Polystyrene Round-Bottom Tubes (Falcon, BD Biosciences, San Jose, CA). Using the Lumat LB 9507 luminometer (EG&G Berthold, Bundoora, VIC, Australia), 100 μ L of Luciferase Assay Reagent (LARII)(Promega Corp., Madison, WI) were injected, followed by a 2.0 sec delay in measurement and a 15.0 sec measurement of *Firefly* luciferase expression. This was followed by the injection of 100 μ L of freshly prepared 1X Stop&Glo® Substrate (Promega Corp., Madison, WI), a 2.0 sec delay in measurement, and a 15.0 sec measurement of *Renilla* luciferase expression.

4.2.7 Differentiation of ATDC5 Cells

Initially, we seeded several 6-well plates (Falcon, BD Biosciences, San Jose, CA) with ATDC5 cells at a cell density of 2.0×10^5 cells/well. We allowed these cells to grow to confluency, at which point we began to culture the cells in differentiating media consisting of DMEM/F-12 HAM nutrient mixture (1:1), 5% FBS, 1% ab/am, 1% Glu, 10 μ g/mL bovine insulin, 3.0×10^{-3} mM sodium selenite. The media would be aspirated, the cells washed and fresh differentiating media added every two days.

4.2.8 *Differentiating ATDC5 cell assay*

Eight days after administration of the differentiating media to the cells, the media was aspirated, the cells washed with 1X PBS, and 2 mL of fresh differentiating media conditioned with 0.5 µg/mL of one IHH-N variant (suspended in concentrated media collected from one of the stably transfected lines of HEK293) were added to each well. The cells were left to incubate under these conditions for six days.

After the five day incubation, the media was aspirated and the cells washed with 1X PBS. 500 µL of 2% PFA was added to each well, and incubated at room temperature for five min. The PFA was discarded, and the cells were treated with 1 mL of 1% Alcian Blue solution in 3% acetic acid, pH 2.5 at room temperature for 30 min. The stain was collected and discarded and the cells were washed twice with ddH₂O. At this point, 100 µL of ddH₂O were added to each well and the cells were photographed using the Axiovert 5100 microscope (Zaner IVF Inc., Vero Beach, FL). Each well contained a single treatment group, and duplicates of each sample group were examined. Three random images were taken per well.

In order to quantify the amount of positive alcian blue staining, the percentage of cells stained per image was calculated. Each picture was analyzed using the ImageJ software (NIH, Bethesda, MD)[92]. The area of the cells staining positive for alcian blue was charted, the area calculated and then measured as a percentage of the total area of the image.

4.3 Results

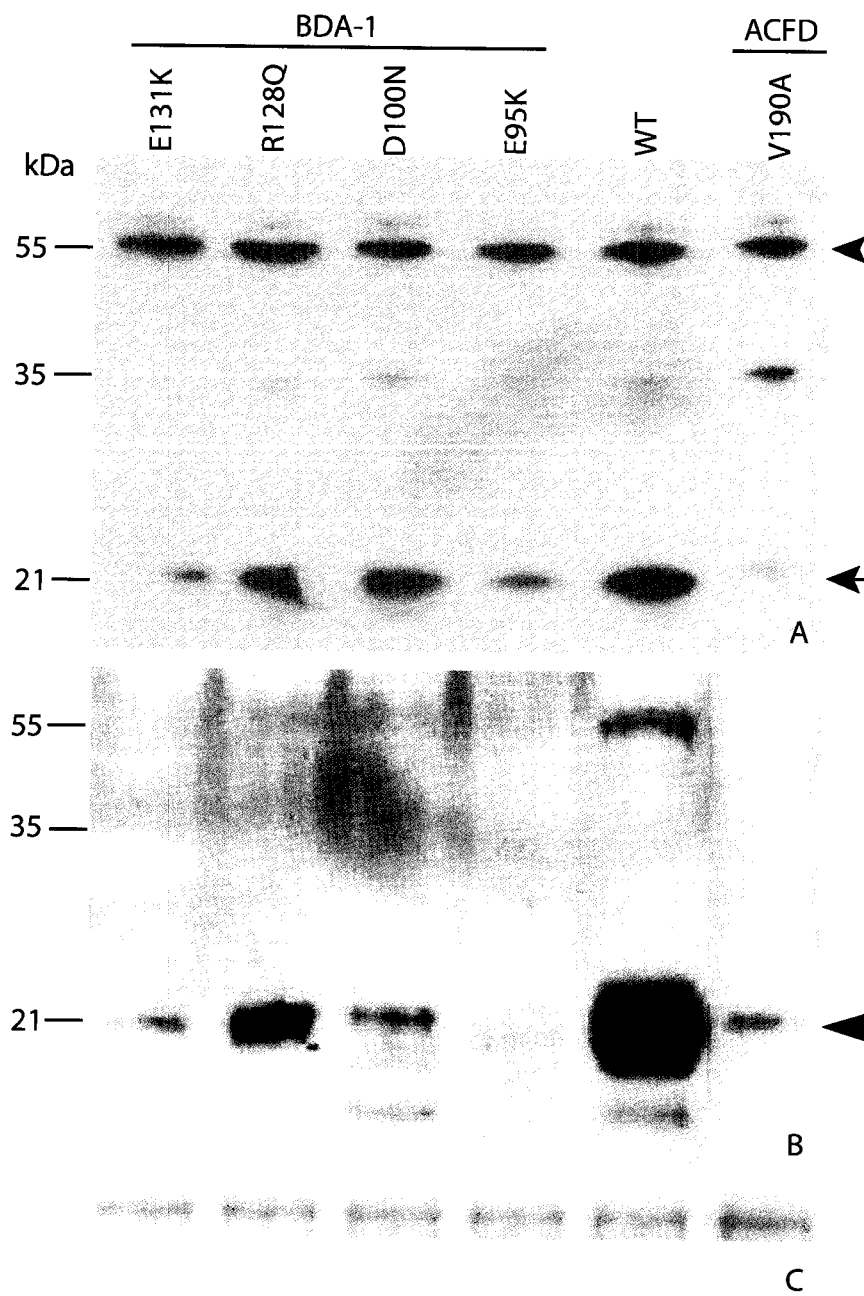
4.3.1 *Analysis of expression of IHH variants in vitro*

During the last few years, genetic studies have associated BDA1 and ACFD with, combined, ten different missense mutations in the human Indian hedgehog gene. As mentioned previously, for the sake of this project only the E95K, D100N, R128Q and E131K BDA1-causing mutations were analyzed, along with the V190A ACFD-causing mutation.

To analyze the effect of these mutations on the processing of IHH and how this could possibly affect its biological activity, we first characterized the expression of IHH^{WT} in comparison to each mutant in separate lines of HEK293 cells stably expressing one of the wild-type or mutant variants of the pcDNAIhh constructs. To confirm successful protein production of IHH-N, a western blot was conducted to analyze both IHH-N secreted in the media and the lysates of the stable HEK293 cells. This approach also provided us the ability to analyze how IHH is processed in each of the mutant lines compared with the wild-type.

Several western blots analyzing the cell lysates were prepared in order to assess general levels of IHH-N contained in each lysate sample (data not shown). Based on the estimated level of full-length IHH precursor protein in each sample, a western blot analysis where the amount of each sample loaded in an additional pre-cast SDS-PAGE gel was normalized for the level of full-length IHH present in each sample previously estimated (figure 4.1A). The antibody detected the full-length precursor protein of 55 kDa in the homogenates, in agreement with the predicted molecular mass of

Figure 4.1. IHH processing and secretion assessed by western blot of cell homogenates and concentrated media. IHH levels in the cell homogenates were normalized for intensity of 55 kDa IHH precursor (**A**), and culture media (concentrated 60X, **B**) collected from HEK293 cells stably transfected with the indicated pcDNAIhh construct. The arrowhead, arrow and flat-backed arrowhead indicate the expected sizes of the IHH precursor, IHH-N and the secreted signalling peptide, respectively. Protein loading was verified by intensity of an unidentified band from a coomassie-stained gel of the concentrated media samples (**C**).



the full-length IHH sequence [93]. The antibody also detected the presence of the 21 kDa N-terminal signalling peptide of 21 kDa, a result in accordance with the expected molecular mass of the signalling peptide [93]. Interestingly, an as yet unknown product of approximately 35 kDa was detected (figure 4.1A). Previous studies analyzing holoprosencephaly (HPE) mutations in *SHH* have shown evidence of a similar product present in western blotted samples of select HPE-causing mutations [82]. This product was visible on autoradiographs of western blots employing anti-sera specific for SHH-C and those specific for SHH-N. This would indicate that the fragment were some type of intermediate product that contained connected sections of both SHH-N and SHH-C, however, the visibility of this product on blots of homogenates for SHH-N was not acknowledged by the authors of the study [82].

The degree to which the N-terminal signalling peptide of IHH was produced, via autocatalytic cleavage, appeared to vary between each of our mutants and the wild-type peptide, relative to the un-cleaved, full-length precursor to which we normalized. Both the D100N and R128Q mutations seemed to produce an amount of IHH-N comparable to the wild-type. In addition to this, both of these mutations showed trace amounts of the 35 kDa *intermediate* product. Contrary to this, both of the glutamate to lysine mutations, E95K and E131K, resulted in less than 50% of the amount of IHH-N, in comparison to IHH^{WT}, as well as no trace of the 35 kDa intermediate product. The V190A mutation resulted in a nearly complete ablation of the production of the N-terminal signalling peptide, and subsequently a significantly increased amount of the 35 kDa protein species in comparison to IHH^{WT}.

An additional western blot was conducted analyzing the amount of IHH-N secreted into the media. Although the signal produced by the IHH^{WT} was clearly discernible on the blots, the amount of IHH-N secreted by all of the mutant cell lines produced a very weak signal, and thus the samples were concentrated 60X in order to produce a stronger signal. Equal volumes of the 60X concentrated sample of media from each cell line was loaded on a pre-cast SDS-PAGE gel and *IHH* expression monitored by western blot analysis using the same polyclonal α -SHH-N antibody (figure 4.1B). An identical SDS-PAGE gel was run and stained with coomassie blue, and an unknown protein band selected in order to demonstrate an equal amount of sample loaded in each well (figure 4.1C).

As expected, the signal produced by blotting a 60X concentrated sample of secreted IHH-N in the media collected from HEK293 cells transfected with pcDNAIhh^{WT} was very strong. A small amount of full-length precursor, migrating with a relative molecular mass of 55 kDa can also be seen in the IHH^{WT} lane. Although relatively equal amounts of IHH-N was produced by the D100N and R128Q mutant species in comparison to the wild-type, the signal resulting from the secreted mutated peptide was significantly lower than its wild-type counterpart. Furthermore, the signal from both the E95K and E131K mutants and the V190A, ACFD-causing mutation, were nearly absent, indicating highly decreased secretion of any produced IHH-N.

4.3.2 Immunohistochemistry of stably-transfected HEK293 cells

In order to find an explanation for the variations observed with the banding patterns, we decided to analyze the subcellular localization of IHH by immunohistochemistry

(IHC) of our HEK(IHH) stably transfected cell lines, seeded on culture slides. 24 hrs post-transfection, the cells in each well were washed, fixed with 2% PFA, and probed with antibodies for both SHH-N and KDEL. KDEL signifies the amino acid sequence, Lys-Asp-Glu-Leu, the most common retention sequence of resident proteins of the endoplasmic reticulum (ER), making KDEL an excellent marker for the ER. These antibodies were tagged with a secondary antibody conjugated to a fluorophore; so that the red signal signified the localization of the ER-marker, KDEL, while the green signal signified the localization of IHH-N. Nuclear DNA was visualized through the aid of DAPI staining, which provides a blue signal when visualized.

As expected, an intense green signal, evenly distributed across the plane of the cell image, was observed in the HEK(IHH^{WT}) line, in accordance with the presence of both processed IHH-N and the full-length IHH precursor [94] (figure 4.2b). In addition, there also appeared to be an even more intense signal that could be visualized at the cell membrane barrier separating each cell. Our equivalent stain of non-permabilized HEK(IHH) cells proved inconclusive (data not shown). Therefore, although possible, it was not determined that these more intense signals at the cell membrane were, in fact, secreted IHH-N that had bound to the membrane of adjacent cells. The red signal, corresponding to the ER and the golgi, was also very strong and was located throughout the cell, however, often highly localized to a specific region of the cell – as evidenced in the mock-transfected HEK293, stained for both IHH-N and KDEL (figure 4.2). Furthermore, our mock-transfected HEK293 cells showed no evidence of positive green staining, indicating that the basal level of any hedgehog expression in HEK293 cells is undetectable by IHC, and that any positive green staining that we observed was the result

Figure 4.2. Immunohistochemistry of HEK293 cells stably transfected with one of pcDNAIhh constructs. Un-transfected HEK293 cells were stained as a negative control. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.4% Triton X-100 in PBS with 10% horse serum. The cells were stained with polyclonal rabbit α -SHH and polyclonal mouse α -KDEL. The secondary immunoreaction consisted of Alexa Fluor 488 donkey a-rabbit and Alexa Fluor 594 donkey α -mouse. All images are under the 40X, oil immersion, objective. Exposure times for each filter were normalized for each group.

Controls	BDA-1	ACFD	
negative	IhhE95K	IhhD100N	IhhV190A
IhhWT	IhhR128Q	IhhE131K	

of over-expression of our stably-transfected constructs.

The line expressing the ACFD-causing mutation, V190A [HEK(IHH^{V190A})], showed a somewhat similar staining pattern to that of the HEK(IHH^{WT}). That is, the cells displayed a very strong green signal, particularly along the cell membrane, in the plane of image (figure 4.2). However, unlike the HEK(IHH^{WT}) line, the positive green, mutant IHH staining pattern was inconsistent. Each cell that stained positive for IHH-N appeared to be expressing equal amounts of *IHH*. However, by simple examination, approximately 25% of the cells in each photographed region displayed very little to no positive hedgehog staining. Similarly, when immunostained, the HEK(IHH^{D100N}) line showed distinct variations from the stained HEK(IHH^{WT}) line. But where the level of IHH production in the HEK(IHH^{V190A}) cells appeared equal from cell to cell, there was a great variability of IHH production between each cell in the HEK(IHH^{D100N}) line, in a given field of view. The intensity of the green hedgehog signal from some cells was comparable to those seen in the HEK(IHH^{WT}) line, while others appeared to show no green, hedgehog signal whatsoever (figure 4.2). The HEK(IHH^{R128Q}) line also displayed a similar trend. However, in addition there appeared to be a strong localization of the IHH signal in approximately a quarter of the cells imaged (figure 4.2). These intensely localized signals stained almost solely green, that is they did not show the characteristic yellow stain indicative of co-localization of IHH with the ER or golgi.

The immunostaining of both the HEK(IHH^{E95K}) and HEK(IHH^{E131K}) produced the results most drastically variable from their wild-type counterpart. The staining of the HEK(IHH^{E131K}) showed a very low level of positive staining for hedgehog in all cells, while nuclear staining and staining for the ER/golgi complex was comparable to the

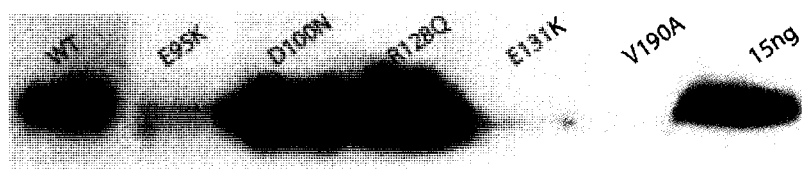
stained mock-injected HEK293 control (figure 4.2). Staining of the HEK(IHH^{E95K}) line was remarkably different from the results observed in stained HEK(IHH^{E131K}) cells, despite the fact they share the same change in residues. Unlike any other cell line, the immunohistochemical signal corresponding to hedgehog was apparent in every cell and was highly localized in a small region of the cytosol that, similar to the staining seen in the HEK(IHH^{R128Q}) line, did not appear to co-localize with the ER, according to staining for the ER marker, KDEL (figure 4.2).

4.3.3 *Functional characterization of missense mutations located within the IHH-N domain*

We had determined that these mutations in *IHH* lead to reduced production, cellular distribution and subsequent secretion of the N-terminal signalling peptide, which alone would undoubtedly affect normal development in the growth plate. However, we wanted to examine the activity of each of these compounds in order to determine if the mutations had a negative effect on the signalling capability. Analysis of signalling activity could also provide insight into potential functional groups of residues in IHH-N. We chose to do so through a luciferase-based assay employing the Shh-LIGHT2 cell line; the clonal NIH 3T3 cell line stably incorporating a Gli-dependent *Firefly* luciferase reporter and a constitutive *Renilla* luciferase reporter [85].

It is likely, based on the decreased secretion levels observed in our previously detailed analysis, that significantly less mutant IHH-N is secreted *in vivo*. Meaning that there is not likely to be equal amounts of mutant and wild-type IHH-N in the growth plate (when considering patients with BDA1 – an autosomal dominant disorder). However,

Figure 4.3. Western blot of IHH-N in concentrated media used for densitometry quantitation. Media collected from the stable cell lines was concentrated 60X and electrophoresed on a 4-12% gradient gel. Proteins were blotted with the polyclonal rabbit α -SHH-N H-160 (Santa Cruz, Santa Cruz, CA). The amount of IHH-N in each sample was calculated by densitometry relative to the amount intensity of the signal from the 15 ng of recombinant mouse SHH-N (indicated as 15 ng), using the program Alpha Imager (Alpha Innotech Corp., San Leandro, CA).



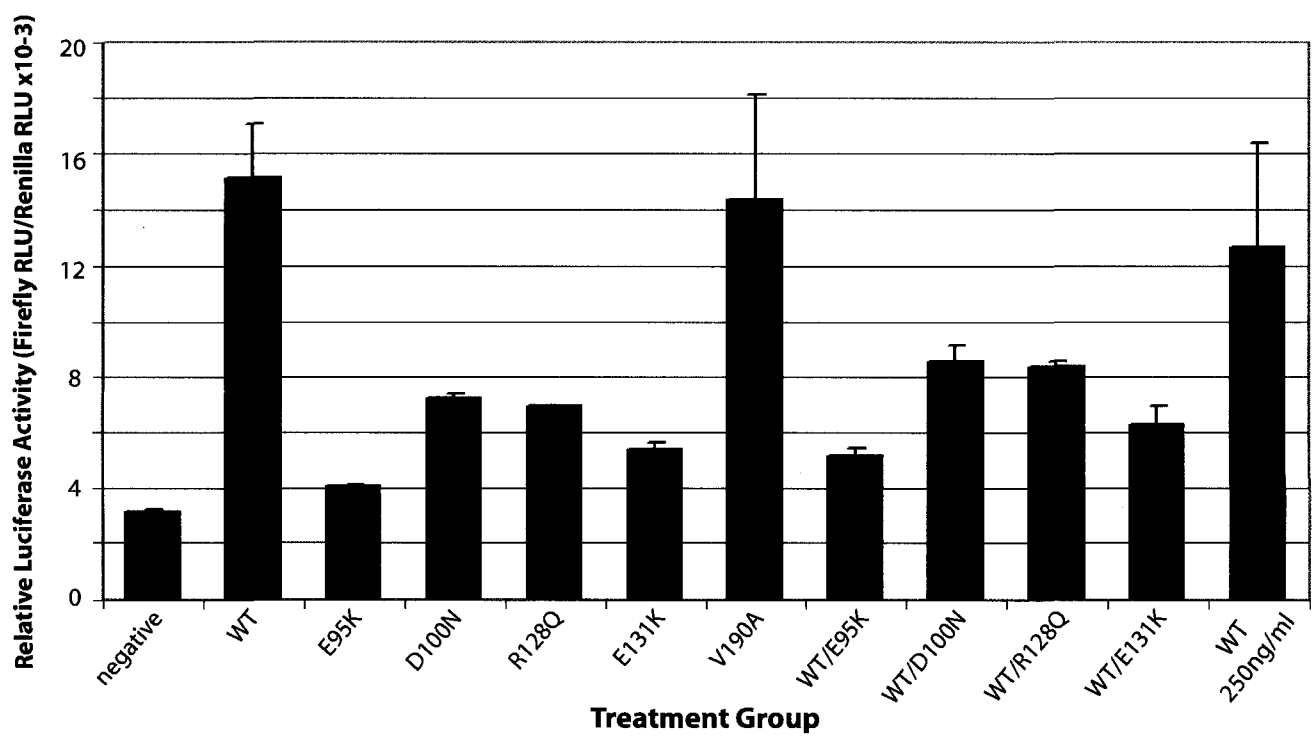
our goal was to accurately quantify the activity of mutant IHH-N relative to the wild-type. Therefore, we opted to treat the reporter cell lines with equal amounts of either mutant, or wild-type IHH-N. Therefore, culture media from the stably-transfected HEK293 cells was collected and, due to the low levels of mutant IHH-N in the media, concentrated 90X. The concentration of IHH-N in each of these samples was determined by densitometry of a western blot analyzing aliquots of each sample in comparison to a known quantity of SHH-N. The blot used for the densitometric analysis is pictured in figure 4.3.

IHH-N, suspended in concentrated HEK(IHH) media, was added to LIGHT2 growth media, to a final concentration of 500 ng/mL, and applied to LIGHT2 cells. The cells were incubated under these conditions for 28 hours. At which point, the cells were washed, lysed and the luciferase activity measured by luminometer. A summary of the relative luciferase activity (a ratio of the induced *Firefly* luciferase activity to the constitutive expression of *Renilla* luciferase) of each sample tested is illustrated in figure 4.4.

The V190A mutation was the only mutant that induced a comparable level of relative luciferase activity to that of IHH^{WT} (at 94.9% of IHH^{WT} activity). All of the BDA1 causing mutations displayed between 26.9% and 47.9% the level of activity level observed in cells treated with IHH^{WT}. In particular, both of the Glu to Lys mutations, IHH^{E95K} and IHH^{E131K}, displayed the lowest relative activity at 26.9% and 35.3% of IHH^{WT} levels, respectively.

As BDA1 is an autosomal dominant disease, and although significantly less mutated protein is secreted from the cell than the wild-type variant, and considering the

Figure 4.4. Tabulated data of LIGHT2 IHH-activity assay. Luciferase reporter activity of Hedgehog-responsive LIGHT2 cells treated with LIGHT2 media conditioned with media collected from lines of HEK293 cells stably transfected with one of either wildtype (WT) or mutant IHH-expressing pcDNAIhh constructs. The collected media was concentrated 60X, and the amount of IHH-N in each media sample was calculated using densitometry analysis of a western blot. The amount of IHH used to condition the media was normalized to 0.5 ug/mcv L. In order to analyze the situation of co-expression of both WT and mutant IHH in the case of BDA-1 patients, LIGHT2 cells were treated with media collected from both WT IHH and one of the BDA1 mutants. Cells were incubated with the conditioned media for a period of 28 hours. Tabulated data shows the relative luciferase activity of Hh-induced *Firefly* luciferase when controlled for by the constitutively expressed *Renilla* luciferase. Data from each sample group is the mean +/- S.E. ($p < 0.05$) from three independent experiments (with the exception of the 250 ng/mL group, which was calculated from duplicate trials conducted in parallel).



results from our previous sections, it is conceivable that individuals expressing one of the BDA1 mutations may only have traces of the mutated protein alongside the wild-type variant in the developing growth plate. Therefore, in order to analyze the effect that both IHH^{WT} and one of the BDA1-causing mutants would have on signal transduction, equal amounts of both to a final IHH-N concentration of 500 ng/mL were used to condition LIGHT2 media and treat hedgehog-responsive LIGHT2 cells. Interestingly, the presence of an equal amount of IHH^{WT} incubating with one of the BDA1-mutants yielded an average increase in luciferase activity (relative to the wild-type) of $8.0 \pm 1.4\%$ ($p < 0.05$), averaging 47.0% of the luciferase activity induced by 500 ng/mL of IHH^{WT}. In contrast, a control treatment of only 250 ng/mL of IHH^{WT} induced luciferase activity at 84.1% of the levels of 500 ng/mL of IHH^{WT}. It is important to note that there were only two trials of those samples that were treated with 250 ng/mL of IHH^{WT}, as opposed to three for the other treatment groups, and the second tested sample of 250 ng/mL of IHH^{WT} was months later than the first, and the integrity of the sample may have been in question. This would attribute to the larger standard deviation on this sample group. Even considering this fact, the samples treated with a combination of IHH^{WT}/IHH^{E95K}, IHH^{WT}/IHH^{R128Q}, and IHH^{WT}/IHH^{E131K} showed significantly less activity than those cells treated with 250 ng/mL of IHH^{WT} alone. Therefore, incubation of equal amounts of wild-type IHH-N and one of the BDA1-associated mutants provided strong evidence to suggest a dominant negative effect of all of the BDA1-associated mutants over the wild-type variant.

4.3.4 *Treatment of differentiating ATDC5 cells with mutant IHH attenuates differentiation*

To coincide with the reporter-cell-based study in LIGHT2 cells, we opted to analyze whether a similar administration of IHH-N-conditioned media could alter the program of chondrocyte differentiation. We elected to use the mouse chondroprogenitor cell line, ATDC5, as our model to examine chondrocyte differentiation.

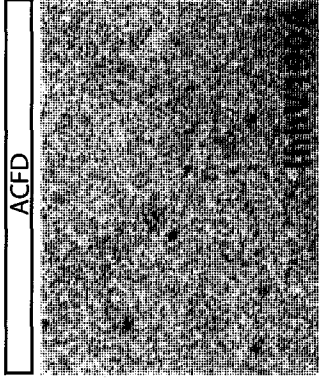
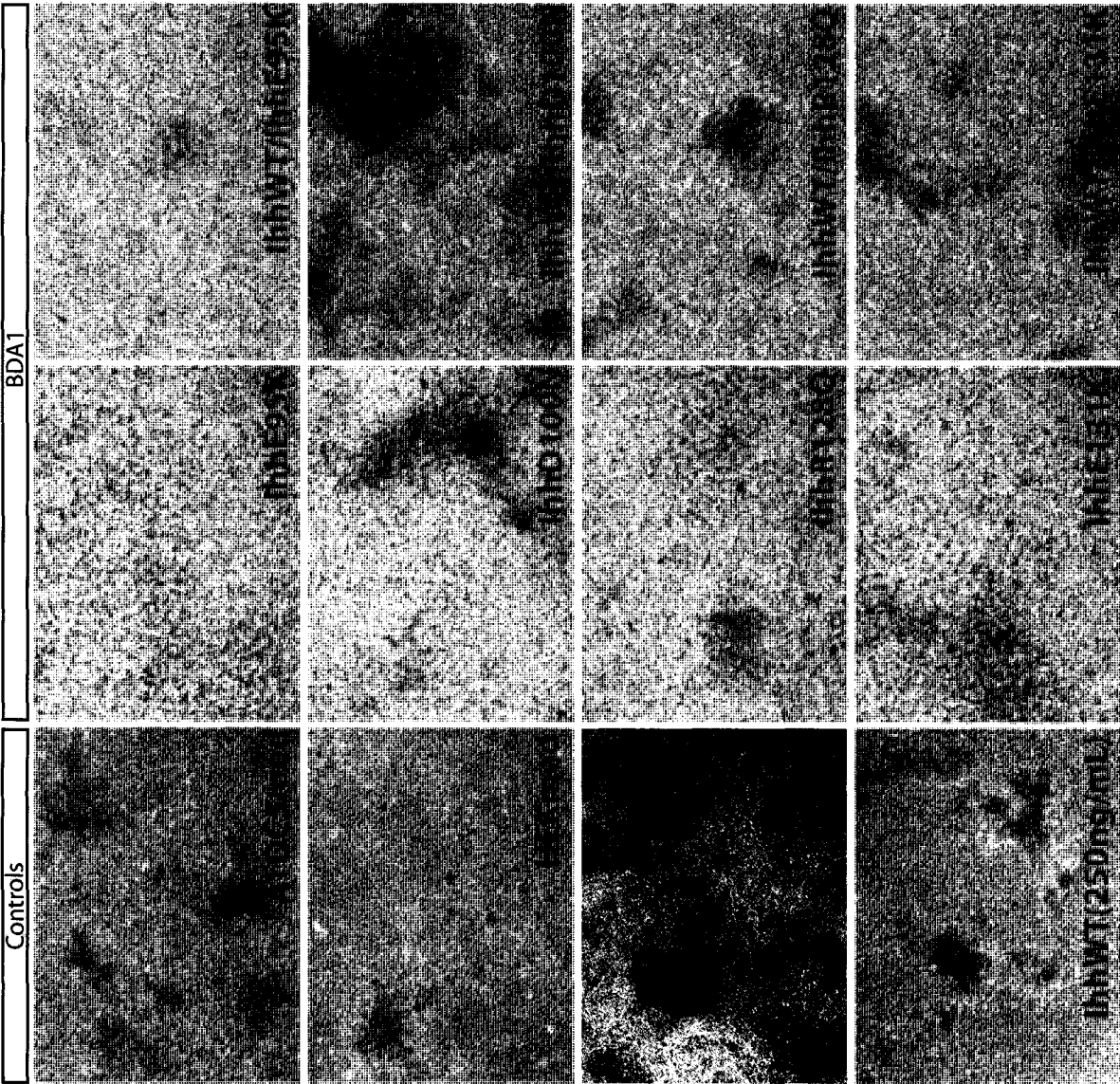
Once differentiation is induced via infusion of the media with insulin, expression of *IHH* can be noted as early as day six, and a 10 fold spike in *IHH* expression is witnessed between days six and 11 of the differentiation process [88]. *IHH* is an essential component to promote chondrocyte proliferation, and thus the development of overgrown, cartilaginous nodules [87, 89]. It was our intent to examine whether the dominant-negative effect of the BDA1-causing mutant proteins could also be observed when a similar experiment was conducted employing ATDC5 cells in the early stages of differentiation.

ATDC5 cells were cultured in 6-well plates. Once confluent, the media was conditioned with bovine insulin and sodium selenite to induce differentiation from pre-hypertrophic to hypertrophic chondrocytes. Eight days after the differentiation media was administered, the media was conditioned with 500 ng/mL of one of, or a combination of, the wild-type or mutant IHH-N (same quantified amount suspended in media collected from HEK(IHH) stable cell lines). Following five days of incubation in this IHH-N-conditioned media, the media was aspirated and the cells were stained with a 3% alcian blue solution to measure the degree of GAG production, more specifically ColII production as a marker for degree of differentiation [51, 86, 87]. The experiment was

only conducted once, however two wells of the cell culture plate were treated with the same sample. For each well five random photographs of alcian blue staining were taken. Following this, the ImageJ program was used to calculate the area of the field-of-view that was stained positive for alcian blue.

Most notably, the cells treated with IHH^{WT} displayed not only significant nodular condensation, but also extensive alcian blue-positive GAG staining (figure 4.5). When analyzed by ImageJ, on average 63.3% of the IHH^{WT}-treated cells stained positive for increased GAG production. In comparison, only 16.4% of the cells treated with unconditioned ATDC5 differentiating media stained positive for GAG. Therefore, it would appear that treatment with much larger amounts of IHH^{WT}, in fact, accelerated the differentiation process. Treatment of the cells with any of the BDA1-associated mutations, or the V190A mutation showed little to no condensation, and very little alcian blue-positive staining (figure 4.5). Particularly, the E95K and V190A mutations showed very little evidence of GAG production, with 1.6% and 0.8% of cells staining positive, respectively – a possible indication of inhibited differentiation. The remaining mutations, R128Q and E131K, showed close to the same level of positive staining (7.8% and 9.2%, respectively) as those cells treated with media collected from untransfected HEK293 cells (9.8%; administered at the same time as IHH-conditioned media, eight days into the chemical- induced differentiation process). Interestingly, the cells treated with IHH^{D100N} showed a level of staining comparable to that treated with ATDC5 differentiating media only – at 16.6% - suggesting that differentiation in this case, unlike the other mutants, was not attenuated. When the differentiating ATDC5 cells were treated with a 1:1 combination of IHH^{WT} and one of the BDA1 mutations (to a final IHH concentration of

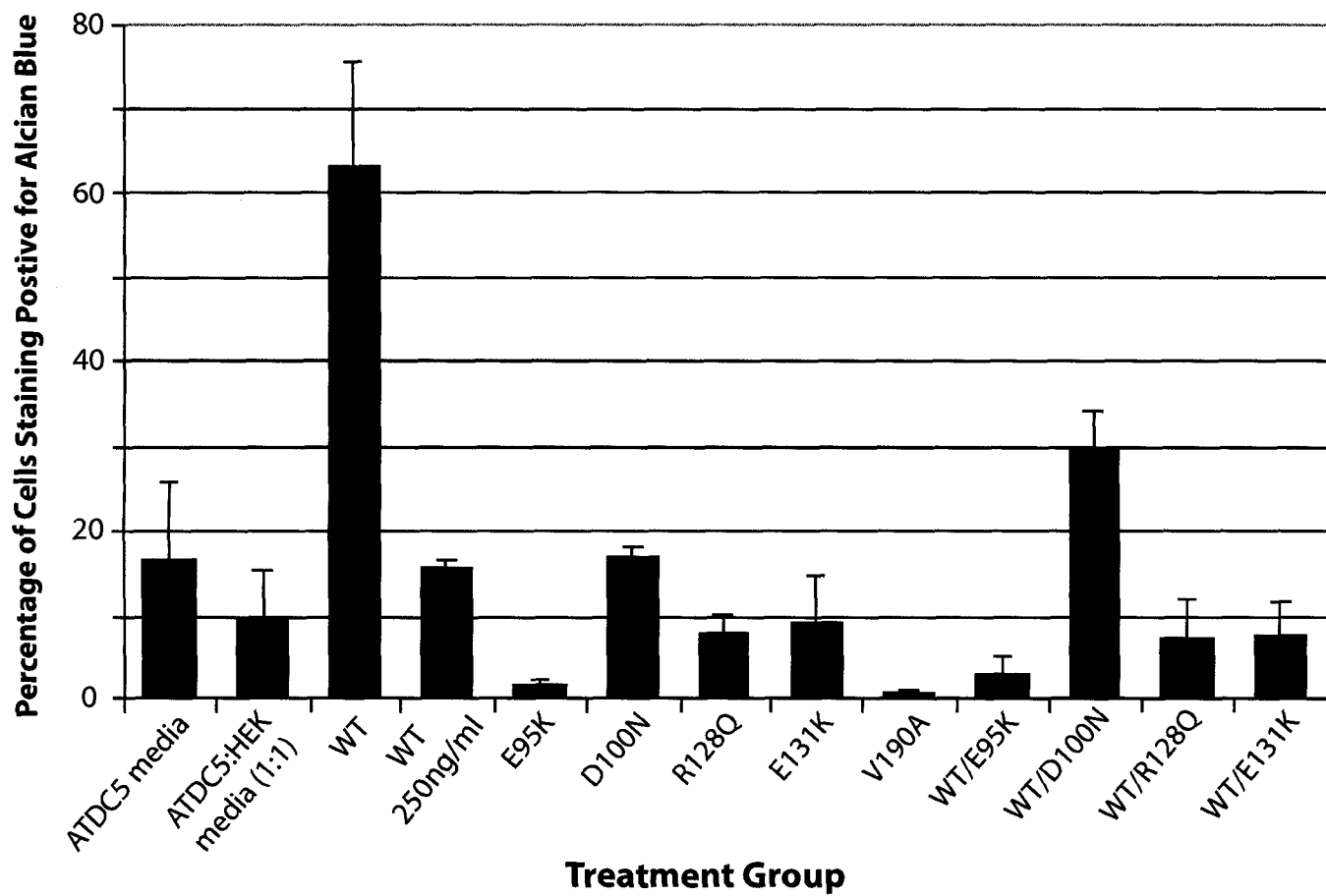
Figure 4.5. Differentiating ATDC5 cells treated with IHH-N. ATDC5 cell differentiation was induced by treatment with insulin. Eight days into the differentiation process, the media was conditioned with 500 ng/mL either WT or mutant IHH-N, or an equal combination of IHH^{WT} and one of the BDA1-associated mutations up to 500 ng/mL (indicated above). Five days after conditioning of media with IHH-N, cells were stained with alcian blue and photographed. Control groups were treated with ATDC5 differentiating media only (ATDC5med), equal amounts of ATDC5 differentiating media and media from un-transfected HEK293 cells (HEKmed), or differentiation media conditioned with 250 ng/mL of IHH^{WT}.



500 ng/mL), there was a significant change in the amount of alcian blue-positive staining in comparison to the cells treated with the mutant protein only. Furthermore, cells treated with the combinations of IHH^{WT}/IHH^{E95K} (2.9%), IHH^{WT}/IHH^{R128Q} (7.1%), and IHH^{WT}/IHH^{E131K} (7.5%) displayed significantly less alcian blue staining than the control group treated with 250 ng/mL of IHH^{WT} only (15.6%; figure 4.6), and also showed no significant increase from those cells treated with the BDA1-causing mutant only. On the contrary, the IHH^{WT}/IHH^{D100N} treated cells showed a significant increase in the amount of stained cells, at 29.5%, from the cells treated with IHH^{D100N} only (16.6%; figure 4.6). This result would suggest that, unlike our other BDA1- and ACFD-causing mutations, in a chondrogenic model, the IHH^{D100N} mutation does not confer a dominant negative effect over the wild-type variant, but has also maintained effective signalling function.

Therefore, it was evident that on their own, all mutations appeared to be incapable of not only inducing IHH signalling and advancing chondrocyte differentiation *in vivo* comparable to that witnessed in the IHH^{WT}-treated cells, but it also appeared to attenuate this differentiation in comparison to the control group that was treated with unconditioned, ATDC5 differentiating media. Furthermore, consistent with what we witnessed in the LIGHT2 assay, with the exception of the D100N mutation, it appears that the BDA1 mutations may possess a dominant-negative effect over the wild-type variant of protein in equal molar amounts, and that this effect significantly delays chondrocyte differentiation.

Figure 4.6. Average calculated area of positive alcian blue staining in differentiating ATDC5 cell assay. Results were obtained by calculating the area of each image using the ImageJ software. A total of six images were examined for each sample group - three images from each of duplicate treatment groups. Data from each sample group is the mean $\pm$ S.E. ($p < 0.05$) from each of the images examined.



4.4 Discussion

Indian hedgehog is one of the principal regulators of endochondral ossification [19, 29]. In conjunction with several co-factors, acting on different tissues, Ihh regulates the rate of proliferation and differentiation of chondrocytes in the developing long bone. It is our goal to understand the processes governing this pathway, and it is our belief that through analyzing how this system is altered in the presence of mutated IHH, that we will further understand how IHH governs its downstream transcription factors and thus the potential consequences in the developing long bone.

Very little research has previously been conducted to model the activity of recombinant Indian hedgehog in any system, *in vitro* or *in vivo*. In fact, there is a distinct possibility that we are the first group to do so. In addition, it is also possible that we are the first group to characterize a panel of mutations in *IHH*; in this case, mutations associated with brachydactyly type A1 and acrocapitofemoral dysplasia. After successfully developing the first full-length clone of *IHH*, our group successfully developed multiple lines of HEK293 cells stably expressing one of either the wild-type or BDA1/ACFD-associated mutant recombinant proteins.

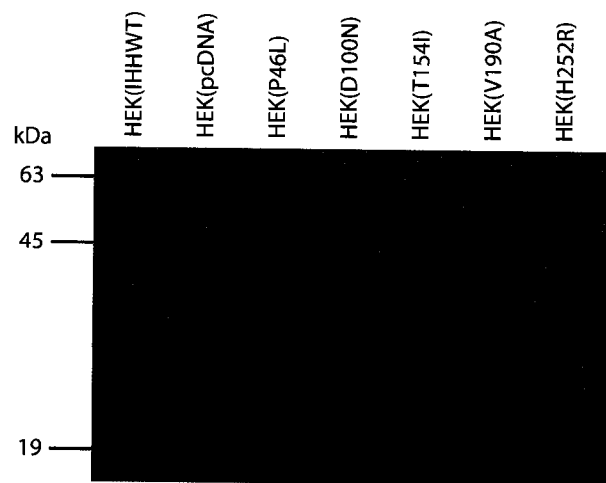
BDA1-associated mutations in IHH alter the processing and subsequent secretion of IHH-N

Shortly after the initial submission of this manuscript, one of my colleagues in the lab of Dr. Dennis Bulman, Kelly Patterson, conducted a western blot analysis of a selection of the same, along with some different, BDA1-associated mutants. Similar to

the study that I had conducted, Kelly produced new lines of HEK293 cells, stably transfected with pcDNA constructs expressing one of the mutant variants or wild-type *IHH*. Similar to my own study, Kelly examined between 20 and 60 μ g of total protein from cell lysates for each sample. The PVDF membrane was blotted with the same polyclonal rabbit α -SHH-N IgG H-160 (Santa Cruz, Santa Cruz, CA; to a final concentration of 1:2000), and a goat anti-rabbit secondary antibody (1:20000). The autoradiograph of this blot is pictured in figure 4.7 (K. Patterson, *personal communication*).

From figure 4.7 it is evident that in every lane there is a protein band corresponding to a size of 63 kDa, which is present in all lanes, including that of the mock-transfected HEK293 cell line. Therefore, what I had claimed to be a 55 kDa full-length *IHH* precursor protein, was in fact a HEK293 protein which cross-reacted with our polyclonal α -SHH-N IgG. Considering this fact, some of our observations need to be re-analyzed. Firstly, Ms. Patterson only examined three of the same *IHH* variants as myself; *IHH*^{WT}, *IHH*^{D100N} and *IHH*^{V190A}. And in these lines, a band is evident at 45 kDa, which I had indicated was the 35 kDa anomalous protein fragment. This is, in fact, the true full-length precursor protein. As the size of 45 kDa is consistent with a great deal of that found in the literature regarding *IHH* [reviewed in 19]. However, when we compare lanes 1, 4 and 6 from figure 4.7, we see a very low presence of the precursor in the *IHH*^{WT} sample in lane 1, significantly more precursor in the *IHH*^{D100N} sample in lane 4, and even more presence of the precursor in the *IHH*^{V190A} sample in lane 6. Furthermore, there is a corresponding lack of *IHH*-N production in each of the mutant samples in comparison to *IHH*^{WT}. Although we did reference the wrong protein band as the

Figure 4.7. IHH processing and secretion by western blot of cell homogenates of stably transfected HEK293 cells. Between 20 and 66 μ g of protein was loaded into each well, the identity of the cell homogenate sample is indicated above the lane. The HEK(pcDNA) sample indicates the HEK293 cells transfected with empty vector, pcDNA3.1.



precursor, the general trend of IHH-N production is consistent. The mutant variants analyzed by Ms. Patterson produce less IHH-N than IHH^{WT}.

IHH is produced primarily in pre-hypertrophic endochondral cells during endochondral ossification. The full-length, 45 kDa, precursor protein is autocatalytically cleaved to produce the 19 kDa N-terminal signalling peptide, IHH-N, and an approximately 30 kDa C-terminal peptide, IHH-C. Through this process IHH-N is modified by the addition of palmitate at the N-terminal region and a covalently-bonded cholesterol at the C-terminal region [24]. Considering the high similarity with SHH-N, and that palmitoylated SHH-N is necessary to facilitate the multimerization of multiple signalling peptides, it is likely the same claim can be made regarding IHH-N [23, 24]. The multimer is then secreted by the cell and, through a chaperoning system, ushered to its target cells.

To obtain a deeper insight into the mechanism by which these mutated forms of the protein convey their pathogenic effects, we opted to analyze the processing and secretion of the signalling peptide, IHH-N, in each line of HEK(IHH) cells. Both the D100N and the R128Q mutations have relatively small changes in the electronegativity of the respective side chains of the original residues. Although, in both cases the change in residues is potentially significant, as these residues are highly conserved across numerous species (table 1.1). The D100N mutation sees the protein donating aspartate changed to a neutral asparagine, and the R128Q mutations sees the basic arginine changed to a neutral glutamine. These changes could potentially alter the local pH in the region of the protein in which it resides.

In both the cases of the D100N and the R128Q mutation there was approximately 50% less IHH-N produced, relative to the WT, and consistent with this pattern, significantly less D100N and R128Q IHH-N was secreted, relative to the wild-type. Therefore, it is possible that since these residues are located on the surface of the protein, the D100N and R128Q mutations have reduced the ability of the protein to either multimerize and be secreted by the cell, or reduce the protein's ability to localize to the transmembrane protein *dispatched*.

The two remaining BDA1-causing mutations seem to confer a similar type of change in behaviour from the wild-type protein, although much more pronounced. Both the E95K and the E131K mutations result in a change from the proton-donating residue, glutamate to the protein-accepting residue, lysine; a seemingly drastic change to the local pH of the protein in that region. Of all of the BDA1-causing mutations, these two displayed the greatest decrease in the amount of IHH-N production, relative to the wild-type (figure 4.1A), and the most significantly decreased amount of secretion of IHH-N (figure 4.1B), in comparison to the wild-type.

All of the BDA1-causing mutations reside in a clustered region of the protein near to the surface [17]. This region also contains the His139 residue, a residue conserved in all hedgehogs that has been implicated in Zn^{2+} coordination [59]. It is possible that the slight decrease in the local pH of the protein, in the case of the R128Q and the D100N mutations, and the relatively large change in local pH as a result of the E95K and E131K mutations, could alter the ability of the triad residues, His145, Asp152 and His185 from forming the pocket necessary to bind the Zn^{+2} ion, thus affecting the conformation of the protein, and decreasing the level of autocatalytic cleavage. Furthermore, a critical Lysine

residue at position 144, directly adjacent to His145, is homologous to SHHLys139, which has been shown, along with Asp152 and His185, to be critical in facilitating intermolecular interactions between molecules of SHH, forming the soluble, multimerized form of SHH-N; denoted as s-SHH-Np [24]. Furthermore, in studies analyzing mutations to His140 and Glu147 in SHH, mutants have shown a loss of Zn^{2+} binding and subsequently exhibit misfolding of the protein, and eventual proteolytic degradation [95]. Therefore, a change in the local pH as a result of these BDA1 mutations would likely disrupt the ability of this region to interact with other IHH molecules, subsequently reducing multimerization, and thus resulting in the observed significant decrease in the amount of secreted IHH-N, or it could also prevent the binding of the Zn^{2+} ion, resulting in the misfolding and eventual degradation of the protein.

As it resides very close to the His185 residue, it is likely that the ACFD-causing, V190A mutation also disrupts the conformation of the triad residues; His145, Asp152, and His185. Both valine and alanine have neutral side chains, so it is unlikely that a change in pH could disrupt the interactions in this pocket, however, not only is alanine more hydrophilic than valine, but it can also produce a stable free-radical. It is possible that the introduction of this different species sterically affects the region, thus reducing the ability of the triad to bind Zn^{2+} or to interact with other IHH-N peptides, and ultimately reducing IHH-N production and multimerization, respectively.

The effects of the mutations on the biochemical properties of IHH are also reflected to a certain extent in the immunohistochemical analysis of the HEK(IHH) cells, stably expressing one of the mutant constructs. When analyzed by IHC, IHH^{WT} appeared throughout the cell. Notably, there is an increased intensity of signal at the cell

membrane borders between adjacent cells. It is uncertain whether this is IHH^{WT} residing within the cell or whether this is in fact IHH^{WT} that has been secreted and has since adhered to the surface of neighbouring cells. Meanwhile, both of the HEK(IHH^{D100N}) and the HEK(IHH^{R128Q}) lines contained cells with varying levels of expression of *IHH*, from low to moderate to intense positive staining. A similar trend was apparent in the HEK(IHH^{V190A}) cells that were immunostained. The presence of IHH did not appear to be specifically localized to any particular region of the cell. It is possible that, in the case of the IHH^{D100N} and IHH^{R128Q} mutants, the low levels of IHH-N visible in the secreted media and the inconsistent immunostaining patterns could reflect protein instability. If the proteins are recognized by the cell as unstable or foreign, they could be preferentially targeted for degradation. This would explain why we see such significantly less positive immunostaining in the HEK(IHH^{D100N}) and HEK(IHH^{R128Q}) cell lines.

Our initial hypothesis regarding the BDA1-causing mutations, was that the mutant proteins would attenuate or retard the processing of the IHH precursor to the IHH-N signalling peptide, and that this attenuation would occur in the natural physiological location of processing, the endoplasmic reticulum. Therefore, we hypothesized that this attenuation may be visible in the cell as a co-localization of the Ihh signal with the ER marker. Contrary to what was seen in the HEK(IHH^{D100N}) and HEK(IHH^{R128Q}) lines, the staining pattern seen in the HEK(IHH^{E95K}) and HEK(IHH^{E131K}) was very different. As stated previously, the HEK(IHH^{E131K}) cells displayed a very low level of staining in all cells, with a small percentage of the cells displaying a localized signal that did not co-localize with the ER. Similarly, when stained, the cells of the HEK(IHH^{E95K}) line displayed a strong and distinctly localized signal. Like the HEK(IHH^{E131K}) line, the

signal did not appear to co-localize with the ER. There are several possible explanations for this result. For example, it is possible that the concentrated signal is an indication of the mutated protein's presence in the lysosome, and an indication of its toxicity to the cell. Or, in the case of what was seen in the HEK(IHH^{E95K}) line, the mutant protein could sequester in one area similar to the cases of mutant α -synuclein, which sequesters to form the main component of Lewy bodies, the second most common nerve cell pathology in alzheimer's disease (AD)[96]. It is clear that, with respect to the localisation and sub-cellular processing and trafficking of both WT and mutant IHH-N, more analysis is necessary.

BDA1-mutations significantly decrease the activity of nascent protein

In this study, our group has modelled a series of mutations in Indian hedgehog associated with BDA1 and ACFD. After stable transfection of HEK293 cells, media containing the secreted signalling peptide was collected and concentrated 60X and the quantity of IHH-N in each sample was calculated by densitometric analysis of the intensity of the blotted signal with the intensity of a known quantity of SHH-N. We have shown that, when present in equal molar amounts, the activity of IHH-N possessing a BDA1-causing mutation in comparison with its wild-type counterpart is significantly less. What was particularly interesting was that when the cells were incubated in equal molar amounts of IHH^{WT} and one of the BDA1 mutants, the level of IHH-induced activity was not significantly increased in comparison to those cells treated with only the mutant peptide.

Several studies have been conducted to analyze the activity of Sonic Hedgehog and its various associated mutations [24, 82, 97, 98]. In these assays, the activity of mutations including W122, H145, and C188 (the IHH-homologous positions are indicated), residing in close proximity to our R128, E131 and V190 positions, were analyzed. In each instance, these mutants exhibited less than 5% of the level of activity of wild-type SHH-N. Although these results differ significantly from ours, it is important to note that in each instance where the activity was analyzed, the examiners treated the reporter cells with equal volume amounts of media from cells transfected with a construct expressing *SHH*. Meanwhile, based on their western blots, the amount of mutated peptide secreted by each transfected cell line was significantly lower than those lines expressing the wild-type variant [99, 100]. Therefore, it is impossible to assess the activity of the mutant peptide in comparison to the wild-type if the reporter cells are treated with unequal amounts.

In the case of the BDA1-causing mutations, based on various 3D models of the highly conserved human SHH, all four of the mutated residues lie at the surface of the protein [4, 17, 82]. Therefore, we postulate that the mutations can disrupt the ability of Zn^{2+} ion to bind, thus preventing proper folding and auto-proteolytic processing of the protein. Subsequently, in the instance that the misfolded protein is in fact secreted from the cell, as the mutations are located on the surface of the protein, it is possible that the misfolding caused by the mutations reduces the affinity of the peptide for the target receptor. Conversely, it is also possible that the affinity of the mutated protein to the receptor may not be affected, but rather the ability of the mutated protein to confer upon the target the necessary conformational change in Ptch that would induce the signal

transduction cascade. In either instance, this would yield a lower activity than treatment of the cells with IHH^{WT} alone. This result is exemplified in both the IHH^{E95K} and IHH^{E131K} samples. In both cases the activity was lower than the other two BDA1 mutations tested. This is likely due to the fact that, in the E95K and E131K mutations, the residue changes to a proton-accepting group from a proton-donating group, a change that would likely affect protein folding, and thus protein-protein interaction, greater than the other two BDA1 mutants.

Interestingly, when the reporter cells were treated with an equal molar amount of both IHH^{WT} and one of the BDA1 mutants, there was only a slight (~8%) increase in the reporter activity when compared to the cells treated with only the mutant protein, indicating that the mutant proteins displayed a dominant-negative effect over IHH^{WT}. Seeing as how each respective protein sample would have been collected separately, and knowing that IHH (by homology with SHH) multimerizes through concentration of IHH-Np on lipid rafts of the producing cell [101], it would be very unlikely that multimers consisting of a combination of mutant and wild-type peptides with decreased affinity for the target receptor could form. Therefore, these results suggest that the mutant proteins maintain their affinity for the target receptor, Ptch, but do not retain their ability to completely transduce the signal. It is possible that they do not retain the ability to confer the conformational change upon the receptor that would initiate the transduction cascade, or the mutant proteins may bind to Ptch and somehow alter its trafficking within the membrane. Therefore, when bound, the mutant proteins not only fail to induce a signal, but also sterically block wild-type IHH from binding and initiating the cascade.

In the case of the ACFD-causing, V190A mutation, the activity of the mutated peptide is not significantly different from that observed from the reporter cells treated with an equal molar amount of IHH^{WT}. This result lends credence to the argument that acrocapitofemoral dysplasia arises not from an inability of IHH to bind to or induce a response in the target cell, but rather as a result of an insufficiency in the amount of IHH-N secreted, even when considering that there are two ACFD alleles present in the individual [16]. Although it is uncertain exactly where, the addition of the cholesterol moiety takes place at the C-terminus of the N-terminal signalling peptide, with the V190A mutation sitting very close to this C-terminal end of IHH-N, it is conceivable that it could obstruct the addition of the cholesterol moiety to IHH-N. In addition, some *in vitro* studies indicate that cholesterol modification may precede palmitoylation [91]. Therefore, if the V190A mutation prevents cholesterol addition and the subsequent palmitoylation, it is possible that the protein would not be able to efficiently multimerize in order to signal over the distance of several cellular diameters.

In order to examine these theories, we opted to test them in a chondrogenic system - by conducting an analysis of differentiating chondrocytic ATDC5 cells that were treated with either IHH^{WT}, one of the mutants or a combination thereof.

Treatment of differentiating chondrogenic precursors with BDAI- or ACFD-associated IHH mutants attenuates the differentiation process by decreasing proliferation

There are two principal and distinct steps in the process of chondrogenic differentiation. Primarily, regulated by local factors, the cells undergo mesenchymal condensation, giving rise to proliferating chondrocytes – this is known as early-phase

differentiation. Secondly, the cells undergo a transition to hypertrophic chondrocytes, which is followed by the second stage of late-phase differentiation, matrix mineralization [89]. As was previously indicated, based on an ATDC5 transcription profiling study conducted by Chen *et. al.*, *IHH* expression can first be detected between four and six days after insulin-induced differentiation begins [88]. Akiyama *et. al.* demonstrated, this early-phase expression of *IHH* is largely responsible for stimulating the late-phase chondrogenic differentiation [89]. Furthermore, it has also been demonstrated that retroviral overexpression of *SHH* in micromass cultures of chicken limb bud cells can increase cellular proliferation, and confer upon the cells characteristics of hypertrophic chondrocytes – including positive alcian blue staining [102]. Therefore, it was our goal to test whether the dominant-negative effect that we witnessed in the LIGHT2 activity assay would be reflected in a chondrogenic model, i.e. would the mutant variants of *IHH* exhibit a reduced capacity to both induce cellular proliferation, and coordinate late-phase differentiation?

Briefly, the cells were incubated in differentiation media containing insulin for a period of eight days prior to the media being conditioned with *IHH*. By doing this, we could assure that the cellular machinery necessary to transduce the hedgehog signal would be expressed. Using a simple RT-PCR analysis on various target genes in undifferentiated ATDC5 cells we did find that *Ptch* is in fact expressed in these cells (data not shown). Also, having shown that BDA1 mutations appear to have a dominant-negative effect over the wild-type species *in vitro*, we wanted to test our chondrogenic model at a moment when *IHH* expression is at its peak, therefore between days 8 and 11

[88]. In this manner we could test whether the mutants would be able to significantly affect differentiation.

Interestingly, the cells treated with 500ng/mL of IHH^{WT} displayed significantly more positive alcian blue staining but also displayed significantly more cellular overgrowth and development of cartilaginous nodules than all other sample groups. In the developing long-bone, IHH secreted by hypertrophic chondrocytes is shuttled to, amongst other targets, proliferative columnar chondrocytes [19]. Activation of the hedgehog pathway delays differentiation and ensures continued proliferation of cells, and subsequently the extension of the growth plate [19, 54, 103]. Seeing as how differentiation had been induced for eight days prior to the exposure to IHH, by the 8-13 day point of insulin-induced differentiation, other markers of the differentiation process, namely BMP-2, Col II and Col X, would be expressed at high levels [88, 90]. Therefore, it is likely that treatment with large amounts of IHH^{WT} increased the rate of proliferation of these cells, as evidenced by the increased presence of overgrowth and cartilaginous nodules. Therefore, an increase in the amount of cells expressing drivers of differentiation, such as BMPs and FGFs, would lead to an increase in the amount of these signals in the media which would ultimately result in an increased rate of differentiation. This is apparent in our results that showed both an increase in overgrowth and an increase in positive alcian blue staining in those cells treated with IHH^{WT}. In order to more accurately assess proliferation, it would be beneficial to repeat these studies and employ a BrdU cellular proliferation assay.

Furthermore, in accordance with what we observed in the LIGHT2 model, the cells treated with media conditioned with the BDA1 mutants showed significantly less

positive alcian blue staining than those cells treated with IHH^{WT}. However, with the exception of those cells treated with IHH^{E95K}, there was no significant difference from the samples treated with differentiating media only. Therefore, it is likely that the BDA1 mutants, as in the LIGHT2 model, do not induce Hh signalling. However, as the cells are still being exposed to insulin in the media, the differentiation of the cells continues as it would if no IHH had been added to the media. In the case of the IHH^{E95K}-treated cells, the level of positive alcian blue staining was significantly lower than all of the control groups, including the group treated with equal volume amounts of ATDC5 differentiating media and HEK293 media. In order to fully explain this phenomenon, further analysis is necessary.

When the differentiating cells are treated with a combination in equal molar amounts of IHH^{WT} and one of either IHH^{E95K}, IHH^{R128Q} and IHH^{E131K}, there is no significant change in the amount of positive alcian blue staining, and therefore no change in the amount of hedgehog signalling induced. Previous studies have shown that inhibition of IHH signalling, through over-activation of PTH/PTHrP receptors in ATDC5 cells, inhibits the chondrogenic differentiation of the cells and subsequent growth of cartilage nodules [87]. Therefore, we can infer that our result is another indication of attenuated or inhibited IHH signalling. This lends further credence to the argument that these mutations in this crucial cleft, residing on the surface of the protein, could lead to misfolding of the protein, increased proteolysis, and decreased secretion of IHH. And, that what little peptide that is secreted, binds to its receptor, Ptch, but is unable induce conformational change and thus prevents transduction of the signal.

With respect to the IHH^{D100N} mutation, unlike the activity pattern observed in the LIGHT2 model, when cells were treated with an equal amount of IHH^{WT} and IHH^{D100N} there was a marked significant increase in the activity level. It is important to also note that in the LIGHT2 model, the activity of the D100N mutant was the highest of all the BDA1 mutations. This could suggest that the D100N mutation does not result in the same marked deviation from the normal tertiary structure of the peptide that is observed in the other BDA1-causing mutants, and that in a chondrogenic model, IHH^{D100N} does not sterically hinder the binding of IHH^{WT} to the receptor Ptch.

With respect to the IHH^{V190A} mutation, there is a marked difference in the results obtained in both *in vitro* systems. This is likely because the concentration of IHH^{V190A} in the media collected from the HEK(IHH^{V190A}) cell lines was extremely low. Therefore, we were forced to incubate the differentiating ATDC5 cells in a volume of media consisting almost entirely of concentrated media from HEK(IHH^{V190A}) that contained neither insulin, nor an adequate amount of IHH^{V190A}. As a result, it appears as though the cells were arrested in early-phase differentiation. Further study is necessary to truly ascertain the properties of the V190A mutation in IHH.

In vitro Summary

To recapitulate, all of the BDA1 mutations that we analyzed – E95K, D100N, R128Q, and E131K – introduce a residue with properties different to those of the native residue, and that all mutations lie in relatively close proximity to residues necessary for facilitation of Zn²⁺ coordination. These mutant proteins likely disrupt protein folding, which subsequently reduces the amount of IHH precursor being processed into IHH-N.

Furthermore, we have shown that the mutated IHH-N variants have a significantly lower level of secretion relative to IHH^{WT}, and we have inferred that this is a result of a decreased tendency to multimerize as a result of the mis-folding of the protein and subsequent increased probability of proteolysis. Finally, the secreted BDA1-associated mutant IHH not only has a significantly lower ability to induce IHH signalling, but also appears to confer a dominant-negative effect over IHH^{WT}.

Chapter 5

Analysis of angiogenesis in the zebrafish, *Danio rerio* and an *In Vivo* characterization of BDA1- and ACFD-causing mutations in Human *Indian hedgehog* in the zebrafish model

5.1 Introduction

Hedgehog signalling plays a vital role in signalling across species of vertebrates and arthropods. Various hedgehog proteins are responsible for the induction and patterning of multiple tissues and organs in both phyla [100]. Indian hedgehog in particular has been shown to play a role in the formation of haematopoietic progenitors [99], development of the gut [104], angiogenesis [61, 66, 105], and both endochondral and intramembranous ossification [19, 29, 34]. These key functions of Indian hedgehog are conserved across species [34]. In the zebrafish (*Danio rerio*), for example, it has been shown that *ihha* (the Indian hedgehog ortholog) plays a key role in both endochondral and intramembranous ossification in the head and flat cells surrounding cartilaginous structures [77]. The sequence of the zebrafish Ihha signalling peptide displays 93% similarity and 90% exact amino-acid conservation with murine Ihh [77]. The mature transcript is 1327 bp in length, which codes for 413 amino acids [77].

The signalling pathway of zebrafish Ihha, is very similar to that observed in other vertebrate Hh signalling. Ihha produced, primarily in the notochord in the early stages of zebrafish development, is localized and secreted through the aid of dispatched, *disp1* [106]. The protein targets its receptor patched (Ptch), which, when activated, relieves inhibition of Smoothened (Smo) [34, 107]. Once inhibition has been relieved, Smo mediates an intracellular process that regulates the processing of the zebrafish homologs of the transcriptional activator Glis; Gli1 and Gli2, and the transcriptional repressor Gli3 [34, 44, 108]. In addition to this, there are additional players seemingly unique to zebrafish that play a regulatory role downstream of *ihha*; e.g. β -Arrestin 2, and Scube2 [44, 109]. In total, there are five hedgehog family members in the zebrafish; *Sonic*

hedgehog, *shh*; *Indian hedgehog*, *ihha*, *desert hedgehog*, *dhh*; *tiggy-winkle hedgehog*, *twhh*; and *echidna hedgehog*; *ehh/ihhb* [74, 75, 77].

The zebrafish provides a highly tractable model for the analysis of hedgehog signalling in vertebrate development. The transparent embryo ideally facilitates the use of the anti-sense morpholino (MO) technology. A morpholino is a short-sequence oligonucleotide that, in place of a phosphate-ribose backbone, possesses a phosphorodiamidate-morpholine ring backbone. The sequence is designed to be complementary to a sequence flanking the start site of the transcript of interest. When the MO binds to its complementing sequence, it sterically hinders the binding of the ribosomal complex, thus preventing translation of the message. In this instance, we have designed an MO anti-sense to the start site of the *ihha* transcript. The binding of these oligonucleotides is highly specific. A variation of only three nucleotides in the *ihha*-MO sequence would make it incapable of binding to the start site of the *ihha* transcript. An alignment of the *ihha*-MO sequence with the family of hedgehog genes, along with our *in vitro* transcribed human *IHH*^{WT}, is illustrated in table 5.1.

In addition to analyzing the expression pattern of *ihha* in the developing zebrafish, we wanted to examine whether over-expression of human *IHH* would be able to rescue the morphant phenotype, and whether there would be any significant difference in the degree of rescue between the morphants injected with *IHH*^{WT} or those injected with one of the BDA1- or ACFD-associated mutations. Ultimately, we discovered that over-expression of human *IHH* was able to improve the morphant phenotype, and that the wild-type was able to do so more effectively than the mutant transcripts. Furthermore, we observed an unexpected phenotype in the zebrafish morphant; namely a decrease in

Table 5.1. Alignment of various hedgehog genes with *ihha*-MO. The start codon for each hedgehog of *Danio rerio* and for our synthetic *IHH* mRNA are aligned. The identity of each sequence is indicated on the right. Letters in red indicate regions of overlap with the morpholino. Letters in blue indicate the locations on the *ihha*-MO that were randomly changed for the mis-matched control morpholino. Abbreviations: *dhh*: desert hedgehog; *ihha*: Indian hedgehog α ; *ihha*-MO: Indian hedgehog α - morpholino; *shh*: sonic hedgehog; *twhh*: tiggy-winkle hedgehog; *ihhb*: Echidna hedgehog; *Hsa IHH*: *Homo sapiens* Indian Hedgehog.

Start

CTAGCAAAC~~T~~CCACGCGCGTCAATGACGTTGGCTCCTTGGTTAAGGCTGG
GAGTGCCCGGCCGCTTGC~~G~~GTGGAATGCGTCTCCCCGTGGTGT~~T~~TGG
CGCTTGC~~G~~GTGGAATGCGTCTCC
AGATT~~C~~AGCGTGCGGCAAATGCGGCTTTTGACGAGAGTGC~~T~~GC~~T~~GGTGT
ATGGACGTAAGGCTGCATCT
ATGAGACTCTCCACGGCGGC
TTGTTCTTTTTGCAGGATCCATGTCTCCCGCCCGGCTCCGG

dhh
ihha
ihha-MO
shh
twhh
ihhb
Hsa IHH

physical size, a slight bend in the trunk and tail of the embryo, rounded as opposed to chevron-shaped somites, and edema of the cardiac chamber. Considering that *Ihh* has been shown to play a role in angiogenesis [61, 62, 66, 105], we decided to examine the morphant using the transgenic *fli1*:EGFP zebrafish line [110]. This line was engineered to express EGFP under the control of the *fli1* promoter. *fli1* is a known endothelial marker in the mouse [111], which has also been shown to be expressed in zebrafish vascular development [81]. Therefore, when analyzed under UV light, expression of EGFP allows for visualization of the developing vasculature. Furthermore, although blood circulation starts at 24 hpf, zebrafish embryos can survive up to 7 dpf without a functional vasculature or heart beat, as they absorb oxygen through their skin [112, 113]. This makes the *fli1*:EGFP an ideal model through which to analyze vasculature development.

Through our analysis we have provided additional evidence to support the concept of functional redundancy between Hedgehog genes across vertebrate species. And, we have also shown, through an experiment analyzing the injection of the BDA1- and ACFD-associated mutant transcripts alone, that *in vivo*, the BDA1-associated mutations can confer a dominant-negative effect over endogenous *Ihha* and result in altered development of the zebrafish larvae.

5.2 Materials and Methods

5.2.1 Design and development of *ihha* morpholino

Morpholino phosphorodiamidate oligonucleotides (MO), were designed by Gene Tools (Philomath, OR). The MOs are designed to inhibit translation of *Danio rerio ihha* mRNA (Genbank accession number BC133983). We used one MO, designed to bind to the start site and a portion of the 5' UTR of *ihha*, with the following sequence: *ihha*-MO: 5' – GGGAGACGCATTCACCCCAAGCGG – 3'. In addition to this, a mismatched morpholino was designed to act as a control with the following sequence: *ihha*-MM-MO: 5' – GGGAGAGGCATTCAGCCCATGCGG – 3'. To determine the efficacy of morpholino distribution, both the *ihha*-MO and *ihha*-MM-MO were fluorescein-labeled.

5.2.2 Production of *pCS2Ihh* constructs

It was our intention to excise our *IHH* cDNA out of pcDNAIhhWT using the flanking restriction sites *Bam*HI and *Apa*I and insert the subsequent restriction digested product into a likewise-digested pCS2+ plasmid. However, to do so would excise the essential SV40 polyA tail region from the plasmid, which is essential for adequate translation of our synthetic mRNA. Therefore, in a 20 µL volume, including 2 µL of 10X Buffer M (Amersham Biosciences; GE Healthcare, Buckinghamshire, UK), 800 ng of pCS2+ was digested with 1 µL of *Xba*I. The reaction mixture was incubated at 37°C for one hour in an MBS Satellite 0.2G thermal cycler (Thermo Electron Corporation, Milford, MA). Separately, 800 ng of each of the pcDNAIhh constructs was digested in a reaction volume of 20 µL, including 2 µL of NEBuffer #4 (New England Biolabs), 1 µL

10% BSA, and 1 μ L of *Apa*I – the reaction was incubated at 25°C for one hour in the thermal cycler. To both the digested pcDNAIhh constructs and the digested pCS2+ plasmid, we added 1 μ L of Klenow enzyme, 5 μ L of NEBuffer #2 (New England Biolabs) and 1 μ L of 7.5 μ M dNTPs. The reactions were incubated at 30°C for one hour. After this, to each tube, 5 μ L of 10X K Buffer (Amersham Biosciences; GE Healthcare, Buckinghamshire, UK), and 1 μ L of *Bam*HI was added, and the reactions incubated at 30°C for one hour. The products were separated on a 1% agarose gel, and the appropriate bands were excised and purified using the GE Healthcare illustra GFX PCR DNA and Gel band purification kit (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's specifications. Five μ L of each purified sample was electrophoresed on another 1.0% agarose gel for 25 minutes at 100V. This gel was subsequently photographed under UV light and the concentration of each sample was calculated based on comparison of band intensity with bands of the HighRanger Plus 100bp DNA ladder (Norgen Biotech, Thorold, ON). These digested and purified products were ligated in a 30 μ L reaction with 50ng of pCS2+ and, stoichiometrically, 4 times that amount of insert (one of either the WT or mutant *IHH* cDNA), along with 3.0 μ L of the 10X ligase buffer, and 1.0 μ L of 1U/ μ L T4 DNA ligase (both from Invitrogen, Carlsbad, CA). This reaction mixture was incubated at 16°C in the MBS Satellite 0.2G thermal cycler (Thermo Electron Corporation, Milford, MA) for 16 hours. The following day, the ligated products were transformed in competent *E. coli* DH5 α (Invitrogen, Carlsbad, CA) by heat-shock at 42°C. An inoculated suspension of the transformants was plated on a Luria-Bertani (LB)-agar plate, containing ampicillin (1 μ g/mL), and incubated overnight at 37°C. Colonies were selected and used to inoculate a 5 mL seeding culture, which was

incubated overnight at 37°C until the optical density (OD) at 600 nm (OD₆₀₀) reached 1.0. The plasmid was isolated using the GenElute™ Plasmid Miniprep kit (Sigma, St. Louis, MO), according to the manufacturer's specifications. Successful production of each pCS2IhhWT construct was confirmed by digestion of the preparation of plasmids with *EagI*.

5.2.3 *In vitro* transcription of pCS2Ihh variants

Capped *IHH* mRNA was made by *in vitro* transcription using an Sp6 polymerase (mMessage Machine; Ambion Inc, Applied Biosystems, Foster City, CA), according to manufacturer's specifications. The pCS2IhhWT plasmid was linearized with *Bam*HI. The *in vitro* transcribed RNA was re-suspended in PCR-grade H₂O for storage. At the time of injection, the mRNA solution was diluted in 1X Danieau solution [58mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, 5 mM Hepes, pH 7.6].

5.2.4 *Zebrafish* husbandry

For this study we used the transgenic *fli1*:EGFP line of *Danio rerio* – generation of this line is described in Lawson and Weinstein *et. al.* [114]. The strains were maintained in a continuously recirculating system with 14 hr light and 10 hr dark cycle at a constant temperature of 28.5°C. Zebrafish were fed on a regular basis. Fish to be bred were separated in the late afternoon or evening into males and females in their own respective crossing tanks containing the filter insert. The following morning the male fish were added to the crossing tank containing the female fish. At the time of

fertilization and release, the embryos would fall through the perforated insert and rest at the bottom of the tank. Embryos were collected and placed in a Petri dish.

5.2.5 *Morpholino and RNA injection*

For the ‘rescue’ experiment, solutions were prepared of either 1 µg/µl *ihha*-MO and 0.1 µg/µl synthetic *IHH* mRNA, or 1 µg/µl *ihha*-MO alone, diluted in 1X Danieau solution [58mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, 5 mM Hepes, pH 7.6]. The sequence of the synthetic *IHH* mRNA contained very little overlap with the *ihha*-MO (Table 5.1) - insufficient to bind to the synthetic message and prevent translation. The second experimental group was injected with synthetic *IHH* mRNA only. A solution of synthetic mRNA to a final concentration of 35 ng/µL in the same 1X Danieau solution was prepared.

A 1.0 mm injection needle was prepared by pulling a 10 cm borosilicate capillary pipette (1.0 mm outside, and 0.5 mm inside diameter; Sutter Instrument company, Novato, CA) using a P-87 Flammig/Brown micropipette puller (Sutter Instrument company, Novato, CA).

Using a micromanipulator and a Narishige IM300 microinjector (Narishige International USA, Inc., East Meadow, NY) set to the following settings: the volume per injection was calculated to be 3 nL. Embryos were injected at the 1-to-2 cell stage with the capped-mRNA/*ihha*-MO mixture using the same micromanipulator and microinjector set to the same settings. The solution was injected into the yolk sac, as previously described [115]. For each sample group, between 50 and 80 embryos were injected with either 3 ng of *ihha*-MO alone or in combination with 700 pg of either wild-type or mutant

IHH mRNA. Embryos injected with *IHH* mRNA alone, were injected with 100 pg of either *IHH*^{WT} mRNA or one of the BDA1- or ACFD-associated mutant species.

5.2.6 *Classification of the knockdown phenotype*

After injection, the embryos were sorted according to sample injected, and separated into respective Petri dishes. To each dish, phenylthiourea (PTU) was added to a final concentration of 0.001%, approximately 3 hours post-fertilization. The following morning, 24 hours post-fertilization, the embryos were analyzed under UV light, and the few embryos that were not fluorescing to the same intensity as most were removed. The remaining embryos were dechorionated chemically by the addition of pronase to the embryo water to a final concentration of 0.003%. Once all embryos were dechorionated, they were transferred to another Petri dish with fresh embryo water containing 0.001% PTU. Each day, dead embryos were removed. 3 days post-fertilization (dpf) the embryos were examined using the Leica dissection microscope (Leica MZ FLIII) with integrated FLUOIII filter system. Photographs were captured using an attached Sony 3CC colour video digital camera system and accompanying Axiovision software.

The two major experiments employing the *ihha*-MO and the synthetic *IHH* mRNA were classified by two different systems. For the co-injection or rescue experiment, a four-class system was employed. Those fish showing no signs of an altered phenotype were classified as ‘class 1’ (figure 5.1). Those showing characteristics of a mildly altered phenotype, e.g. slightly decreased size, some aberrant vascular branching, are classified as class 2 (figure 5.2). Those classified as class 3 (figure 5.3) possess typically the same signs of an altered phenotype as class 2 but to a much greater

Figure 5.1. Class 1 larvae. **A** and **A'** show fluorescence and brightfield images, respectively, of lateral view of an uninjected control *fli1*:EGFP larvae, 3 days post-fertilization. Images display phenotypically normal embryonic development; expected body size, full development of the dorsal fin (DF) and ventral fold (VF; indicated by white arrows), normal development of the cardiac chamber (cc). Note the size and regular organized pattern of the caudal vein plexus (CV). White box in **A** and **A'** panels represent sections of embryo examined under higher magnification in panels **B** and **C**, respectively. The arrows in panel **B** show the presence of fully developed dorsal longitudinal anastomotic vessel (DLAV), parachordal vessels (PAV) and intersegmental vessels (SeV), which are indicative of a class one, phenotypically normal phenotype. Panel **C** shows the characteristic chevron pattern of somites (black arrow), typically possible through proper development of intersegmental vessels (SeV), schematically represented in panel **D** (Modified from Ny *et. al.*, 2006). FP, floor plate; My, myoseptum; NC, notochord; S, somites. A darkfield image embryonic midsection of a class 1 IHH^{WT} rescue attempt, 4 days post-fertilization (**E**). The light fluorescent across the organism indicates the presence of the morpholino.

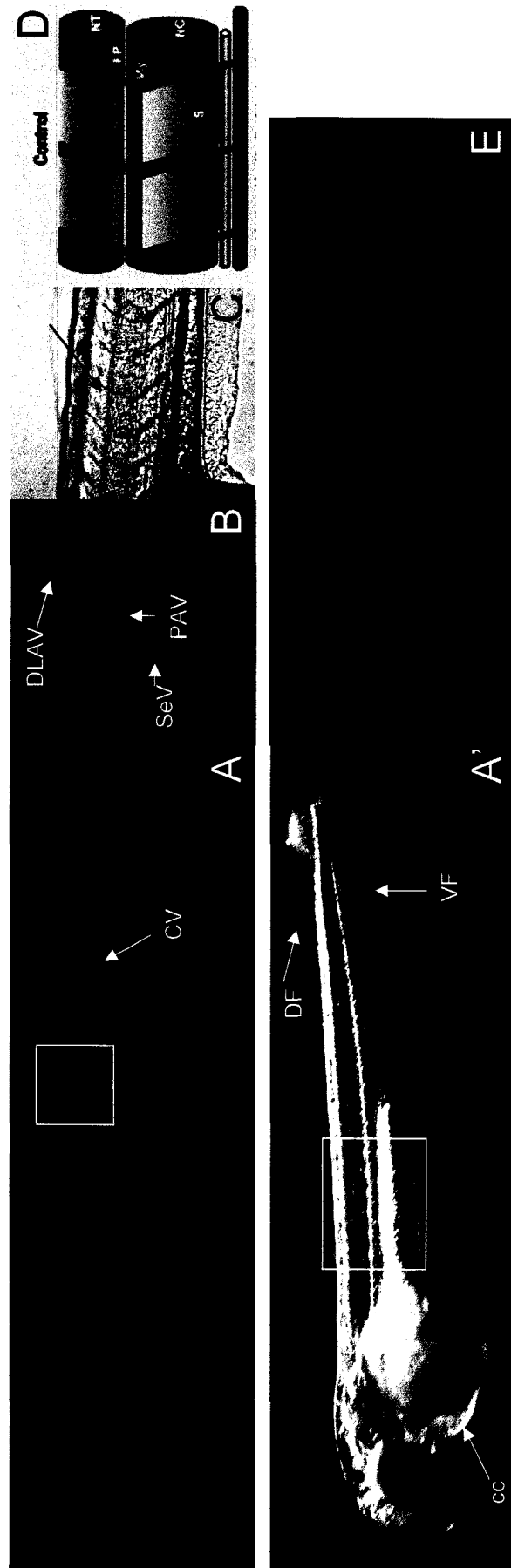


Figure 5.2. Class 2 larvae. Images of 3 dpf larvae injected with MO plus IHH^{E131K}(**A**), IHH^{D100N}(**B**), IHH^{R128N}(**C**), all larvae were classified as class 2. These appear predominantly phenotypically normal, however, possess one or two delays in development. Some embryos may display a slight bend in the trunk (**A**), a somewhat altered intersegmental vessel (ISV) branching pattern (**B**), or a somewhat decreased physiological size (**C**) in comparison to a class 1, phenotypically normal embryo (**D**). An embryo can be defined as class 2 if it displays aberration of somite segmentation in the trunk (**E**, black arrow) in comparison, again, to the class 1 phenotype (**F**). If an embryo appears almost completely phenotypically normal, and yet displays altered branching of ISVs, they are also classified as a class 2 embryo (**G**). Note also the loss of PAV branching (**G**). SeV, intersegmental vessel; DLAV, dorsal longitudinal anastomotic vessel.

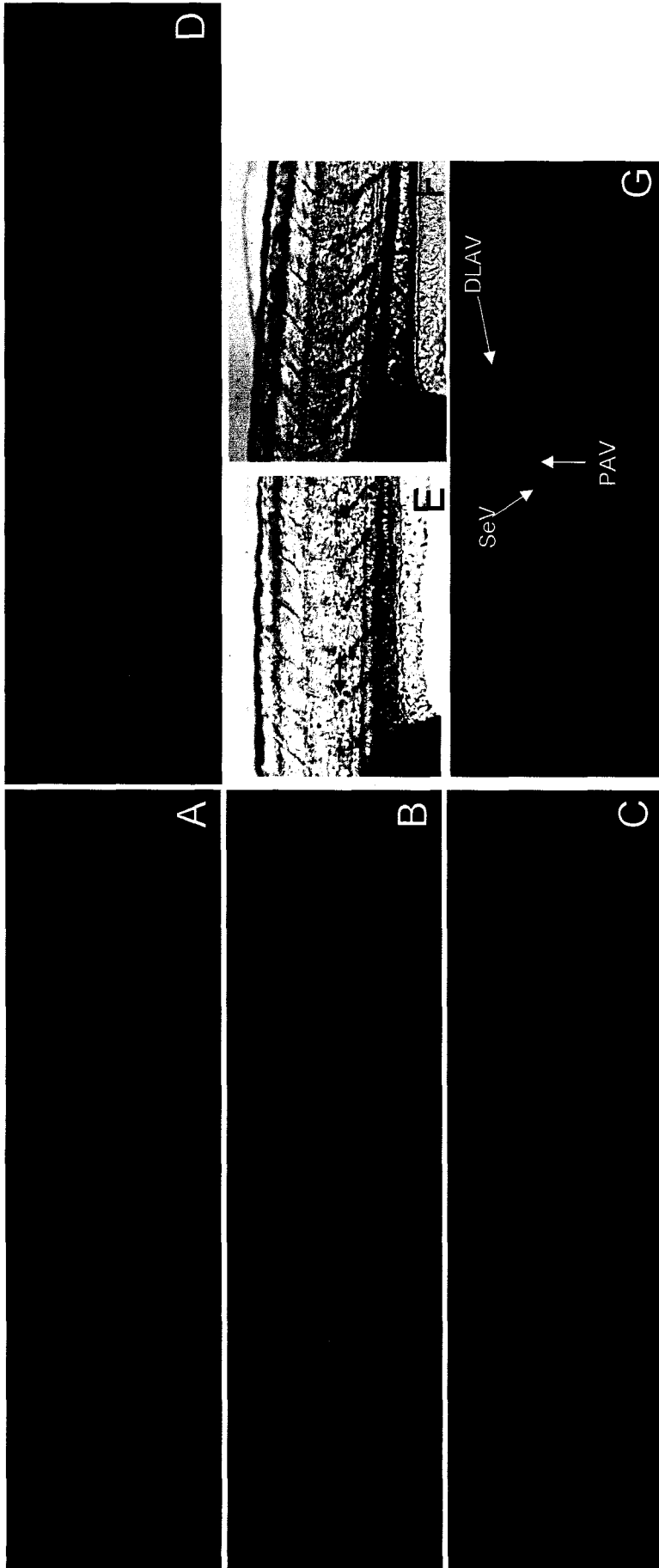


Figure 5.3. Class 3 larvae. Brightfield (**A** and **B**) and fluorescent (**A'** and **B'**) images of two different larvae classified as class 3. The classification of a larva as class 3 is dependent upon the presence of two or more developmental abnormalities but not so extreme in phenotype as to be classified as a class 4. Some larvae may display normal growth in size and fin development, yet loss of somite segmentation and extremely altered vasculature and slight hypertrophy in the cardiac chamber (cc), as visible in panel **A** (somites and cardiac chamber indicated by black arrows) and panel **A'** (white arrow indicates altered vasculature). Other embryos may display a decrease in size, altered fin development (**B**, black arrow), edema of the cardiac chamber (cc, **B**, black arrow), loss of somite segregation (S, **B**, black arrow) and an extremely under-developed vasculature (**B'**, white arrow), in comparison to a class 1 embryo (**C**), and yet this phenotype is not extreme enough to warrant a class 4 classification. Panel **D** shows the possible altered vasculature development of a class 3 embryo - note though the normal development of the dorsal aorta (DA). Panel **E** illustrates a schematic representation of how altered ISV branching can result in the loss of the characteristic chevron pattern of the somites (Modified from Ny *et. al.*, 2006).

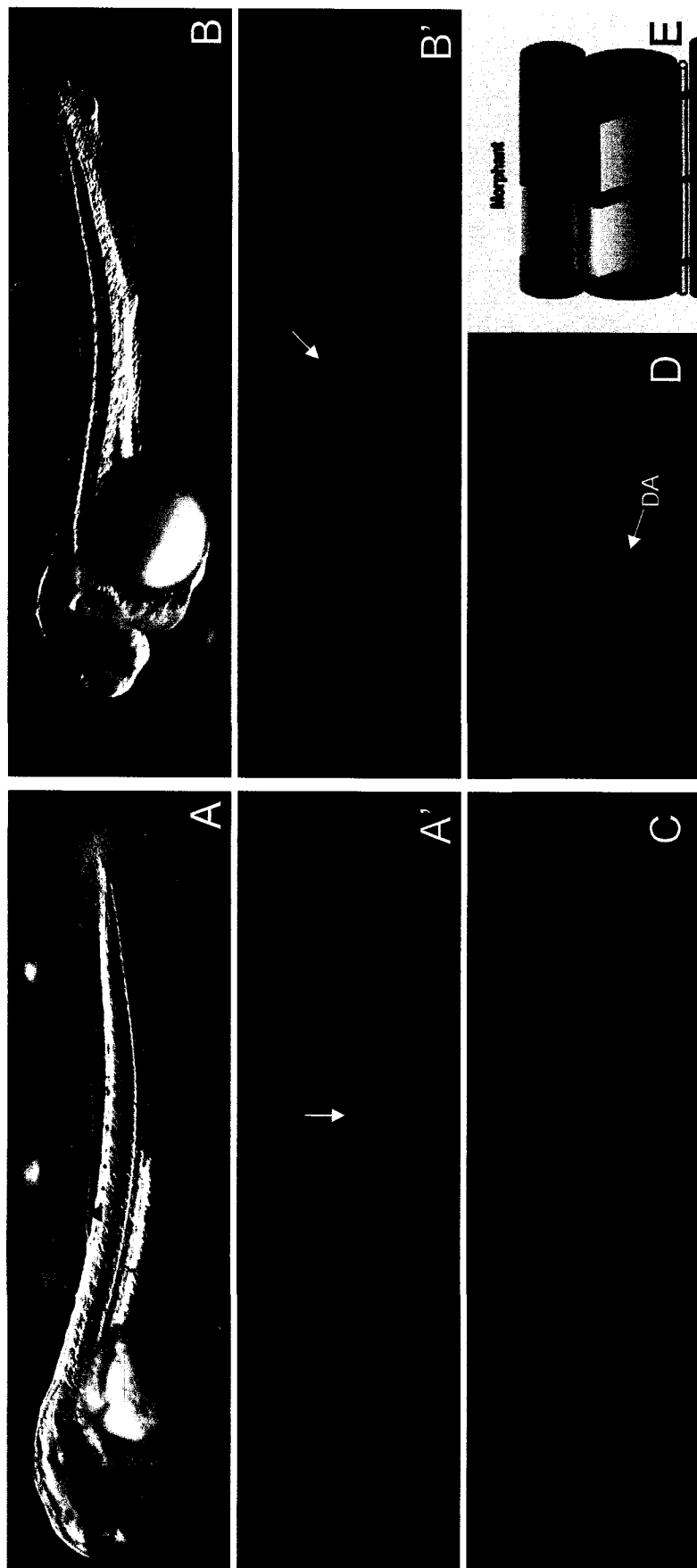
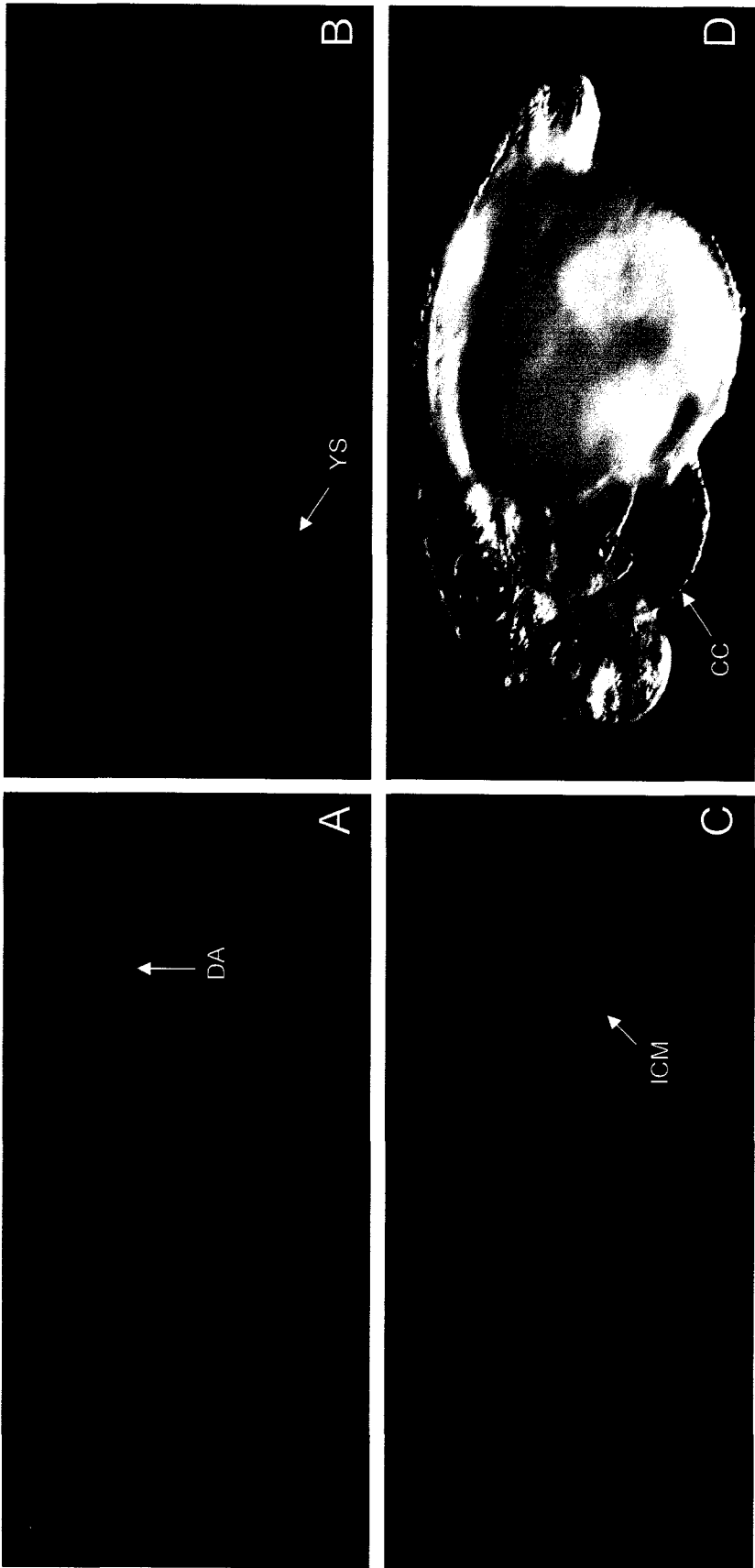


Figure 5.4. Class 4 larvae. Fluorescent (**A**, **B**, **C**) and brightfield (**D**) images of class 4 larvae. These larvae display extreme developmental abnormalities as a result of morpholino knockdown. All cases display a significant decrease in size, often complemented by a comparatively enlarged yolk sac (YS, **B**, white arrow), a bend in the trunk of the embryo (evident in all panels), a severely altered vascular branching pattern (**A**, white arrow), hypertrophy of the intermediate cell mass (ICM, **C**, white arrow), edema of the cardiac chamber (CC, **D**, white arrow), or separation of the two chambers of the heart (**D**, black arrow). Note in panel **A**, the normal development of the dorsal aorta (DA).



degree. Finally, class 4 larvae are those that are severely affected (figure 5.4). All classification assessments were made blind – once all of the embryos for a certain sample group were injected with the designated solution, tape was used to cover the identity of the group written on the Petri dish, and a third party randomly assorted the plates and numbered them. Once the assessment was completed, I removed the tape and recorded the sample group.

In the second experimental group, analyzing the effects of injection of synthetic mRNA alone, larvae were classified by a three-class system. Larvae were classified as either phenotypically normal (class 1), mildly affected (class 2), or severely affected (class 3). This classification system was employed to more closely resemble the classification systems in the literature. Also, in this experiment the differences between each class was much more distinct than in the rescue experiment. The class 1 larvae are completely normal in their development, the class 2 larvae resemble class 3 larvae from the rescue experiment (figure 5.3), and the class 3 larvae resemble the class 4 larvae from the rescue experiment (figure 5.4).

5.2.7 *Alcian Blue staining*

At 5 dpf, a selection of larvae - some un-injected, and some injected with either *ihha*-MO alone or a combination of *ihha*-MO and wild-type *IHH* mRNA – were fixed in 4% paraformaldehyde (PFA) and stored at 4°C overnight. The following day the fixed embryos were washed multiple times with 1X PBS. Larvae, in eppendorf tubes, were treated with 1 mL of 10% H₂O₂, and 25 µL of 2M KOH, and left exposed to the air at room temperature for one hour. At this point the larvae were again washed multiple

times with 1X PBS. The PBS solution was aspirated and 1 mL of 0.1% alcian blue solution in 70% ethanol and 1% HCl was added to each tube and the tubes were incubated on a rocking platform overnight. The following morning the alcian blue solution was aspirated and discarded and 1 mL of acidic ethanol solution (5% HCl in 70% ethanol) was added to each tube and incubated for five hours at room temperature. The larvae were then dehydrated by successive 20 min incubations on a rocking platform in 85%, and then 100% ethanol. The larvae were stored in 80% glycerol until the time of examination.

5.3 Results

5.3.1 *ihha* knockdown phenotype in *Danio rerio*

It was our intention to analyze the effects of over-expression of injected synthetic human *IHH* mRNA on an *ihha*-null background in the developing zebrafish. In order to achieve that status, 3 ng of *ihha*-MO were injected into each embryo at the 1-to-2 cell stage immediately following fertilization. Initially, our group opted to analyze the effects of the *ihha*-MO by analyzing an injected wild-type embryo at the 3 dpf stage. We had initially expected not to see any sign of altered development until at least 4 dpf, as that is the point, according to previously published literature, in larval development that *ihha* is first detected [77]. However, the *ihha*-knockdown phenotype displayed numerous signs of altered development; most notably a decrease in size (figure 5.2a,b&c), a slight bend in the trunk and tail of the embryo (figure 5.3a&b), altered somitic segregation (figure 5.2e&f,) and edema of the cardiac chamber (figure 5.4). Interestingly, the embryos that

showed a bending in the trunk and tail did not bend in the same direction as the trunks of *sonic-you* (*syu*) zebrafish mutants lacking *shha* [116, 117]. Furthermore, the disruption of the chevron patterning of the somites did not produce the same ‘U-shaped’ pattern typical of the ‘you-type mutants’, or strains of zebrafish containing a mutation in one of the hedgehog signalling pathway members [116]. The result we observed was more of a loss of the distinct barrier between each somite. In the embryos most severely affected by knockdown of *ihha* expression, separation of the two chambers of the heart was evident (figure 5.4d). Consequently, there was a bizarre pattern of sequential contraction of each chamber – an indication of the extreme stress being put on the circulatory system of the morphant embryo. As previously indicated, hedgehog signalling has been implicated in development of the vasculature in vertebrates. Most vasculature development-related hedgehog research has focused on *SHH* [118, 119], some recent studies have shown that *IHH* also plays a role in angiogenesis [61, 62, 66, 105]. Therefore, we used transgenic *fli1:EGFP* line in an attempt to examine whether the significantly altered development was a result of aberrant development of the vasculature.

When analyzed at 3 dpf, the morphant larvae displayed a significantly altered vascular phenotype when analyzed under UV light. In comparison to the un-injected control group, morphant larvae displayed altered vasculature development to varying degrees. In the milder cases, individual morphant larvae showed at first a loss of the branching of the parachordal vessels (PAV), which connect the intersegmental vessels [79] (figure 5.1b vs. figure 5.2g). Those morphant larvae that were severely affected by the knockdown of *ihha* displayed ectopic segmental vessel sprouts from the dorsal aorta (DA) that were dispersed throughout the trunk, or incomplete development of the dorsal

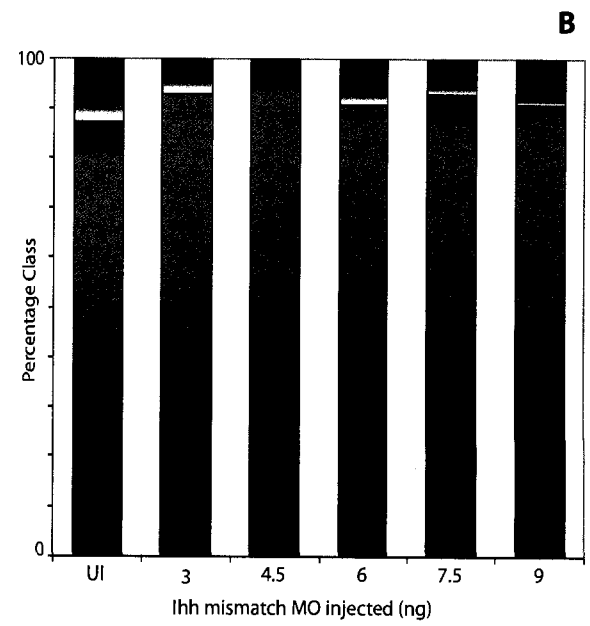
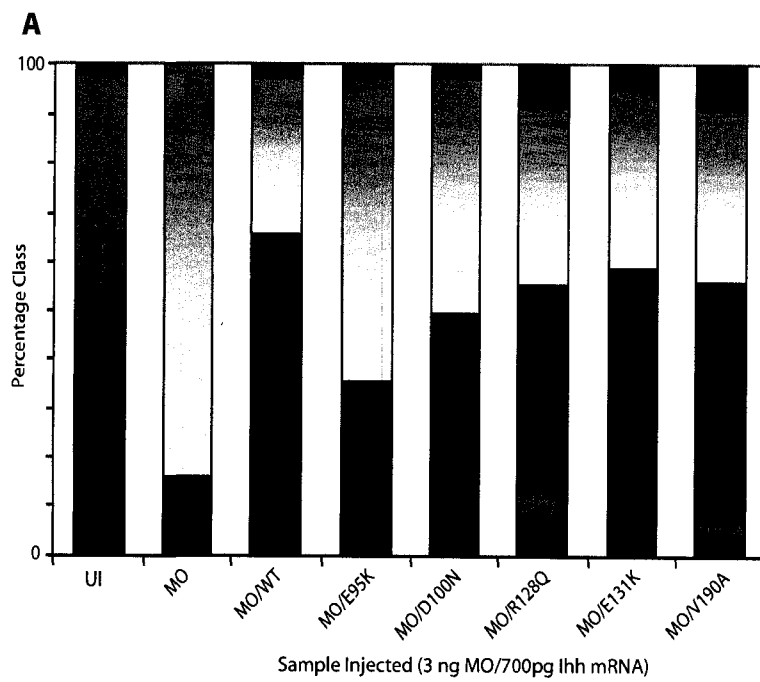
longitudinal anastomotic vessel (DLAV) (figure 5.3d). In the more severe cases, morphant embryos showed a near complete absence of the intersegmental vessel (SeV) vessel sprouting, and subsequently no development of the DLAV – a consequence of anastomosis of SeV. Interestingly, in all cases, the development of the DA was normal. As the DA is the product of vasculogenesis, or the *de novo* generation of blood vessels, and the branching SeV, PAV and DLAV are a product of angiogenesis – a process in which hedgehog activity has been previously indicated [61, 62, 66, 105] – it is likely that these results indicate that despite previous indications [77], *ihha* is expressed prior to 4 dpf and is involved in angiogenesis in the early stages of zebrafish development.

5.3.2 *IHH overexpression on an ihha-null background in Danio rerio flil:EGFP*

As mentioned, multiple embryos in each sample group were injected with 100 pg of in vitro transcribed *IHH* mRNA – one of either the mutant variants or wild-type samples. In each group, equal level and distribution of fluorescence throughout the embryo was evident, as a result of excitation of the fluorescein molecule attached to the 3' end of the MO, indicating the successful introduction of the *ihha*-MO. In a preliminary experiment we homogenized the zebrafish embryos and analyzed protein expression by western blot. We did confirm that there was in fact expression of our synthetic *IHH* mRNA (data not shown).

As indicated previously, within these sample groups, there was a distinct range of affected phenotypes. It was our initial belief that translation of synthetic human *IHH* mRNA would compensate for the loss of *ihha* expression in those cells expressing the key players of the Hedgehog signalling network. Therefore we classified the degree to

Figure 5.5. *Ihha*-MO/*IHH* mRNA rescue experiment. Tabulated representation of the percentage of each class per each group of injected embryos for the rescue experiment (A) and the mis-matched *ihha*-MO control group (B). In the rescue experiment (A), each group was injected with a combination of 3 ng of *ihha*-MO and 700 pg of synthetic *IHH* mRNA (indicated in each lane). In the mis-matched control group, embryos were injected with the mis-matched *ihha* control morpholino - the amount injected is indicated in each lane in ng. Each group was analyzed and classification assessed at 3 dpf. Colour coding used throughout diagram: light blue, class1; dark blue, class 2; yellow, class 3; red, class 4.



which the phenotype of each larvae was affected in a 4-class system described above – with phenotypically normal larvae classified as class one (figure 5.1), mildly affected larvae classified as class 2 (figure 5.2), moderately affected larvae; class 3 (figure 5.3), and the most severely affected were classified as class 4 (figure 5.4). Between 50 and 80 embryos were injected in each sample group. Nearly all of the uninjected control group was exclusively class 1 phenotypically normal larvae (98%) (figure 5.5a). Conversely, the group of embryos injected with 3 ng of *ihha*-MO only, nearly 4 out of 5 embryos were class three, moderately affected. When embryos were injected with a combination of 3 ng of *ihha*-MO and 700 pg of *IHH*^{WT} mRNA, there was a greater than 4-fold improvement in the number of mildly affected, class two larvae, and a 3-fold decrease in the amount of moderately affected larvae, class three larvae. This evidence showed that, although unable to completely rescue the phenotype to that observed in the un-injected control embryos, there was a marked improvement in the distribution of the affected phenotypes from the group injected with 3 ng of *ihha*-MO only. In fact, this general trend was evident in all groups injected with a combination of MO and *IHH* mRNA. However, no group showed the same combination of either phenotypically normal class 1, or mildly affected class 2 embryos as those injected with the combination of *ihha*-MO and *IHH*^{WT} mRNA (figure 5.5a). In each case of embryos injected with both *ihha*-MO and one of *IHH*^{D100N}, *IHH*^{R128Q}, *IHH*^{E131K}, or *IHH*^{V190A} mRNA were almost equally distributed between class 2 and class 3 affected embryos, with a small amount of class 1 and class 4 in select examples (figure 5.5a). However, the group of embryos injected with a combination of *ihha*-MO and *IHH*^{E95K} mRNA showed the least degree of rescue

from the morphant phenotype, with 33% class 2, 65% class 3 and 2 % class 4 larvae (figure 5.5a).

To show that the morphant phenotype was the result of a knock-down of *ihha* expression and not of the presence of the MO itself, we designed a ‘mis-matched’ MO with only three nucleic acids altered randomly throughout the *ihha*-MO sequence. The location of the changed nucleotides is illustrated in table 4.1. Five different sample groups were injected with different total amounts of *ihha*-MM-MO ranging from 3 to 9 ng. In each case, there was no difference in the distribution of phenotypes when compared to the un-injected control group (figure 5.5b). A BLAST search of the *ihha*-MO was conducted and the only sequence with homology greater than 80% that flanked a start sequence were the other zebrafish hedgehog orthologs. In each case, the sequence showed greater variation to the *ihha* sequence than the mis-matched MO. This would indicate that the morphant phenotype is specifically a result of the knockdown of *ihha* expression and not of the presence of the MO itself or knockdown of an unintended target gene.

5.3.3 *Homo sapiens IHH mutants have dominant negative effect on Danio rerio ihha*

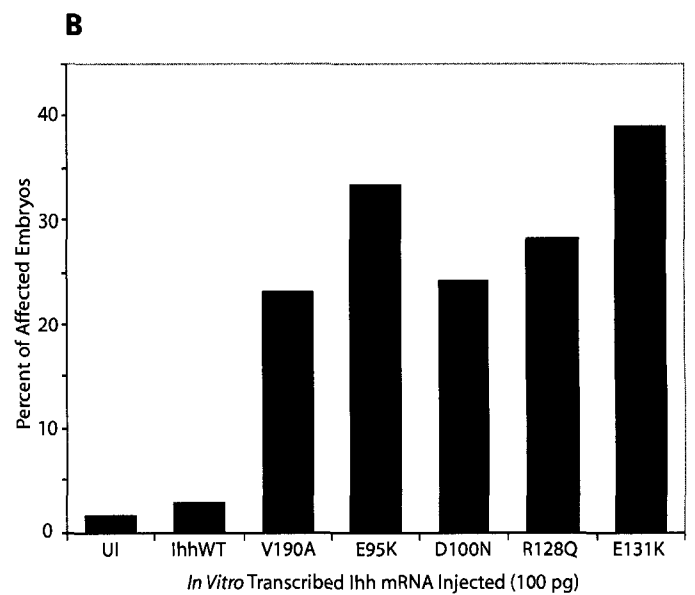
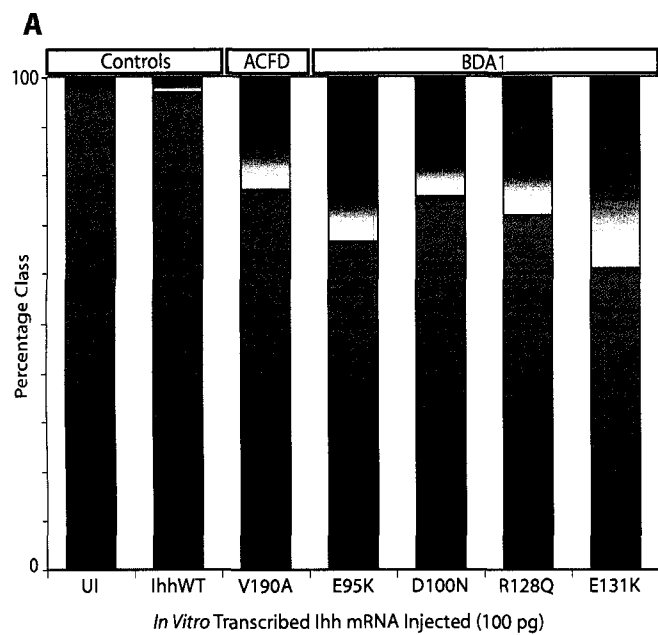
We have demonstrated that over-expression of *in vitro*-transcribed *IHH* mRNA can generally improve the phenotype of zebrafish embryos injected with *ihha*-MO, however, it was also clear that over-expression was not sufficient to completely rescue the wild-type phenotype. We wanted to analyze whether over-expression of human *IHH* mRNA by itself had a detrimental effect on the development of the embryo and whether

there was a difference in the degree to which the embryo was affected between those injected with *IHH*^{WT} or one of the BDA1- or ACFD-associated *IHH* mutants.

Each *fli1*:EGFP embryo was injected with 100 pg of *in vitro* transcribed *Ihh* mRNA in the yolk sac at the 1-to-2 cell stage, immediately following fertilization. Considering that *ihha* expression is not detectable in the zebrafish by *in situ* hybridization prior to 4 dpf, we chose to inject a low quantity of 100 pg so as not to inflict a greater developmental reaction as a result of human *IHH* over-expression. Injection of the sample suspended in the phenol red in the 1X Danieau solution facilitated confirmation of injection. Multiple other studies have demonstrated that mRNA is ubiquitously distributed. Unlike the previous rescue experiment, in this instance we opted to use a 3-class system for classification; class 1: phenotypically normal; class 2: mildly affected; class 3: severely affected.

Of the uninjected control group, only 1.7% showed an altered development in any way (either class 2 or class 3). Interestingly, only 2.9% of the group injected with 100 pg of *IHH*^{WT} mRNA showed any signs of altered development; not significantly different from the un-injected control group (figure 5.6a&b). The group of embryos injected with one of the BDA1-causing mutations showed a greater degree of being affected by over-expression of the mutant message. The percentage of affected (class 2 and class 3) larvae ranged from 24.2% and 28.1% in the *IHH*^{D100N} and *IHH*^{R128Q} groups, respectively, to 33.3% and 38.9% in the *IHH*^{E95K} and *IHH*^{E131K} groups, respectively (figure 5.6b). Interestingly, this pattern, of the *IHH*^{E95K} and *IHH*^{E131K} groups showing a greater significant difference from the wild-type group than the other two BDA1-associated mutant groups is consistent with the results we obtained from our *in vitro* analysis of the

Figure 5.6. Over-expression of synthetic *IHH* mRNA in *Danio rerio*. Tabulated representation of the percentage of each class group per each group of embryos injected with 100 pg of the indicated *in vitro* transcribed *IHH* mRNA. Panel **A** shows the distribution of the percentage of larvae presenting each phenotypic classification per injection group. Panel **B** illustrates the percentage of each injection group that was affected, either mildly or severely. each group was analyzed and classification assessed at 3 dpf. Colour coding used in panel A: light blue, phenotypically normal; yellow, mildly affected; red, severely affected.



mutant peptide. The fact that injection of *IHH*^{WT} mRNA appears to have little to no effect on the development of the zebrafish embryo, contrary to the injection of the BDA1-associated mutations is a further indication that these mutants could confer a dominant negative effect over endogenous *Ihha* that is targeting the same cells.

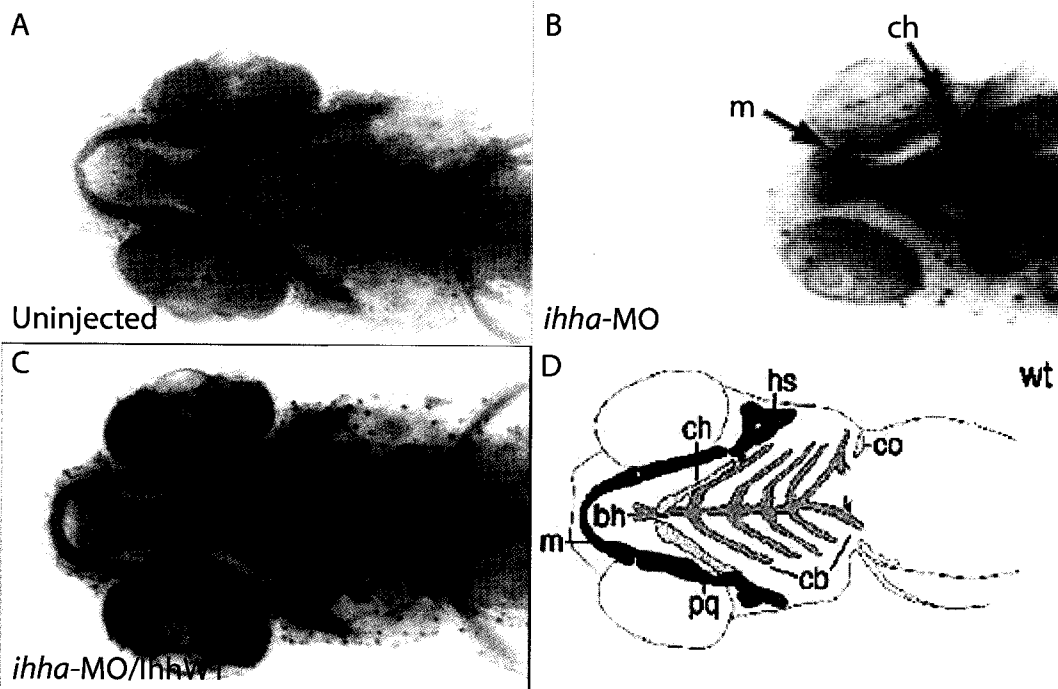
Lastly, of the group injected with *IHH*^{V190A} mRNA, 23.1% were either mildly or severely affected by over-expression of the mutant *IHH* mRNA, a number significantly different from the percentage affected by injection of *IHH*^{WT} mRNA, and not significantly different from those injected with either *IHH*^{D100N} or *IHH*^{R128Q} mRNA (figure 5.6a&b).

5.3.4 'Knockdown' of *ihha* in zebrafish results in altered endochondral ossification

As it has been shown previously that Indian hedgehog plays a crucial role in the development of both the axial and appendicular skeleton in not only humans, but the zebrafish as well, we chose to examine whether knockdown of *ihha* expression would prevent adequate development of early skeletal elements [77]. Several of the larvae used for the rescue experiment were maintained up to 5 dpf, at which point the larvae were fixed in PFA, dehydrated and stained with alcian blue, which adheres to glycosaminoglycans (GAGs), present in high amounts in collagen type II and type X – highly expressed during endochondral ossification [77, 120].

The stained larvae were visualized under a microscope and the images obtained are illustrated in figure 5.7. The morphant phenotype clearly displays incomplete extension of Meckel's cartilage (m) and the palatoquadrate (pq). It is also evident that there is aberrant development of the ceratohyal cartilage (ch), and although not clearly

Figure 5.7. Visualization of skeletal development via alcian blue staining of 5 dpf zebrafish larvae. Un-injected control larva (**A**), *ihha*-morphant larva (**B**), and a morphant larva rescued by synthetic *IHH*^{WT} mRNA (**C**) were fixed at 5 dpf, dehydrated and stained with alcian blue. These images were compared to a colour-coded diagram of a stained larval head (**D**) (modified from Pitrowski et.al., 1996). Abbreviations used throughout figures: bh, basihyal; ch, ceratohyal; co, coracoid of pectoral girdle; cb, ceratobranchial; hs, hyosymplectic; m, Meckel's cartilage; pq, palatoquadrate.



discernible from the image in figure 5.7b, it can be assumed, based on the physical development of the head, that there is also altered development of the ceratobranchial cartilage (cb). Figure 5.7d shows a colour-coded diagram of a stained larva head at 5 dpf, modified from Piotrowski *et. al.* [121]. Figure 5.7c illustrates an image of the alcian blue-stained head of a larva injected with 3 ng of *ihha*-MO and 700 pg of *IHH* mRNA. The phenotype of the skeletal development does not seem at all different from the uninjected control group. And yet, considering that when the *fli1*:EGFP morphant was analyzed there was significantly altered vasculature development, it can not be conclusively proven that the abnormal development of the larva skeleton is a consequence of the knockdown of *ihha* translation directly on endochondral ossification, or a consequence of the abnormal vasculature development. Further analysis is necessary.

5.4 Discussion

Ihha plays a critical role in early Danio rerio angiogenesis

It was our initial intention to examine if and how over-expression of human *IHH* mRNA could rescue the phenotype of *ihha*-morphant zebrafish embryos. We hypothesized that the BDA1- and ACFD-associated mutations would be unable to rescue the morphant phenotype to the same degree as wild-type synthetic *IHH* mRNA. Avaron *et. al.* had previously demonstrated that *ihha* expression in zebrafish larvae can first be detected in 2 to 3 cells located bilateral to the rostral end of the notochord of the parachordal cartilage of the four-day-old larvae [77]. Despite this fact, in our earliest

experiments analyzing the *ihha*-morphant, an altered phenotype was noticeable as early as 24 hpf. When analyzing wild-type embryos injected with 3 ng of *ihha*-MO we observed – to varying degrees in each larva – a decrease in physical size, a slight bend in the trunk and tail of the larvae, altered somitic segmentation, edema of the cardiac chamber, and in the most extreme cases, separation of the chambers of the heart. This led us to believe that *ihha* could in fact be expressed earlier than originally suggested [77]. Interestingly, the larvae that showed a bending in the trunk and tail did not bend in the same direction as the trunks of *sonic-you* (*syu*) zebrafish mutants lacking *shha* [116, 117]. This would suggest that the hedgehog signalling pathway obstructed is different from that which is abrogated in *shha*-mutants.

It has been shown previously that *shha* expression in the zebrafish larvae is crucial for arterial differentiation, and the promotion of angiogenic blood vessel growth [21, 63, 114]. Furthermore, through various *in vivo* studies in mice and through bioinformatics analysis, *Ihh* expression has also been shown to play a significant role in angiogenesis in other species [61, 63, 122, 123]. Therefore, we hypothesized that the significantly altered phenotype that we observed in the *ihha*-morphant was a result of a knockdown of *ihha* expression in the earliest stages of development that had a negative effect on angiogenesis in the zebrafish larvae, resulting in the other phenotypes we observed. Therefore, we examined the knockdown of *ihha* expression in the transgenic *flil*:EGFP line. The vascular phenotype that we observed - altered SeV and PAV branching and incomplete development of the DLA_V – provides evidence that it is likely that low-level expression of *ihha* is essential for early stages of angiogenesis. However,

in order to ultimately confirm our hypothesis, more specific in situ hybridization studies analyzing early larval vasculature are necessary.

Interestingly, in the embryos classified as class 2 and class 3 in the rescue experiment, there was normal development of the DA (figure 5.3b'). Despite the fact that the branching of the SeVs appeared disjoint and less organized, while the development of the DLAV was incomplete. This is consistent with results seen in studies analyzing the *Ihh*-deficient murine embryonic yolk sac. *Ihh* is an essential component regulating angiogenesis in the murine yolk sac [124, 125]. *Ihh* mutant yolk sacs form normal endothelial-cell-containing blood islands and a primary capillary plexus, and do also show a limited ability to remodel blood vessels [61]. And yet, those vessels that form in the absence of *Ihh* signalling are smaller, less organized and appear flattened or collapsed [61]. Therefore, it is likely that *ihha* is a key player promoting angiogenesis, or the remodelling of existing blood vessels to form mature vasculature, in the earliest stages of development of the zebrafish, *Danio rerio*.

With regards to what role *IHH* may play in angiogenesis in the developing long bone; studies analyzing murine embryos have shown that both *Ihh* and *vascular endothelial growth factor* (*vegf*) are produced in the visceral endoderm layer, and may act in concert to promote vascular remodelling [65]. This is supported by a bioinformatics study, which employed a one-dimensional finite element to show that *Ihh*, *PTHrP* and *vegf* act together to regulate tissue differentiation in the developing long bone [122]. Furthermore, chondrocytes from *Ihh*^{-/-} mice pre-maturely switch from anti-angiogenic to angiogenic, as they express *vegf*, and are thus invaded aberrantly by blood vessels [66]. Subsequently, vessel expansion is impaired, ultimately resulting in a degraded cartilage

matrix devoid of blood vessels [66]. In our previously hypothesized model of IHH signalling in the long bones of ACFD patients, we suggested that there is a significantly reduced amount of IHH signalling peptide in the growth plate as a result of decreased auto-proteolytic processing and a decrease in the amount of secretion. Although the activity of the V190A mutant is on par with the wild-type, the significantly lower level of signalling peptide in the growth plate results in a reduced rate of chondrocytic proliferation and accelerated rate of differentiation. It is plausible that in addition to this fact, the reduced level of IHH-N in the growth plate also partially relieves the inhibition of *vegf* expression and thus facilitates a pre-mature transition of chondrocytes from an anti-angiogenic state to an angiogenic state that would result in an aberrant invasion of vessels and eventual degraded cartilage matrix in the long bones of ACFD patients, similar to that seen in the *Ihh*^{-/-} mouse. This could also explain the generally smaller stature of ACFD patients – a phenotype similar to that we saw in the *ihha*-morphant zebrafish.

Additional studies are necessary to conclusively determine the role of *ihha* in the early stages of angiogenesis in the zebrafish larvae. *In situ* hybridization (ISH) analysis of *ihha* has been capable of detecting expression no earlier than 4 dpf [77]. It is important to conduct a reverse transcriptase PCR on mRNA collected from homogenized zebrafish larvae at various developmental stages. This could elucidate more precisely the exact moment onset of *ihha* expression, although localization of such a signal would be lost. Furthermore, *in situ* hybridization analysis of the expression of various angiogenic markers (*vegf*, angiopoietin-1 and -2) on cross-sectional tissue from both the wild-type and *ihha*-morphant larvae could shed some light on the role of *ihha* in angiogenesis in the

zebrafish. Also, it is uncertain whether hedgehog proteins, governing angiogenesis, act directly on endothelial cells, or act through a hedgehog-responsive intermediary element [123]. A study examining known hedgehog target genes could be advantageous for elucidating this information.

Over-expression of Human IHH in the zebrafish, Danio rerio

The gene knockdown approach provides a novel strategy to identify potential signalling pathways of a candidate gene. In our rescue experiment, we injected 3 ng of *ihha*-MO along with 700 pg of synthetic *IHH* mRNA. As expected, the BDA1- and ACFD-associated *IHH* mutants were unable to rescue the morphant phenotype as effectively as the synthetic *IHH*^{WT} mRNA. However, the set of embryos injected with *IHH*^{WT} mRNA, although appearing to show the greatest degree of improvement from the group injected with *ihha*-MO only, demonstrated that over-expression of human *IHH* is unable to rescue the knockdown phenotype completely. Previous studies have indicated that *Ptch* is expressed throughout the developing embryo as early as 10 hpf, along with *shha* and additional hedgehog players including *Smo* [126]. However, *ihha* expression has not been detected until 4 dpf, and even at this point, it is only expressed in a restricted region of the notochord in the parachordal cartilage [77]. When 700 pg of human *IHH* mRNA is injected into an embryo, it is ultimately equally distributed throughout the larvae. Therefore, the increased amount of hedgehog signalling is likely the reason behind the inability to fully recover the wild-type phenotype. Our synthetic message would be equally divided between each generation of cells. At every stage, every cell would seemingly be producing the same amount of human Indian Hedgehog. Therefore,

there would be an increase in *IHH* secreted from each cell, and thus an increase in the positive proliferative effects (i.e. expression of zebrafish homologs of *Foxf1*, *Ccnd2*) [47, 48, 105] but also an increase in the Hh-inhibitory effects; PTHrP and Hip [19, 29, 34]. When these regulatory signals relay back to the hedgehog-expressing cells there would likely be inhibition of all endogenous hedgehog expression, and yet there would synthetic *IHH* mRNA, present in the cytoplasm, would continue to be translated. This pattern of hedgehog signalling would likely be altered significantly from the natural state, which is likely the reason why we were unable to see a complete restoration of the wild-type phenotype.

Previous studies have shown a high degree of functional redundancy of hedgehog genes in zebrafish [79, 80]. We have, seemingly for the first time, provided evidence that expression of human *IHH* can compensate for the loss of zebrafish *ihha*. Therefore this ability for human *IHH* to functionally substitute for the expression of the endogenous expression of the highly conserved zebrafish *ihha* is further evidence that the essential function of Indian hedgehog in controlling cell fate and tissue patterning through controlling cellular proliferation and differentiation is preserved between fish and mammals and thus arose at a distant point back on the evolutionary chain.

With regards to our control study where we analyzed over-expression of synthetic *IHH* mRNA, we injected only 100 pg as opposed to the 700 pg of synthetic mRNA that was injected in the rescue experiments. Therefore, as the amount of mRNA in each cell is divided by a factor of two with each cell division, the 100 pg should not last as long into development as the 700 pg did in the rescue in experiment. However, it was clear that mutant *IHH* mRNA conferred a negative effect on the phenotype of the developing

larvae. The embryos injected with *IHH*^{WT} mRNA displayed no significant difference from the un-injected control embryos, while 23% to 39% of the embryos injected with one of the BDA1- or ACFD-associated mutant mRNA showed either mild or severely affected development. This result is consistent with the results we saw *in vitro*. In both the LIGHT2, and the differentiating ATDC5 experiments, when the cells were treated with a combination of *IHH*^{WT} and one of the BDA1-associated mutants there was little increase in reporter activity from that detected from the cells treated with mutant *IHH* only. Clearly, in the cells injected with *IHH*^{WT}, the signalling peptide was able to complement endogenous hedgehog signalling to the point that it did not hinder the development of the embryo as a result of over-expression of hedgehog inhibiting factors, or gross alteration of the expression levels of target genes downstream of *ihha*.

From our results obtained in chapter 4, we suggested that in the growth plate of BDA1-affected individuals, significantly less IHH-N is produced and secreted, and thus the amount of IHH-N in the growth plate is significantly less than the wild-type. However, the mutant peptide is still able to confer a phenotype as it possesses dominant-negative properties over the wild-type. We deduced that it is likely that the BDA1-associated mutant proteins are able to bind to the receptor protein, Ptch, but not induce signal transduction. Furthermore, these bound signalling peptides subsequently sterically hinder any additional IHH-N signal from binding to the receptor. The results of this experiment provide further evidence to suggest that the activity of mutant IHH-N has a negative effect on the normal signalling activity of endogenous *Ihha* activity, however it does not confirm the theory that mutant IHH inhibits the binding of *IHH*^{WT} to Ptch. In lower amounts (in comparison to our rescue experiment), human *IHH*^{WT} does not

significantly alter hedgehog signalling in the developing zebrafish, and yet, when present in those same amounts, the mutant *IHH* mRNAs presumably produce proteins that can sterically hinder the binding of additional hedgehogs to their receptors and thus results in decreased hedgehog signalling and ultimately in the increased prevalence of altered phenotypes in the groups injected with mutant *IHH* mRNA.

Additional analysis is necessary to better understand these processes. In particular, more accurate and reproducible results analyzing the translation of the message, production and secretion of the protein in each sample group, time trials analyzing expression and distribution of the message over a period of 4 days, and ISH or RT-PCR analyzing expression of key hedgehog players in each sample group.

Chapter 6. Conclusions

Summary of Findings

It has been over a century since Farabee initially classified brachydactyly type A1 as a disorder that was transmitted through an autosomal dominant mode of inheritance. It has been almost ten years since mutations to Indian hedgehog (IHH) were revealed to cause BDA1. Furthermore, in 2003, Mortier *et. al.*, discovered that mutations in IHH lead to another bone development disorder, acrocapitofemoral dysplasia (ACFD) [16, 17]. And, until now, no study has been conducted to analyze how those mutations lead to the pathogenesis of the disease. We are the first group to examine the characteristics of mutant *IHH*. Moreover, we have provided insight into the various properties of different regions of IHH-N.

We have shown that all of the BDA1 mutations that we analyzed – E95K, D100N, R128Q, and E131K – introduce a residue with properties different to those of the native residue, and that all mutations lie in relatively close proximity to residues necessary for facilitation of Zn^{2+} coordination [59]. These mutant proteins likely disrupt protein folding, which subsequently reduces the amount of IHH precursor being processed into IHH-N. Furthermore, we have shown that the mutant IHH-N displays a significantly lower level of secretion relative to IHH^{WT} , and we have inferred that this is a result of a decreased tendency to multimerize as a result of the mis-folding of the protein. Subsequently, we have shown that what little BDA1-associated mutant IHH secreted not only has a significantly lower ability to induce IHH signalling, but also appears to confer a dominant-negative effect over IHH^{WT} . In the case of the ACFD-associated $\text{IHH}^{\text{V190A}}$

mutant, the activity level is comparable to that observed in IHH^{WT} . Finally, *in vivo* over-expression of *IHH* in a zebrafish model is unable to completely rescue an *ihha*-morphant phenotype. However, over-expression of mutant *IHH* in the zebrafish confers a negative phenotype, while over-expression of IHH^{WT} does not. Also, the knockdown of *ihha* expression in the zebrafish resulted in aberrant and incomplete branching of blood vessels during vasculature development, indicating *ihha* plays a critical role in early zebrafish angiogenesis.

Brachydactyly Type A1 Model

We propose that in patients with BDA1 resulting from the possession of one allelic copy of IHH^{E95K} , IHH^{D100N} , IHH^{R128Q} , or IHH^{E131K} , will display a mal-development of all bones, but in particular in the phalanges of the hands and feet, as a consequence of a series of factors. Firstly, the mutant species of *IHH* will produce a precursor protein that is likely mis-folded that does not produce the same amount of *IHH*-N as the wild-type species. In addition to this, these mutations are all located in a highly conserved region in close proximity to a pocket of the protein that has been shown to be critical in facilitating intramolecular interactions and multimerization of the homologous *SHH* [24]. Ultimately, significantly less mutant *IHH*-N is secreted by the cell, and significantly less mutant *IHH*-N will be present in the growth plate relative to the wild-type. However, as the phenotype of the $IHH^{+/-}$ mouse appears to be not at all different from the $IHH^{+/+}$ mouse, it is unlikely that the pathogenesis of BDA1 is a simple result of haploinsufficiency of *IHH* [102]. During endochondral ossification of the appendicular skeleton, the growth plate in most bones is large, and as the mutant proteins likely do not

multimerize to the same degree than their wild-type counterparts, it is plausible that the motility of the mutant morphogen is limited and mutant IHH-N would be unable to travel very far into the zone of proliferating chondrocytes or to the perichondrium of the adjacent bone collar. Therefore, in the longer bones of the developing skeleton the majority of the message travelling to the periarticular perichondrium and the proliferating chondrocytes would be IHH^{WT} (and additional SHH). And despite the fact that there is seemingly only half of the amount of IHH^{WT}, there would likely be enough IHH signal to promote normal development of the majority of the appendicular skeleton. However, the small amount of reduced motility mutant IHH-N does, as we have shown, confer a dominant-negative effect over the wild-type. Therefore, in those regions of these developing long bones directly adjacent to the IHH-secreting cells there would likely be decreased proliferation and eventual attenuation of endochondral ossification. This would account for why BDA1-affected family members are of a generally shorter stature than non-affected family members [4]. Finally, when considering the growth plate of the phalanges in the hands and feet, the area of each of the proliferating chondrocytes are significantly smaller than those in the longer bones of the appendicular skeleton. In these smaller growth plates, the effect of the mutant IHH species is amplified, as long distance signalling becomes less of a necessity. More mutant IHH-N can reach the terminal ends of this smaller growth plate and subsequently block more IHH^{WT} signal from being transduced, which is why the effect of the mutant protein is much more profound in the phalanges of the hands and feet than in the rest of the appendicular (and axial) skeleton.

Acrocapitofemoral Dysplasia Model

With regards to the single ACFD-causing mutation we analyzed, IHH^{V190A}, the argument for how the mutant protein contributes to the pathogenesis is somewhat different from that of the BDA1-associated mutations. Like the BDA1 mutations, the V190A mutation displayed a significantly reduced level of autocatalytic processing and a significantly lower presence in the media, indicating decreased secretion, in comparison to IHH^{WT}. Furthermore, the V190A mutation lies in close proximity to the highly conserved, His185 residue, reportedly essential for facilitating multimerization of the protein [24]. Therefore it is likely that, similar to the BDA1-associated mutations, the V190A mutation disrupts the ability of the protein to multimerize and thus it is unable to travel across long distances in the growth plate. And yet, unlike BDA1, ACFD is an autosomal recessive skeletal dysplasia [16, 17]. Therefore, in the patient affected with ACFD caused by the V190A mutation, in the developing long bone there is only the mutant variant of IHH. However, unlike the BDA1-associated mutations, the results we obtained in the LIGHT2 activity assay suggest that the V190A mutation has maintained the ability to bind to its target and transduce a signal comparable to IHH^{WT}. This would suggest that signalling is affected partly by a decreased quantity of signal. However, it is also possible that the V190A mutation obstructs the addition of the cholesterol moiety and possibly palmitoylation, as the mutation resides at the C-terminus of IHH-N, the site of cholesterol addition, which precedes palmitoylation. This would explain the significantly decreased levels of IHH-N production and secretion. The alteration of post-translational modifications would also undoubtedly decrease the ability of the protein to multimerize and thus reduce its motility in the growth plate. Therefore, reduced motility

of IHH^{V190A} in the growth plate, and an overall reduced amount of IHH signal, would ultimately cause proliferating chondrocytes proximal to the IHH-producing pre-hypertrophic chondrocytes to pre-maturely differentiate. This is in accordance with the postulation of Hellemans *et. al*, who suggested that an increased rate of differentiation, throughout both the appendicular and axial skeleton, was the principal factor driving the pre-mature closure of the growth plate and the development of cone-shaped epiphyses in hands, proximal femur, and to a lesser extent, at the shoulders, knees and ankles [17]. Furthermore, the knockdown of *ihha* expression in zebrafish demonstrated what a critical role *ihha* played in angiogenesis. Therefore, it is also plausible that a significant decrease in the total level of IHH production could lead to altered vascular branching in the early stages of development. This could also explain why the phenotype of ACFD patients is much more severe than those affected with BDA1. It is also important to note that of the six parents of the three individuals affected with ACFD, three display only mild shortening of the middle phalanges of the hands (the shortened digit varies), while the other three parents reveal no obvious signs of a BDA-related phenotype. This is in accordance with our findings that, unlike the BDA1-associated mutations, IHH^{V190A} maintains its natural level of activity, and exerts no dominant-negative effect over IHH^{WT}.

We are the first group to analyze how a series missense mutations in the human Indian hedgehog gene give rise brachydactyly type A1 and acrocapitofemoral dysplasia. Although we have presented a set of data that has provided us with a novel insight into the biochemistry of IHH, further analysis is necessary to more explicitly determine how the altered properties of the mutated proteins affects the function of the signalling

peptide. More specifically, studies analyzing the post-translational modifications, expression profiling of *IHH*-target genes both *in vitro* and *in vivo*, and competitive binding studies between the wild-type and mutant proteins would be very beneficial. It is also very important to further analyze the motility of the protein, perhaps through live cell-imaging that would track labeled IHH-N. In addition to this, similar types of studies examining the protein *in vivo* could provide novel insight into the hedgehog signalling pathway, in particular multimerization, secretion, and long-distance signalling.

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Contribution of Collaborators

Dr. Marie-Andrée Akimenko (Senior Scientist, Chronic Disease, Ottawa Health and Research Institute, Ottawa, Ontario, Canada): Supplied all zebrafish resources; fish, food, storage, maintenance.

Andrew Seto (Summer student, Ottawa Health and Research Institute, Ottawa, Ontario, Canada): Amplified portion of exon 1 of *IHH*, acquired IMAGE clone (5182642), and successfully introduced the c.G298A mutation by site-directed mutagenesis to *IHH* in the pBSIhhWT construct, thus generating the pBSIhhD100N construct.

Danielle Guay (Laboratory technician, Ottawa Health and Research Institute, Ottawa, Ontario, Canada): Prepared several crosses of male and female zebrafish.

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