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The role of Bone morphogenetic protein signaling in zebrafish caudal fin regeneration

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The role of Bone morphogenetic protein signaling in zebrafish caudal fin regeneration

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Abstract

Zebrafish possess the remarkable ability to regenerate many organs and tissues following damage, including their fins. Understanding the mechanisms behind regeneration in these organisms may be the key to promoting regeneration of tissues in humans. Using the zebrafish caudal fin as a model we examined the role of the Sonic hedgehog (Shh) and Bone morphogenetic protein (Bmp) signaling pathways in fin regeneration. The expression patterns of *shh*, its receptor *ptc1,* and downstream target *bmp2b* within the fin regenerate revealed their potential roles in bone regeneration. To test their involvement we performed a functional analysis by ectopically expressing and inhibiting Shh and Bmp2b signaling within the regenerate. Ectopic expression of *shh* and *bmp2b* within the blastema, using an *in vivo* transfection method, resulted in ectopic bone formation. This effect was blocked when we co-injected *shh* and *chordin*, a Bmp antagonist, indicating that Shh signaling requires a Bmp factor to induce ectopic bone formation. These findings link Shh and Bmp signaling to scleroblast proliferation, differentiation and/or function during fin regeneration. Two Bmp family members are expressed within the regenerate in a pattern suggesting their role in bone regeneration: *bmp2b*, and the newly characterized *bmp6*. On the other hand, *bmp4* expression, in cells of the distal blastema suggests its possible function in growth of the regenerate. To establish a direct role for Bmp signaling, we inhibited signaling by injecting *chordin* into the blastema of individual fin rays, resulting in reduced matrix secretion attributed to a defect in scleroblast function. We identified Bmp targets with known function in bone formation, *runx2a/b* and *col10a1*, which are both expressed in scleroblast cells and downregulated following Bmp inhibition and are likely mediating the function of Bmp signaling in

scleroblast differentiation/function. Interestingly, within the regenerate we detected the expression of 'cartilage-specific' genes involved in endochondral bone formation although fin rays form through dermal ossification, and many of these factors are regulated by Bmp signaling. These findings suggest that fin rays may be made of an intermediate type of bone or that intramembranous and endochondral bones are more closely related than previously thought. Also, inhibition of Shh and Bmp signaling pathways, using cyclopamine and *chordin* misexpression respectively, led to a decrease in blastema proliferation, and a downregulation of the *msxb* and *msxc* transcription factors. Overall our studies indicate a critical role for the Shh and Bmp signaling pathways in fin regeneration, mediating both scleroblast differentiation/function and blastemal proliferation and growth.

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List of Abbreviations

Apical epithelial/epidermal cap (AEC)
Bone morphogenetic protein (Bmp)
Bone morphogenetic protein 2b (Bmp2b)
Bone morphogenetic protein receptor type I (BMPR-I)
Bone morphogenetic protein receptor type II (BMPR-II)
Bone sialoprotein (Bsp)
Bromodeoxyuridine (BrdU)
Captomelic dysplasia (CD)
Chondrocyte like osteoblasts (CLOs)
Collagen type X (Col10al)
Common partner Smads (Co-Smads)
Days post amputation (dpa)
Days post fertilization (dpf)
Days post injection (dpi)
devoid of blastema (*dob*)
Dickoppf1 (Dkk1)
Distal blastema (DB)
Distal most blastema (DMB)
Dominant negative fgfr1 (dnfgfr1)
Enhanced Green Fluorescent Protein (EGFP)
Enhanced Green Fluorescent Protein gene (pCMv5 EGFP)
Expressed sequence tags (ESTs)
Extracellular matrix (ECM)
Fibroblast growth factor (Fgf)
Fibroblast growth factor receptor (Fgfr1)
Hedgehog (hh)
in situ hybridization (ISH)
Indian hedgehog (Ihh)
Indian hedgehog a (Ihha)
Inhibitor Smads (I-Smads)
Matrix metalloproteinases (MMPs)
Mitotic checkpoint gene (Mps1)
Morpholinos (MOs)
nightcap (*ncp*)
Osteopontin (Opn)
Paraformaldehyde (PFA)
Parathyroid hormone related peptide (PTHrP)
Patched1 (ptc1)
Patterning zone (PZ)
pCMV5 (cytomegalovirus 5)
Phosphate buffered saline (PBS)
Phosphohistone H3 (H3P)
Proximal blastema (PB)

PTH/PTHrP receptor (PPR)
Receptor regulated Smads (R-smads)
Room temperature (RT)
Scanning electron micrograph (SEM)
Sonic hedgehog (Shh)
lymphocyte enhancer binding factor 1 (Lef1)
Transforming growth factor-ß (TGF-ß)
Vascular endothelial growth factor (VEGF)
Wingless (Wnt)
Wound fgf (wfgf)
Zone of polarizing activity (ZPA)

Acknowledgments

First and foremost, I would like to extend my thanks to my thesis supervisor Dr. Marie-Andree Akimenko. Her guidance, patience and scientific creativity was greatly appreciated and necessary for my success in the PhD program. I would also like to thank my committee members, Dr. Marc Ekker, Dr. Kursad Turksen and Dr. Margret Sonnenfeld for their advice, time and useful discussions. I would especially like to thank Dr. Fabien Avaron for all of his help with my projects; none of it would have been possible without him. Danielle, Olga, Jing, Luc and Noel have not only been wonderful co-workers but have become some of my closest friends. I would also like to thank Dr. Joel for always making us laugh.

I owe a special thanks to my friends and family for all of their support and encouragement without it I could not have completed my studies. I must thank Sassy, JC and the pups JP and MP for being such incredible friends and companions. Also, Amy and Squishy for being such supportive and wonderful roommates and friends. I would also like to thank some of my best friends Sarah D, Sarah S, Allison, Trish, Mellie, Carly and Laura for being such great people. Finally, all of my thanks go to my Mom and Shirl without their love and support none of this would have been possible, thank you.

Chapter 1: General Introduction

1.1 Regeneration

1.1.1 Organ and tissue regeneration

Many adult invertebrates and lower vertebrates possess the ability to regenerate complex organs or tissues following tissue injury or removal. To date, urodele amphibians are the vertebrates with some of the most extensive regenerative abilities. Adult newts, for example, regenerate their limbs and tail, jaws, ocular tissues including lens and optic nerve, the intestine and heart ventricles (Reyer 1954; O'Steen *et al.* 1962; Ghosh *et al.* 1994; Oberpriller *et al.* 1995; Mitashov 1996; Brockes 1997; Mitsuda *et al.* 2005; Tassava *et al.* 2005). More recently the regenerative capabilities of the zebrafish, a teleost fish, are becoming more elucidated and zebrafish is becoming one of the most ideal organisms to study the regeneration process. Adult zebrafishes possess the remarkable ability to regenerate a number of structures, such as the spinal cord, optic nerve, lens, retina, and parts of the heart, scales and each of the 5 types of fins (Bernhardt *et al.* 1996; Becker *et al.* 1997; Poss *et al.* 2002; Russell 2003). In contrast mammals possess very limited regenerative ability, being capable of regenerating such structures as blood, liver tissue, skin epithelium and gut epithelium, but the majority of organs heal through scarring (reviewed in: (Carlson 2005; Sanchez Alvarado *et al.* 2006). In salamanders, regeneration is possible due to their ability to create a new progenitor cell through a process known as cellular dedifferentiation (Lo *et al.* 1993; Simon *et al.* 1995; Brockes 1997, 2002; Straube *et al.* 2004).

This degree of cellular plasticity is not seen in mammals and may explain the difference in regenerative abilities between salamanders and mammals. The process of dedifferentiation begins when cells at the site of the injury revert to proliferating progenitor cells that will eventually, during the regeneration process, re-differentiate into the cells that will restore the lost structure. For example, following removal of the lens in the newt, the pigmented epithelial cells of the dorsal iris dedifferentiate (exit from quiescent state), lose their pigment, begin to proliferate and then re-differentiate into lens epithelial cells (Eguchi *et al.* 1971, 1988; Tsonis *et al.* 2004). The lost tissue or organ is replaced through a process called epimorphic regeneration which is defined as regeneration involving proliferation (Morgan 1901). Epimorphic regeneration involves the formation of a specialized structure called a blastema, which is a mass of proliferative, pluripotent progenitor cells and is the distinguishing factor between a tissue that can and cannot regenerate. It is believed that the dedifferentiation of mature cells at the site of the injury is the major source of the blastema cells, although it is still unclear if preexisting stem cells may also contribute as well (Lo *et al.* 1993; Brockes 1997, 2002; Yokoyama *et al.* 2000; Echeverri *et al.* 2001). Following injury to the newt limb or tail, a wound epidermis is formed by the migration of non-dividing epidermal cells that cover the wound (Repesh *et al.* 1978; Tanaka 2003). It is thought that a signal secreted from the wound epidermis may induce cells near the site of injury to dedifferentiate and begin to proliferate to form the blastema. As during development, epidermal-mesenchymal interactions between the wound epidermis and blastema are necessary for continued growth of the blastema and differentiation of the cells (Mescher 1976; Tanaka 2003). Therefore the means to understanding the regeneration process and possibly applying it to

mammalian cells is to define what exactly is a blastema, how it is formed and grows, and what signals promote its formation, proliferation and eventual differentiation. One of the most useful models for regeneration studies has been the urodeles (newt and salamanders). To date, regeneration of the limb in these animals has been the most extensively analyzed as the limb has proven to be one of the most useful models due to its size, ready accessibility, ease of surgical and chemical manipulation and the emerging elucidation of its genetics (reviewed in: (Mescher 1976; Mescher 1996; Brockes 1997; Odelberg 2005; Yokoyama 2008). Therefore it will be reviewed below.

1.1.2 Stages of limb regeneration in urodele amphibians

Regeneration of the limbs of urodele amphibians is a local response of cells of the limb stump regardless of the level of the amputation. The process of limb regeneration can be divided into two phases, each of which is made up of several stages. The first phase is considered the regeneration-specific phase and involves the following stages: closure of the wound by epidermal cells, dedifferentiation of stump tissue, transformation of wound epithelium into apical epithelial cap (AEC) that is required for limb outgrowth, migration of dedifferentiated cells to the center of the wound where a blastema forms, and high proliferation of blastema cells. The second phase is the re-development phase which involves growth and patterning of the blastema to eventually replace the lost limb. The blastema cells are thought to be similar to the cells of the limb bud, seen during development, as it goes through morphogenesis, pattern formation and redifferentiation (Turner 1981; Bryant *et al.* 2002). Like the growth and patterning of the limb bud during development, outgrowth of the blastema involves epithelial-mesenchymal interactions.

The second phase of limb regeneration also involves the reactivation of many of the genes involved in the development of the limb bud; these genes will be discussed in more detail below.

1.1.2.1 Wound healing, AEC formation and dedifferentiation

Unlike mammals, where wound healing can take days, following amputation of the newt limb, epithelial cells at the edge of the wound and proximal to the amputation site detach from the basal layer of the epidermis and migrate to close the wound within hours (Norman *et al.* 1967; Repesh *et al.* 1980). Over the next couple of days this wound epidermis thickens, due to continued cell migration, to form a structure referred to as the apical epithelial cap (AEC) (Thornton 1958; Globus *et al.* 1980). In urodele limb regeneration, the first response to amputation or injury is formation of this wound epidermis (AEC) and de-differentiation of stump cells that converts the site into an area that is permissive for redevelopment. The importance of this structure has been demonstrated by the fact that removing the AEC and covering the wound surface with mature skin completely abolishes the regeneration process (Christensen *et al.* 2000). In addition when transplanted, the AEC induces ectopic outgrowth of the limb bud (Mescher 1976). Next, the cells in the stump are believed to receive a signal from the AEC to dedifferentiate. The cartilage, muscle, Schwann cells and dermal and muscle fibroblasts have all been shown to lose their differentiated characteristics to become mesenchymal-like stem cells (Hay *et al.* 1961; Mescher *et al.* 1975; Gardiner *et al.* 1986; Casimir *et al.* 1988; Lo *et al.* 1993; Kumar *et al.* 2000). The de-differentiation of all of these cells types have been shown to contribute to the regeneration process, but

quantitative analysis has shown that they do not all contribute equally, since some tissues such as the dermis contributes more cells than the muscle or skeletal tissue (Muneoka *et al.* 1986; Muneoka *et al.* 1986; Echeverri *et al.* 2001). The de-differentiation stage begins about 4-5 days post-amputation (dpa) and is characterized by general breakdown of internal limb tissues, downregulation of differentiation markers and cell cycle reentry. These cells accumulate under the AEC to form a regeneration blastema which grows by proliferation. Removal of the wound epidermis from the blastema results in limb truncations which are more severe the earlier the epidermis is removed, demonstrating that blastemal proliferation depends on continued interface with the AEC (Goss 1956; Dearlove *et al.* 1974; Saunders *et al.* 1976).

1.1.2.2 Re-development of the lost limb

This stage is characterized by extensive blastemal proliferation, until about 3-4 weeks post-amputation, and eventual differentiation of these cells to replace the lost structure (Tsonis *et al.* 1996; Odelberg 2005). It has been estimated that approximately one thousand progenitor cells grow to hundreds of thousand of undifferentiated cells before the next stage of morphogenesis begins (Stocum 1996). During this stage, the blastemal cells begin to differentiate, the proximal cells differentiating first while the distal blastema continues to proliferate as the regenerating limb keeps growing and re-develops. Regeneration of the newt limb is completed in 5-7 weeks following amputation but will continue to grow over the following months till it reaches the size of the original limb (Tsonis *et al.* 1996). The cartilage producing chondrocyte is one of the first cell types to

differentiate within the regenerate, and the ossification of the bone starts at about 37 dpa (Stock *et al.* 2003).

1.1.3 Genes involved in limb regeneration

Many genes involved in the development of the limb are re-expressed during the regeneration process warranting the classification of this regenerative phase as re-development (Muneoka *et al.* 1982; Torok *et al.* 1998; Roy *et al.* 2000, 2002). As detailed above, the cellular mechanisms governing the regeneration process in the urodele limb can be categorized into two phases: the wound healing and the re-development/re-differentiation phase. These two phases can also be characterized at the molecular level with certain genes and pathways. To date, little is known about the molecular mechanisms responsible for triggering the formation of a wound epidermis (AEC) and for the cellular plasticity seen during the early regeneration phase. Some examples of genes known to play roles during different stages of limb regeneration will be briefly discussed. Matrix metalloproteinases (MMPs) are family of proteases that play a role in remodeling of extracellular matrix (ECM) (Gross *et al.* 1962; Fujimoto *et al.* 2006, 2007). These proteins are capable of degrading nearly all ECM proteins, including collagen and are specifically required for limb regeneration, especially during the initiation phase involving wound epithelium formation and subsequent blastema formation (Yang *et al.* 1994; Vinarsky *et al.* 2005). Growth factors being produced by the wound epidermis and/or blastema are thought to play critical roles in the regeneration process. Firstly, the importance of Bone morphogenetic protein (Bmp) signaling during the early stages of limb regeneration in the *Xenopus* tadpole limb has been demonstrated by inhibiting Bmp

signaling within the regenerate, resulting in the blockage of limb regeneration (Beck *et al.* 2006). During embryonic development, the *Msx* genes are often expressed with a similar pattern and within the same region as *Bmp* genes and have been shown to regulate each others expression (Maeda *et al.* 1997; Scaal *et al.* 2002; Tribulo *et al.* 2003). Msx1 encodes a transcriptional repressor that is responsible for keeping cells in an undifferentiated state while the tissue continues to proliferate and grow (Woloshin *et al.* 1995; Zhang *et al.* 1997; Odelberg *et al.* 2000; Hu *et al.* 2001). *Msx1* expression has also been detected in blastemal cells during urodele limb regeneration, indicating its possible function in keeping these cells in an undifferentiated state during growth of the blastema (Koshiba *et al.* 1998). It was also demonstrated that Bmp signaling activates Msx1 to promote tail regeneration in the axolotl (Beck *et al.* 2006). Secondly, members of the Fibroblast growth factor signaling (Fgf) pathway have been shown to be expressed during limb regeneration, for example in the regenerating newt and *Xenopus* limbs, *fgf-8* expression is detected in the apical epithelium (AEC) and *fgf-10* in the mesenchyme of the blastema (Christen *et al.* 1997; Yokoyama *et al.* 2000). Also, the *fgfr1* receptor is expressed in the blastema cells of the regenerating limb (Poulin *et al.* 1993). Therefore the genes involved in the initiation of limb regeneration mainly have been found to function in; 1) AEC formation and maintenance, 2) de-differentiation and keeping cells in an undifferentiated state, 3) blastema formation and growth mostly through epithelial/mesenchymal interactions as during limb development. This phase is said to be regeneration-specific and some of the genes are not expressed in the same patterns as during development, but have similar function. The next phase of regeneration is devoted to patterning and differentiation of the blastema cells to replace the lost limb

structure. As is indicated by its name, the re-development/re-differentiation phase relies on the expression of many genes and pathways that are involved in growth control and patterning of the developing limb. One important example is a gene which encodes a signaling molecule of the zone of polarizing activity (ZPA) responsible for determining the anterior–posterior axis of the embryonic chicken and mouse limbs called *Sonic hedgehog* (*Shh*) (Riddle *et al.* 1993). Imokawa *et al.*, (1997) set out to demonstrate that a ZPA is present in the developing and regenerating newt limb. They found *Shh* expression, as in the chick and mouse, in the posterior mesenchyme of both the embryonic limb bud and regeneration blastema, suggesting a role for Shh in patterning of the regenerating limb (Imokawa *et al.* 1997). Roy *et al.*, (2002) examined the function of Shh in newt limb regeneration by making use of a pharmacological compound, cyclopamine, which specifically blocks Shh signaling. The treatment of axolotls with cyclopamine during the process of limb regeneration caused a loss of digits similar to that described for the Shh knockout mouse (Roy *et al.* 2002). Ectopic expression of *Shh* was also performed in cells of the anterior blastema of the regenerating limb by injecting a vaccina virus expressing *Shh*. This second functional analysis of Shh resulted in ectopic digits within the regenerated limbs reaffirming the suspected role of Shh in patterning of the regenerate as is seen during development (Roy *et al.* 2000)

1.2 Zebrafish fin regeneration and caudal fin morphology

1.2.1 Zebrafish as a model organism to study regeneration

In the past, most regeneration research has focused on limb and tail regeneration in urodeles. The results have been beneficial to the understanding of the regeneration

process, but the advantages of using small teleost fish are becoming obvious. In the last decade, a variety of genetic and molecular approaches for studying the zebrafish have been established making them a very powerful model to study the molecular mechanisms behind tissue regeneration. These approaches and tools include: the ability to perform large scale mutagenesis screens, transgenesis and gene knockdown by antisense morpholino oligonucleotides, increasing accessibility to zebrafish genome sequencing, and databases containing information about zebrafish cDNA and expressed sequence tags (ESTs) (Driever *et al.* 1996; Haffter *et al.* 1996; Higashijima *et al.* 1997; Nasevicius *et al.* 2000). Most of these above mentioned genetic tools have been widely used for the study of zebrafish embryonic development and now a growing group of researchers are making use of these tools to study regeneration in adult zebrafish. Some of these will be explained in more details in the following sections.

To date, the zebrafish caudal fin has been the most widely used organ for studying regeneration for many reasons, such as its simple structure and limited cell types, its easy accessibility for surgery and the rapidity and reliability of regeneration with most structures being replaced within a few weeks (Poss *et al.* 2003). In comparison to the limbs of urodeles, the morphological structures of the fins of zebrafish are very simple. The fins are devoid of skeletal muscles, except at the very base of the fin, and the major component of the fin are the bony fin rays which are comprised of dermal bone. This type of bone matrix mineralizes directly as bone and does not go through cartilaginous precursor (Montes *et al.* 1982; Becerra *et al.* 1983; Santamaria *et al.* 1991; Geraudie *et al.* 1992). In conclusion, both the advanced genetic tools and the simplicity of the zebrafish

fin make it an excellent model to study both the cellular and molecular mechanisms behind the regeneration process.

1.2.2 Fin morphology and structure

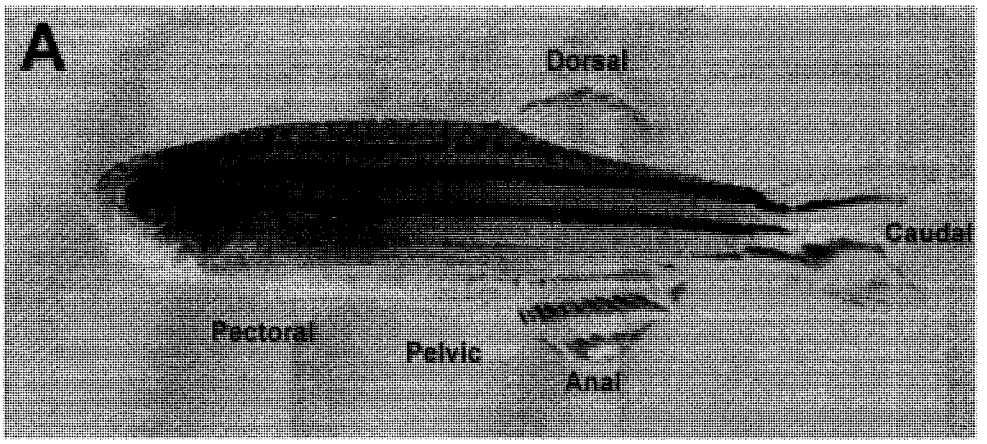
The adult zebrafish has five sets of fins, three unpaired and two paired, all of which possess the ability to regenerate following amputation or injury. The paired fins are the pectoral and pelvic fins which are said to be homologous to the forelimbs and hindlimbs, respectively, of tetrapods (Sordino *et al.* 1995; Ruvinsky *et al.* 2000). The unpaired fins are the dorsal, anal and caudal fins (Fig. 1.1A). The caudal fin is also known as the tail fin and is the most widely used fin for studies focusing on fin regeneration strictly because of its easy accessibility during surgery and its larger size (reviewed in: (Akimenko *et al.* 2003; Poss *et al.* 2003). The zebrafish caudal fin is composed of approximately 18 branching bony fin rays and is divided into two symmetrical lobes called the dorsal and the ventral lobes (Fig. 1.1B). The skeletal element of each bony ray is composed of dermal bone and is called a lepidotrichia. The lepidotrichia does not contain endochondral bone which usually forms by the replacement of a cartilage template by bone matrix; the fin skeleton instead mineralizes directly from bone matrix secreted by scleroblasts (bone-secreting cells), which is also known as intramembranous ossification (Geraudie *et al.* 1982; Sandell *et al.* 1994; Santamaria *et al.* 1996). The fin rays are part of the exoskeleton of the fin and this is the part of the fin that regenerates. On the other hand, the endoskeleton of the zebrafish fin, located at the base, is the support of the fin rays and has a cartilaginous origin and resection of the fin endoskeleton is not followed by regeneration (Grandel *et al.* 1998; Crotwell *et al.* 2004). The fin

exoskeleton is attached to the endoskeleton via ligaments. Each lepidotrichia is composed of two symmetrical concave hemirays that are facing each other (Fig. 1.1C). Each ray or lepidotrichia is made of a series of segments that are connected through ligaments and act as joints (Fig. 1.1D) (Becerra *et al.* 1983). Most rays (except the outermost rays of the caudal fin) bifurcate periodically along the proximo-distal axis of the fin forming 2 sister rays (Fig. 1.1C). A multilayered epidermis surrounds each lepidotrichia and the space between the 2 hemi-rays is filled with connective tissue, the nerves and blood vessels. On the otherhand, the inter-ray tissue that connects all of the rays together contains no bony elements, just connective tissue, nerves and blood vessels (Montes *et al.* 1982; Becerra *et al.* 1983; Santamaria *et al.* 1991; Geraudie *et al.* 1992). The arterial blood vessels run inside the fin rays between the 2 hemirays, whereas the venous vessels are located on the outside of the fin rays in the inter-ray tissue (Fig. 1.1E) (Lawson *et al.* 2002; Huang *et al.* 2003). The fin exoskeleton also consists of unmineralized elastoidin fibrils called actinotrichia located at the distal tip of each fin ray (Fig. 1.1C) (Mari-Beffa *et al.* 1989). As the fish grows from the larval to adult stage the fin rays also grow in size by addition of segments rather than by growth of each segment (Haas 1962; Johnson *et al.* 1999; Iovine *et al.* 2000).

1.2.3 Cellular mechanisms of caudal fin regeneration

Much like limb regeneration in the urodeles, the regeneration process can be broken up into three different stages. The length of time it takes to replace the lost structure is much more rapid in fin compared to limb regeneration and is also species- and temperature-dependent, with the process being quicker at 33°C compared to the normal habitat of

zebrafish which is 28.5°C (Johnson *et al.* 1995). Either way, the process is complete within 2-3 weeks in zebrafish (Fig. 1.2A).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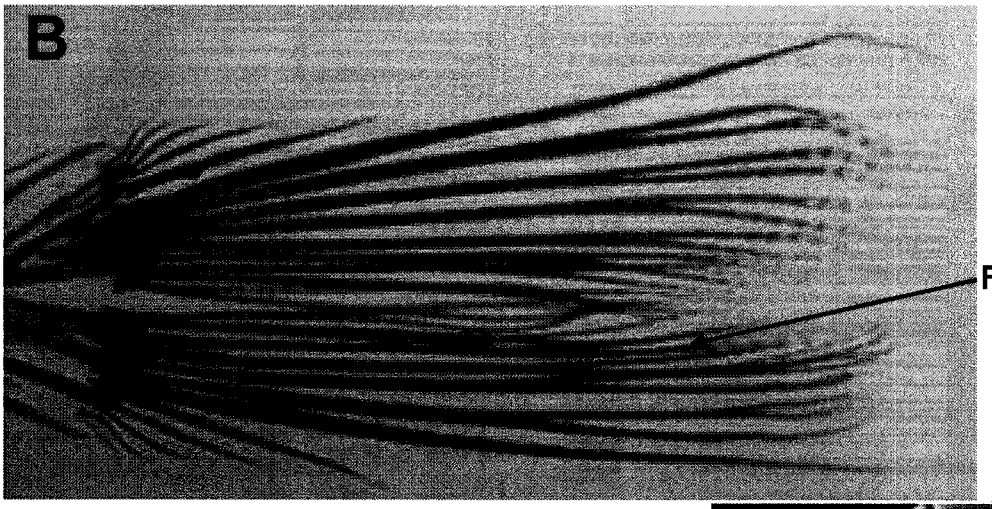
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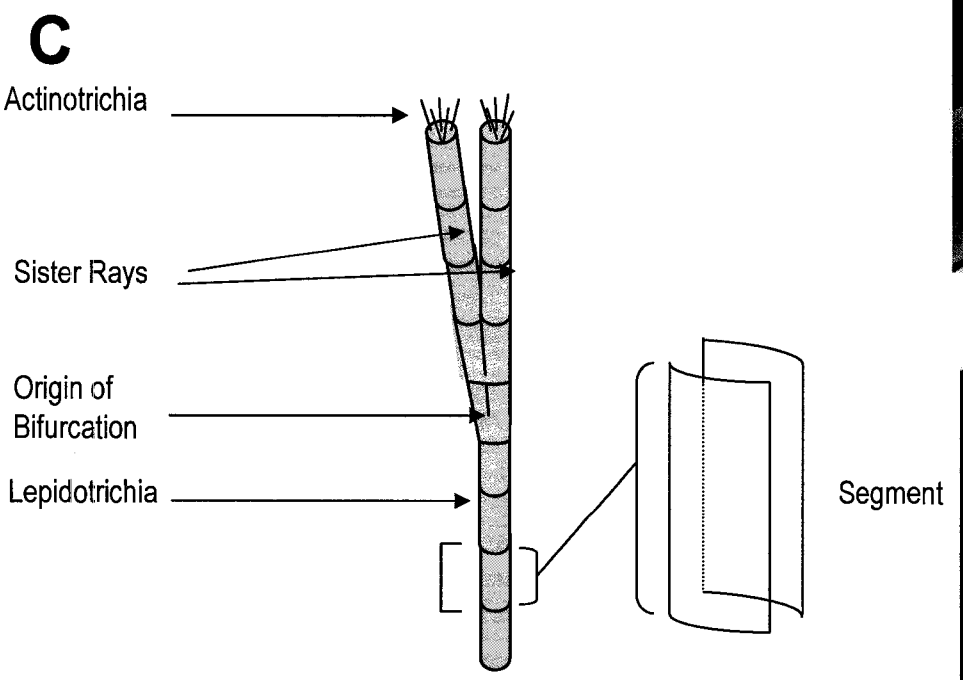
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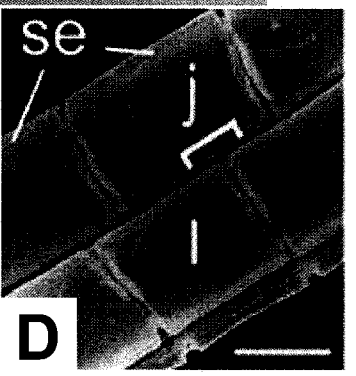
Sister Rays
Origin of
Bifurcation
Lepidotrichia
Segment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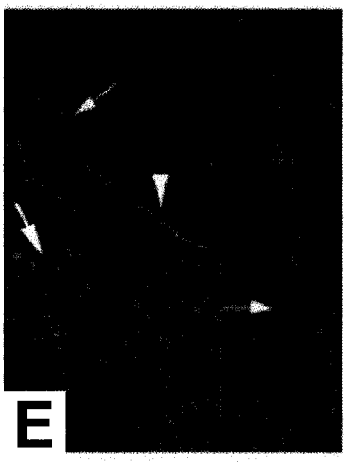
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Figure 1.1

Figure 1.1: Structure of the zebrafish fins

A: Fins of adult zebrafish comprised of 2 sets of paired fins: the pectoral and pelvic fins, and the 3 unpaired fins; the dorsal, anal and caudal fins.

B: Whole mount of a caudal zebrafish fin stained with alizarin red showing the mineralized bony fin rays of dermal origin and also the base of the fin, which is a part of the endoskeleton.

C: Schematic representation of the dermal skeleton of a fin ray (= lepidotrichia). Each segment comprises two parallel hemisegments separated by connective tissue. A ray splits, at the origin of bifurcation into two sister rays.

D: Scanning electron micrograph (SEM) of lepidotrichia showing two segmented hemirays (se) and the joint (j) that attaches each segment. Adapted from (Borday *et al.* 2001)

E: High magnification of a fluorescent photograph of part of the caudal fin of a *fli1:EGFP* transgenic fish showing GFP expression within the vasculature. Red arrows indicate the artery running in center of each ray, while the vein for each ray is located in interray mesenchyme adjacent to the ray (yellow arrow). White arrows indicate intervessel commissures within the same ray connecting the artery to the vein as well as vein to vein of the same ray. White arrowheads show the interray vessels connecting vein to vein of adjacent rays. Adapted from (Huang *et al.* 2003)

Each of these stages is also characterized by the expression of certain genes, most being developmental genes that are being re-expressed during regeneration. These genes and gene families will not be addressed in this section and will be detailed in section 1.3.

1.2.3.1 Wound healing

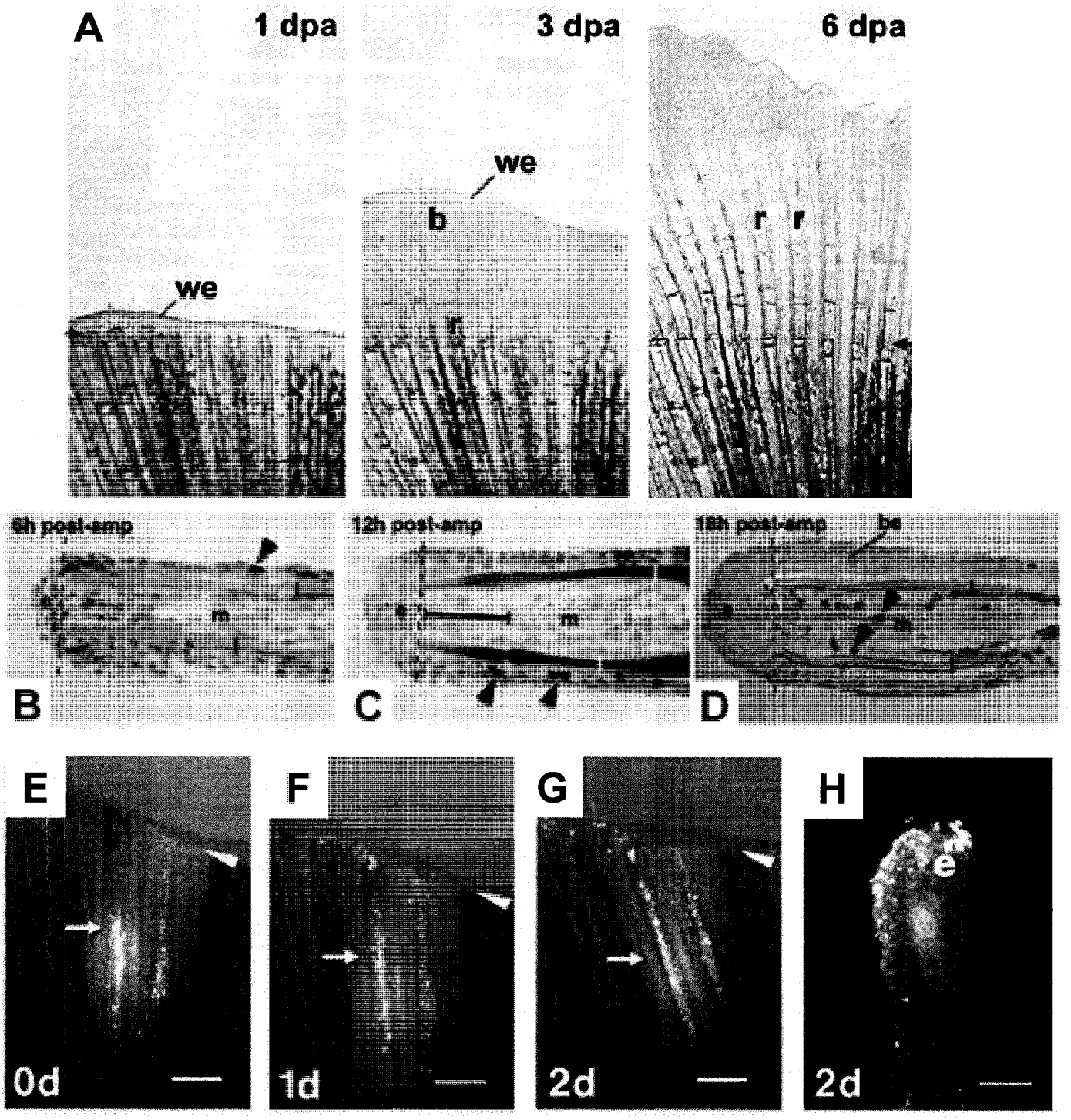
Unlike mammals, following amputation or injury the wound healing process in the fin involves very little bleeding or inflammation. Our laboratory has previously shown that following amputation, epidermal cells of the stump migrate and rearrange within the first few hours to cover the wound and form what is called either the wound epidermis or AEC (Poleo *et al.* 2001) . This migration of epidermal cells continues to happen at a rapid rate resulting in the thickening of the AEC to form a multi layered epidermis. It was determined that this process is governed by cell migration and not proliferation using Bromodeoxyuridine (BrdU) incorporation experiments (Santamaria *et al.* 1996; Poleo *et al.* 2001; Nechiporuk *et al.* 2002). BrdU is a thymidine analogue that incorporates into DNA during S phase and is detected immunocytochemically. It was shown that none of the cells of the AEC/wound epidermis were labeled with BrdU (Fig1.2. B-D). The formation of AEC through cell migration and not proliferation was confirmed using Di-I labeling (a fluorescent lipophilic dye that allows labeling of the cell membrane) where it was injected in the epithelial tissue of the caudal fin along the proximal distal axis between adjacent rays, followed by fin amputation 24 hrs later distal to the site of injection. These labeled stump epithelial cells were shown to migrate distally over the next 2 days and eventually accumulate at the site of injury forming the wound epidermis (Fig. 1.2E-H) (Poleo *et al.* 2001). The importance of studies that focus on the formation

of the wound epidermis during fin regeneration are confirmed by the fact that without this structure regeneration does not occur as is seen during limb regeneration (Goss 1956; Dearlove *et al.* 1974; Saunders *et al.* 1976). This was demonstrated when the regenerating fin was treated with retinoic acid causing apoptosis in the cells of the wound epidermis resulting in abnormal patterning of the fin regenerate and leading to the conclusion that the wound epidermis is vital to proper regeneration of the fin (Ferretti *et al.* 1995). The importance of the wound epidermis for the regeneration process has also been hypothesized based on the expression of developmentally important genes and also its close proximity to the forming blastema cells (Monnot *et al.* 1999; Poss *et al.* 2000a; Poss *et al.* 2000b). The necessity of wound epidermis during regeneration is for epithelial-mesenchymal signaling between itself and the blastemal cells; this interaction is vital for the fin regeneration process (Poss *et al.* 2000b; Whitehead *et al.* 2005).

1.2.3.2 Blastema formation and growth

The event that distinguishes epimorphic regeneration from development is the formation of a blastema. Fin regeneration occurs due to a proliferative mass of mesenchymal cells that forms at the stump of each severed fin ray and eventually gives rise to the new structures of the fin (Mari-Beffa *et al.* 1989; Santamaria *et al.* 1991, 1992; Wagner *et al.* 1992; Becerra *et al.* 1996). Unlike newt limb regeneration, where many studies have pointed to cellular dedifferentiation as the source of blastema cells, it is unclear whether blastema formation in the zebrafish fin involves cellular dedifferentiation, activation of quiescent stem cells, or both processes. To date in zebrafish, experiments to confirm cellular dedifferentiation as the source of blastema cells during fin regeneration are

technically difficult due to the fact that the fin does not contain structural cells with clearly differentiated phenotypes like the myotubes that are present in the newt, although, some evidence as to the source of these blastema cells has been observed. For example, fibroblast-like cells found approximately 1-2 segments proximal to the cut site have been shown to disorganize and orient longitudinally, and a fluorescent dye demonstrated that these cells migrate toward the amputation plane (Poleo *et al.* 2001; Nechiporuk *et al.* 2002).


A
1 dpa
3 dpa
6 dpa
we
we
b
r
r r
5h post-amp
12h post-amp
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be
m
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m
B
C
D
E
F
G
H
0d
1d
2d
2d
e

Figure 1.2

Figure 1.2: Zebrafish caudal fin regeneration: formation of the wound epidermis

A: Whole-mount images of the same caudal fin regenerate at 1 day, 3 days, and 6 days post-amputation (dpa) showing how quickly the lost structures are replaced. The arrows indicate the level of the cut. we, wound epidermis; b, blastema; r, ray.

B-D: Detection of proliferating cells during wound epidermis formation using BrdU labeling. Fish were incubated with BrdU for 6h, fins were then harvested and longitudinal sections were stained with BrdU antibody (black arrowheads are pointing to proliferating cells labeled in brown) and then counterstained with hematoxylin. These experiments were conducted at 33°C. (B) 6 hours post-amputation (hpa) a thin layer of wound epidermis covers the stump region but it contains no proliferating cells, the only labeled cells at this time are epidermal cells immediately proximal to the amputation plane. (C) By 12 hpa there are still no proliferating cells within the regenerated wound epidermis. At this time period we can also see early signs of mesenchymal disorganization (bracket). (D) The first sign of BrdU positive cells within the stump mesenchymal cells occurs at 18 hpa, but there is no proliferating cells found in the wound epidermis. be, basal epidermis; e, epidermis; l, lepidotrichia; m, mesenchyme. Adapted from: (Nechiporuk *et al.* 2002). 'Reproduced with permission of the Company of Biologists"

E-H: Caudal fin amputation triggers epithelial cell migration to the wound epidermis as seen by Di-I labeling. 1 day before amputation Di-I was injected between adjacent rays (arrow) approximately 500-600μm from future amputation plane (arrowhead) and cell migration was observed at (E) 0 dpa, (F) 1 dpa and (G) 2 dpa. Labeled cells are observed by 1 dpa in the wound epidermis (F) and a longitudinal section through a 2 day regenerated caudal fin shows labeled cells in the epidermal cap and lateral epidermis that have migrated from the stump region (H). e, epidermis. Adapted from: (Poleo *et al.* 2001)

In the same study it was shown, using BrdU incorporation, that these fibroblast-like cells or blastemal precursors were re-entering the cell cycle within the first 24 hrs following amputation (Fig. 1.2D) (Poleo *et al.* 2001). At 2 days following amputation, the forming blastema shows high levels of BrdU incorporation as well as mesenchymal cells up to 75μm proximal to the amputation plane (Poleo *et al.* 2001; Nechiporuk *et al.* 2002). Therefore it can be hypothesized that these cells that are re-entering the cell cycle within the stump region following amputation are likely candidates for the cells that will eventually give rise to the undifferentiated mass of cells that comprise the blastema, it is likely that they arise from the dedifferentiation of cells in close proximity to the site of injury, as is seen during newt limb regeneration. This blastema of undifferentiated proliferating cells begins to form and grow underneath the wound epidermis as early as 1-2 dpa (Fig. 1.3A) (Poleo *et al.* 2001; Nechiporuk *et al.* 2002). BrdU analysis performed at 2 and 3 dpa demonstrated that the mesenchymal cells of the distal blastema are highly proliferating allowing for growth of the regenerate (Poleo *et al.* 2001; Nechiporuk *et al.* 2002; Santos-Ruiz *et al.* 2002).

At the same time as the blastema is forming, another layer of cells is also being established, which is called the basal layer of the epidermis. This layer separates the outer epidermis from the blastema and is made up of highly ordered cuboidal cells that are easily distinguished upon sectioning of fin regenerates (Fig. 1.3B). This layer is thought to play major roles in the epithelial-mesenchymal interactions that pattern the newly forming regenerate (Santamaria *et al.* 1996; Laforest *et al.* 1998; Poss *et al.* 2000a; Santos-Ruiz *et al.* 2005).

1.2.3.3 Regenerative outgrowth and differentiation

At 28°C the transition from blastema formation to regenerative outgrowth in the zebrafish caudal fin occurs at 3-4 dpa (Santamaria *et al.* 1996; Poleo *et al.* 2001). In this stage, the regenerate starts to take on a distinct morphological structure with the blastema continuing to grow and proliferate distally while the more proximal cells closer to the amputation plane start to differentiate and replace the lost structures (Fig. 1.3B) (Santamaria *et al.* 1996; Poleo *et al.* 2001; Nechiporuk *et al.* 2002). There are two key events that occur during this phase: the deposition of new bone to replace the lost bony rays and the continued proliferation and growth of the distal blastema which still remains undifferentiated (Santamaria *et al.* 1991, 1996; Poleo *et al.* 2001; Nechiporuk *et al.* 2002). It is important to note that the regeneration blastema itself is hypothesized to be partitioned into two domains, the proximal and distal blastema, with the cells of the distal blastema proliferating at a slower rate than cells of the proximal blastema (Nechiporuk *et al.* 2002). The third region of the regenerate, which is not considered part of the blastema, is located in the proximal regenerate and is where patterning and cell differentiation are occurring.

1.2.4 The regeneration blastema

The proliferation of the mature blastema (from now on referred to as the blastema) that continues to grow distally has been studied in great detail. Nechiporuk *et al.*, (2002) have shown, using an antibody against a specific mitotic marker (anti-phosphohistone H3 (H3P) antibody), that the G2 length during formation of the blastema is greater than 6hr, whereas during outgrowth it is drastically reduced to only 1 hr (at 33°C). This extremely

fast cell cycle rate during the outgrowth phase is not surprising considering that it takes as little as 2-3 weeks for the amputated fin to regain most of its original size. Next, they examined the proliferation properties of the regeneration blastema during the outgrowth phase and found that the blastema is not a homogenous population of proliferating cells, as is seen during blastema formation.

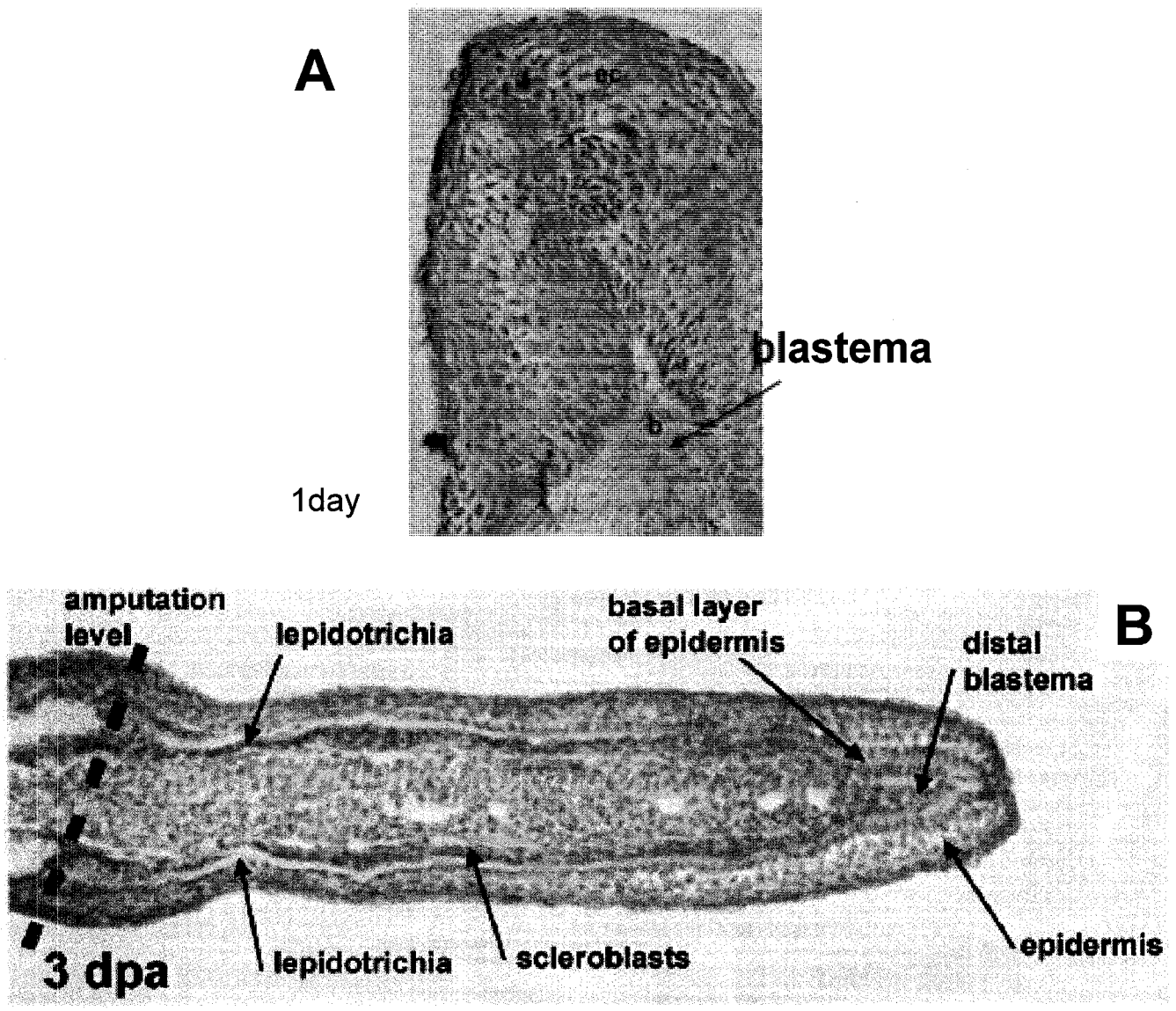

A
blastema
1day
amputation level
lepidotrichia
basal layer of epidermis
distal blastema
B
3 dpa
lepidotrichia
scleroblasts
epidermis

Figure 1.3

Figure 1.3: Process of blastema formation and outgrowth

A: Histological staining of a longitudinal section of a 1 day regenerate showing a small blastema forming underneath the multilayered wound epidermis.

B: Longitudinal section of a 3dpa regenerate stained with hematoxylin and eosin showing the emergence of the distinct morphology and structures of the regenerate. The basal layer of the epidermis, the scleroblasts and the lepidotrichia begin to become evident. Dashed line indicates level of amputation.

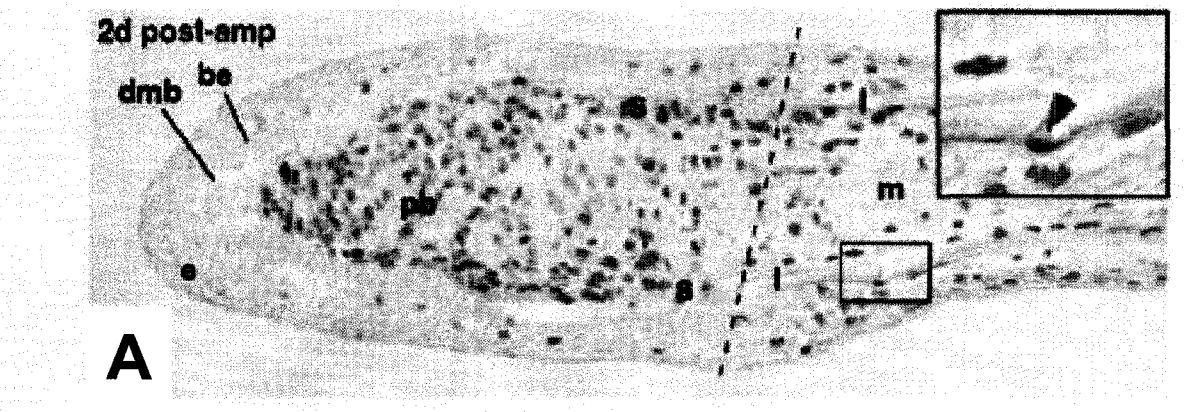
In fact, it is partitioned into two domains with different proliferative properties and possibly distinct functions. This was determined by closely examining BrdU incorporation in the distal-most blastema (DMB) compared to the proximal blastema (PB) at various times during the outgrowth phase. They observed that there was a significant difference in BrdU labeling between distal cells and those immediately proximal to them at 2-3 dpa following amputation at 33°C, with the DMB cells being scarcely labeled (Fig. 1.4A, B). Next, using BrdU pulse chase experiments, it was also shown that both the cells of PB and the DMB arose from early proliferating blastema cells and therefore the slow cycling cells of the DMB did not arise from an unknown stem cell population. Although there is no clear cut boundary between these two proliferation zones, there is a 50 fold gradient of proliferation between the DMB and the PB that is noticeable when proliferating cells are labeled with H3P (Nechiporuk *et al.* 2002). This group also examined the expression of the transcription factor *msxb,* which is thought to maintain cells in an undifferentiated state, and found its expression to become restricted to the DMB during regenerative outgrowth (Woloshin *et al.* 1995; Zhang *et al.* 1997; Odelberg *et al.* 2000; Hu *et al.* 2001; Nechiporuk *et al.* 2002). It was hypothesized that *msxb* expression within this cell population could be necessary to slow cycling DMB cells to prevent them from proliferating and maybe necessary to guide the outgrowth of the regenerate. Based on all of the above mentioned results, Nechiporuk *et al.,* (2002) proposed a compartmentalization model for the fin regenerate during outgrowth, dividing the regenerate into 3 zones: the DMB, the PB and the differentiation zone. The differentiation zone is proximal to the PB and consists of scleroblasts, fibroblasts and differentiating mesenchymal cells. The first two zones are characterized

by *msxb* expression in the non-proliferating cells of the DMB and the expression of zebrafish orthologue of a mitotic checkpoint gene (*mps1*) in the highly proliferative cells of the PB (Fig. 1.4C) (Woloshin *et al.* 1995; Zhang *et al.* 1997; Odelberg *et al.* 2000; Hu *et al.* 2001; Nechiporuk *et al.* 2002, 2003; Poss *et al.* 2002). The third zone will be described in more detail below.

1.2.4.1 The differentiation zone or proximal regenerate

As the regenerate continues to grow distally the lepidotrichia begins to mature in the proximal regenerate closest to the amputation plane. Although there are multiple cell types in the regenerate, the two major and most well characterized cell types of the fin begin to become apparent in this proximal differentiating tissue: the bone matrix secreting scleroblasts and fibroblast-like cells (Santamaria *et al.* 1991; Johnson *et al.* 1995; Mari-Beffa *et al.* 1996; Nechiporuk *et al.* 2003). These cells are easily observable using histological and immuno-staining on a longitudinal section through a fin regenerate in the outgrowth phase. Scleroblasts, for example, can be visualized using an antibody called Zns-5 that specifically labels differentiated scleroblasts (Johnson *et al.* 1995). As during ontogeny of the fin rays, the most mature scleroblasts are found in the most proximal regenerate; therefore, bone matrix mineralization and lepidotrichia regeneration and thickening happen in a proximo-distal direction. In contrast, in the more distal regions newly differentiating scleroblasts align along the basal layer of the epidermis that separates the epidermis from the mesenchyme. In these more distal regions the regenerating lepidotrichia is not visible but scleroblasts are secreting bone matrix into the subepidermal space at the epithelial-mesenchymal interface (Geraudie *et al.* 1982; Landis

et al. 1990; Becerra *et al.* 1996; Santamaria *et al.* 1996; Borday *et al.* 2001). In the most proximal regions of the regenerate closest to the amputation plane, the scleroblasts migrate around the already made bone matrix to the outer surface of the deposited bone. They continue to secrete bone matrix from both sides allowing the lepidotrichia to thicken from both surfaces (Johnson *et al.* 1995; Becerra *et al.* 1996; Santamaria *et al.* 1996). This process occurs throughout the growth of the regenerate in the proximal regions.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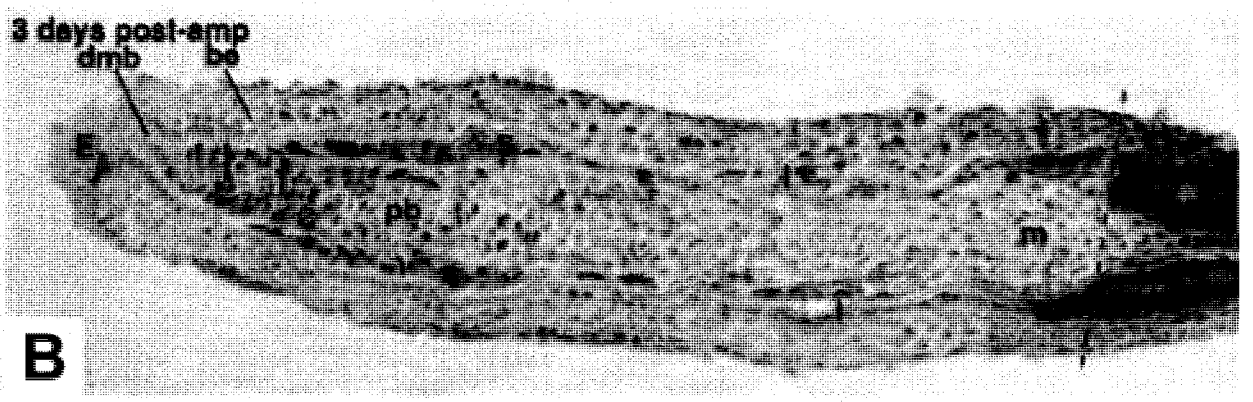
3 days post-amp
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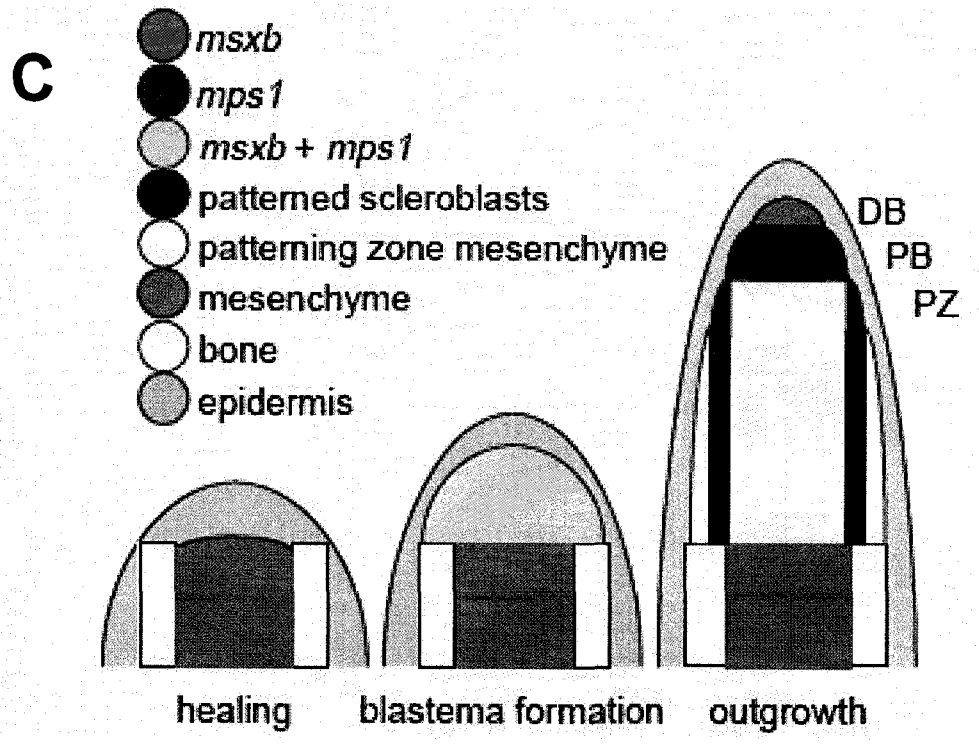

C
msxb
mps1
msxb + mps1
patterned scleroblasts
patterning zone mesenchyme
mesenchyme
bone
epidermis
DB
PB
PZ
healing
blastema formation
outgrowth

Figure 1.4

Figure 1.4: Proliferation during blastema outgrowth

A: At 2dpa following a 6h BrdU treatment, a functional blastema emerges with a large number of BrdU positive cells in the proximal blastema (pb), while it becomes evident that a small group of cells in the most distal blastema (dmb) are unlabeled. Inset shows that scleroblast cells in the stump region are also proliferating. be, basal epidermis; dmb, distal most blastema; pb, proximal blastema; l,lepidotrichia; m, mesenchyme; s, scleroblasts. Dashed line indicates level of amputation. Adapted from Nechiporuk *et al.* 2002).

B: By 3dpa, high levels of BrdU incorporation are seen in the proximal blastema, while the distal blastema still remains unlabeled. By this stage, the distinct regions of the regenerate become apparent: a non-proliferating distal blastema, a highly proliferating proximal blastema and the region where the differentiated scleroblasts begin to replace the lost lepidotrichia. be, basal epidermis; dmb, distal most blastema; pb, proximal blastema; l, lepidotrichia; m, mesenchyme; s, scleroblasts. Dashed line indicates level of amputation. Adapted from: (Nechiporuk *et al.* 2002).

C: Cellular and molecular model for zebrafish fin regeneration: The early stage of blastema formation is characterized by the onset of expression of *msxb* and *mps1*. This is then followed by the outgrowth phase of regeneration when the blastema can be subdivided into 3 domains. The distal blastema cells (DB) expressing *msxb* (orange), the proximal blastema (PB) which expresses *mps1* (blue) and the patterning zone (PZ) where the newly patterned scleroblasts (brown) and differentiating mesenchyme (yellow). Adapted from:(Poss *et al.* 2002).

A-C: 'Reproduced with permission of the Company of Biologists'

1.2.5 Vascularization

The zebrafish fin contains a rich supply of blood vessels. This was demonstrated by Lawson and Weinsten (2002) who created a line of transgenic zebrafish that express EGFP under the control of a regulatory region of the *fli1* gene. This gene is an endothelial cell marker and the transgenic line allows for the visualization of the development of the vasculature *in vivo* (Lawson *et al.* 2002). Each of the fin rays is supplied by one artery that runs inside the lepidotrichia in between the center of the two hemirays. The interray tissue is where the two flanking veins are located on either side of the ray. The arteries and veins of each ray are connected by intervessel commissures (Lawson *et al.* 2002; Huang *et al.* 2003). Using these transgenic fish Huang *et al.*, (2003) demonstrated the blood vessels of the fins also regenerate following amputation or injury. These transgenic fish were used to visualize the regeneration of blood vessels in the adult following caudal fin amputation (summarized in: Huang *et al.* 2003). It was determined that within the first day post amputation, the vessels' ends are healed, then reconnect through anastomosis of sprout extensions from the healed vessel ends and, by 2dpa blood, circulation resumes in these healed ends. The next phase happens between 2-8dpa when numerous thin vessels begin to branch from the bridges that have formed and then begin to make connections with neighboring vessels of the other rays. The blood vessels are then remodeled by pruning of the connections or intervessel commissures to produce definitive arteries and veins. In the late phase of regenerative angiogenesis, the blood vessels continue to sprout from the distal ends as the regenerate continues to grow. Much like the other cell types of the regenerate the origin of the cells that form the regenerating

blood vessels are still unknown, although it is thought that they arise from the pre-existing blood vessels.

1.2.6 Molecular pathways known to be involved in caudal fin regeneration

Much effort is being focused on dissecting the molecular pathways involved in all stages of zebrafish fin regeneration to elucidate the key factors that govern the regeneration process to eventually apply this information to the regeneration of human organs and tissues. Not only do the three zones of the fin regenerate have distinct cellular characteristics as discussed before, each zone is also defined by different molecular factors. As touched upon in the above section, in newt limb regeneration, many of the signaling pathways involved in both fin and limb regeneration are also involved in development of these organs as well as other organs. This, although insightful, poses problems in deciphering the function of many of these pathways during regeneration because of their vital roles in embryogenesis. Mostly due to the fact that the conventional methods of knocking down a gene in zebrafish embryogenesis to decipher its function will not allow the assessment of gene function during regeneration in adult simply because the fish does not survive, many groups have started to hypothesize about the function of many genes in zebrafish regeneration, by simply analyzing their expression patterns within the regenerate and some have taken upon the task of inhibiting their function during the regeneration process. Therefore, several studies in the last decade have shown that many developmental signaling pathways such as the Hedgehog (Hh), Fibroblast growth factor (Fgf), Bone morphogenetic pathway (Bmp) and Wingless (Wnt)

pathways are activated during regeneration. Studies focusing on the signaling pathways that play some of the most vital roles in the regeneration process are described below.

1.2.6.1 Hh and Bmp signaling

The sonic hedgehog signaling pathway is known to play a vital role in the patterning and morphogenesis of many organs and tissues during development (reviewed in: (Ingham *et al.* 2001). Sonic hedgehog (Shh) expression in the zone of polarizing activity (ZPA) of the vertebrate limb buds is critical for the proper patterning of the anterior-posterior axis of the developing limb (Riddle *et al.* 1993). It has also been shown to be expressed within the pectoral fin buds of the zebrafish in a similar posterior domain as that seen in the mouse and chick limb buds (Krauss *et al.* 1993). Laufer *et al.,* (1994) demonstrated that Shh initiates the expression of other signaling molecules within the chick limb bud by misexpressing Shh in the anterior limb bud and inducing an ectopic expression of *Bmp2* within the mesenchyme. Laforest *et al.,* (1998) first characterized the expression of *shh*, its receptor *ptc1*, and a possible downstream target *bmp2b* during both bony ray development and regeneration in the zebrafish fins. It was found that during fin regeneration *shh* and *ptc1* are re-expressed with patterns similar to those observed in the development of larval fin rays. During blastema formation *shh*, *ptc1* and *bmp2b* are all initially expressed in a single domain at the distal stump of the amputated ray. Following the initiation of expression, during the outgrowth and differentiation phase, an imminent bifurcation is signaled by the duplication of the single domain of *shh, bmp2b* and *ptc1* expression into two domains (Fig. 1.5A-C). Upon tissue sectioning it was found that all three genes were expressed in a subset of cells in the basal layer of the epidermis.

Although *shh* expression is restricted to this subset of cells of the basal layer of the epidermis, both *ptc1* and *bmp2b* are also found in the adjacent newly differentiating scleroblasts within the mesenchyme (Fig. 1.5D-F) (Laforest *et al.* 1998). According to the compartmentalization model the expression of *shh* in the basal layer of the epidermis marks the border between the PB and the differentiation zone (Poss *et al.* 2003). The expression patterns of both *shh* and *bmp2b* within the fin regenerate led to the hypothesis that their expression in regions of cell proliferation (mesenchyme) and new bone deposition (scleroblasts) along with their changing expression domains prior to bifurcation implicate them in the formation and patterning of new dermal bone. At this point, no functional analysis had been performed examining the role of Shh and Bmp signaling pathways in fin regeneration.

More recently Avaron *et al.,* (2006) isolated and characterized the expression of another member of the Hedgehog (Hh) family in zebrafish, *ihha*, an orthologue of the mammalian Indian hedgehog (*Ihh*) gene. One of the major roles of Ihh in mammalian development is in endochondral bone development. Ihh is responsible for controlling the maturation of the chondrocytes towards hypertrophy and maintaining a pool of proliferating non-hypertrophic cells (Bitgood *et al.* 1995; Vortkamp *et al.* 1996; St-Jacques *et al.* 1999; Chung *et al.* 2001). In the zebrafish larvae, *ihha* is expressed in mature cartilage cells in the head and fin endoskeleton (Avaron *et al.* 2006). During development of the fin rays, *ihha* was found to be expressed in the scleroblasts. During fin regeneration, *ihha* was also found to be expressed first during blastema formation in two small domains of mesenchymal cells that are hypothesized to be early differentiating scleroblasts. Then, during outgrowth, the expression becomes broader and is detected in

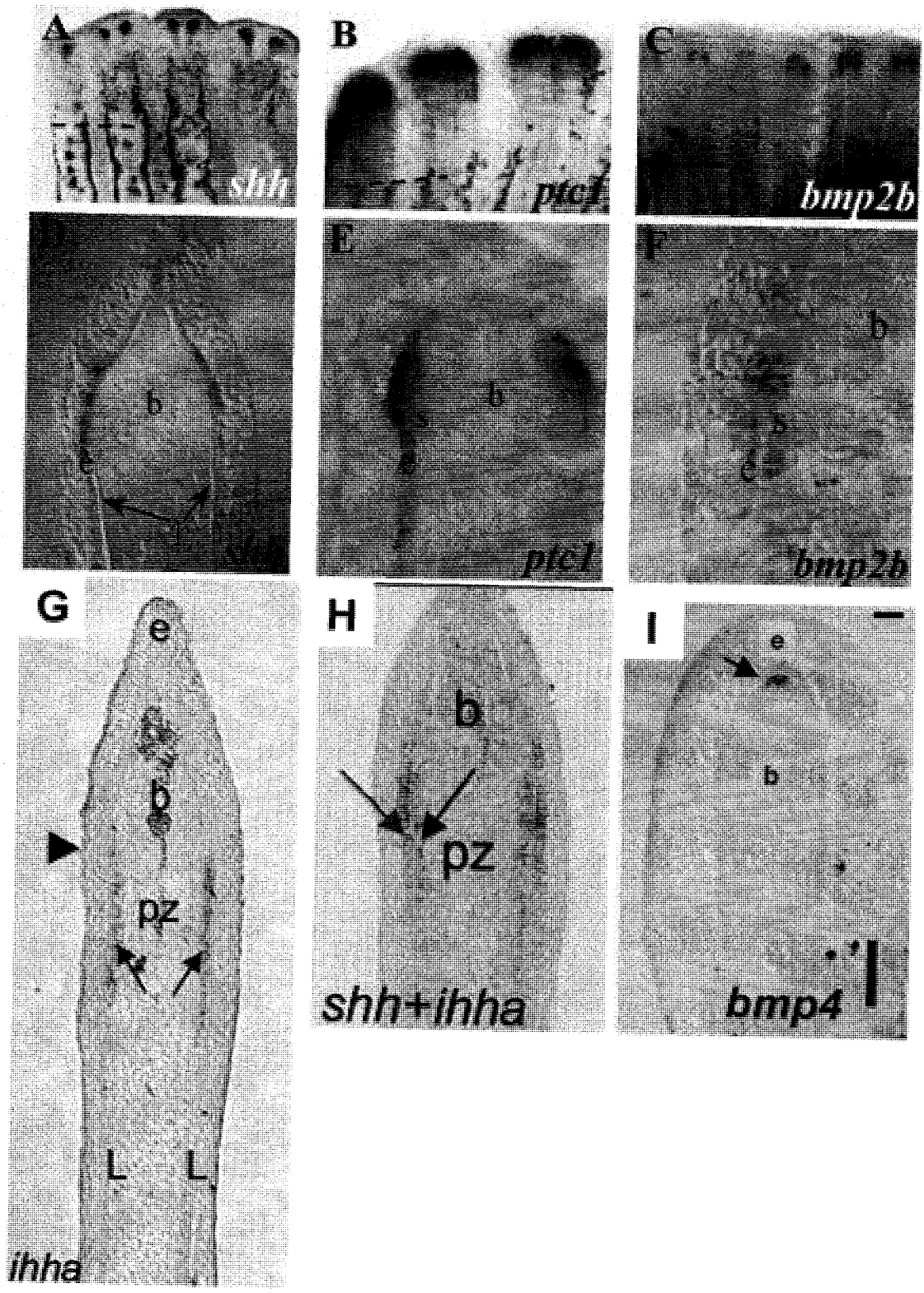
scleroblasts at the level of the differentiation zone (Fig. 1.5G). Co-labeling experiments show that *ihha* expression domain is directly adjacent and has the same proximo-distal limits as *shh* in the basal epithelial layer (Fig. 1.5H) (Avaron *et al.* 2006). Based on these adjacent expression patterns and the fact that *ptc1*, the common receptor of both *shh* and *ihha*, is expressed in the basal epithelial layer and blastema cells, it has been hypothesized that the two Hh signals interact at the epithelial-mesenchymal interface (Avaron *et al.* 2006). The expression pattern of *shh* in basal epithelial cells adjacent to scleroblasts suggests that it may play a role in bone patterning and *ihha* expression in scleroblasts may indicate it is responsible for differentiation and function of the bone-secreting cells possibly acting as an intermediate between Shh and Bmp signaling in bone regeneration. This study was also the first evidence that cartilage-specific genes are expressed in bone secreting cells, which will be discussed in more detail in the following sections.

Previous analyses have shown that at least two members of the *bmp* gene family, *bmp2b* (discussed above) and *bmp4*, are expressed during regeneration of the zebrafish caudal fin (Laforest *et al.* 1998; Murciano *et al.* 2002). *bmp4* expression is restricted to the distal blastema indicating a possible role in controlling blastemal cell proliferation and regenerate outgrowth (Fig. 1.5I) (Murciano *et al.* 2002).

1.2.6.2 Fgf signaling

The Fgf family is a large family of short polypeptides that are released extracellularly and dimerize with heparin to activate specific receptor tyrosine kinases (Fgfrs). During embryogenesis, Fgf signaling plays numerous roles in induction and patterning of many

organs, such as the limb, tooth, brain and heart (Crossley *et al.* 1996; Vogel *et al.* 1996; Zhu *et al.* 1996; Ohuchi *et al.* 1997). It has also been shown to play roles in mammalian wound healing and amphibian and newt limb regeneration (as described above) (Poulin *et al.* 1993; Christen *et al.* 1997; Yokoyama *et al.* 2000). In 2000, Poss *et al.*, (2000b) published the first results showing the expression of members of the Fgf signaling family within the fin regenerate and also performed functional studies elucidating their importance in the regeneration process.


A
shh
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ptc1
C
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b
e
E
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F
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G
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p
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L
ihha
H
b
pz
shh+ihha
I
e
b
bmp4

Figure 1.5

Figure 1.5: Expression of members of the Hh and Bmp signaling families at 4dpa in the fin regenerate.

A-C: Whole-mount *in situ* hybridization done on 4 dpa regenerates showing (A) *sonic hedgehog* (*shh*) expression splitting into 2 domains before a birfurcation event. The downstream targets of Shh signaling pathway; its receptor *patched 1* (*ptc1*) (B) is expressed in a much wider domain and spans the entire width of the fin ray, while (C) *bone morphogenetic protein 2b* (*bmp2b*) is expressed in a much similar domain to *shh*. Dashed line is level of amputation. Adapted from: (Laforest *et al.* 1998)

D-F: Longitudinal sections of whole-mount fins that have been hybridized with *shh*, *ptc1* and *bmp2b*. *shh* expression is restricted to a limited number of cells of the basal epithelial layer (D), whereas *ptc1* (E) and *bmp2b* (F) are expressed not only in the basal epithelial layer but also in the adjacent scleroblast cells. b, blastema; e, epidermis; s, scleroblasts. Adapted from: (Laforest *et al.* 1998)

G-H: *In situ* hybridization done on longitudinal sections of 4dpa fin regenerates showing *ihha* expression (black arrow) in the scleroblasts at the epithelial-mesenchymal interface (G). Double *in situ* hybridization with *shh* (red arrow) and *ihha* (black arrow) demonstrates that *shh* expression in the basal epithelial layer is directly adjacent to *ihha* expression within the scleroblasts (H). b, blastema; e, epidermis; l, lepidotrichia; pz, patterning zone. Adapted from: (Avaron *et al.* 2006)

I: *bmp4* is expressed (black arrow) in the distal most cells of the blastema at 4dpa as is seen on longitudinal sections of a whole-mount *in situ* hybridization. b, blastema; e, epidermis. Adapted from: (Murciano *et al.* 2002)

During blastema formation, *fgfr1* transcripts are detected in fibroblast-like cells between the hemirays just proximal and distal to the amputation plane (Fig. 1.6A, B). Then during the outgrowth phase, transcripts are present in a small population of distal blastema cells and in a large portion of the basal layer of the epidermis (Fig. 1.6C, D). The expression of *fgfr1* in distal blastema cells may correspond to the DMB (Poss *et al.* 2000b). The same group found that a member of the Fgf family designated "Wound fgf" (*wfgf*) was consistently expressed in the distal-most cells of the regeneration epidermis and by RT-PCR it was determined that the expression of *wfgf* was higher during the regenerative outgrowth than during blastema formation (Fig. 1.6E, F) (Poss *et al.* 2000b). The function of Fgf signaling during fin regeneration was analyzed using a pharmacological inhibitor of Fgf signaling, SU5402. It was found that treating animals with SU5402, immediately following amputation resulted in a complete regenerative block (Fig. 1.6G, H). This specific inhibition of Fgfr1 resulted in a block of blastema morphogenesis and proliferation but did not affect the formation of the wound epidermis or disorganization, proliferation, and migration of mesenchymal cells proximal to the amputation plane (Poss *et al.* 2000b). Therefore these results suggest that FgfR1 signaling is necessary for blastema formation but is not essential for earlier stages of regeneration. These results were confirmed when Lee *et al.*, (2005) made a zebrafish transgenic line, *hsp70:dnfgfr1*, that harbors a dominant negative *fgfr1-egfp* fusion gene (*dn-fgfr1*) driven by the zebrafish heat-inducible hsp70 promoter. Heat shocking of the transgenic fish activates the expression of *dn-fgfr* which inhibits Fgf signaling. It was shown that daily heat shocking of adult fish following fin amputation, to maintain the

expression of *dn-fgfr1,* completely blocked blastema formation and fin regeneration (Lee *et al.* 2005).

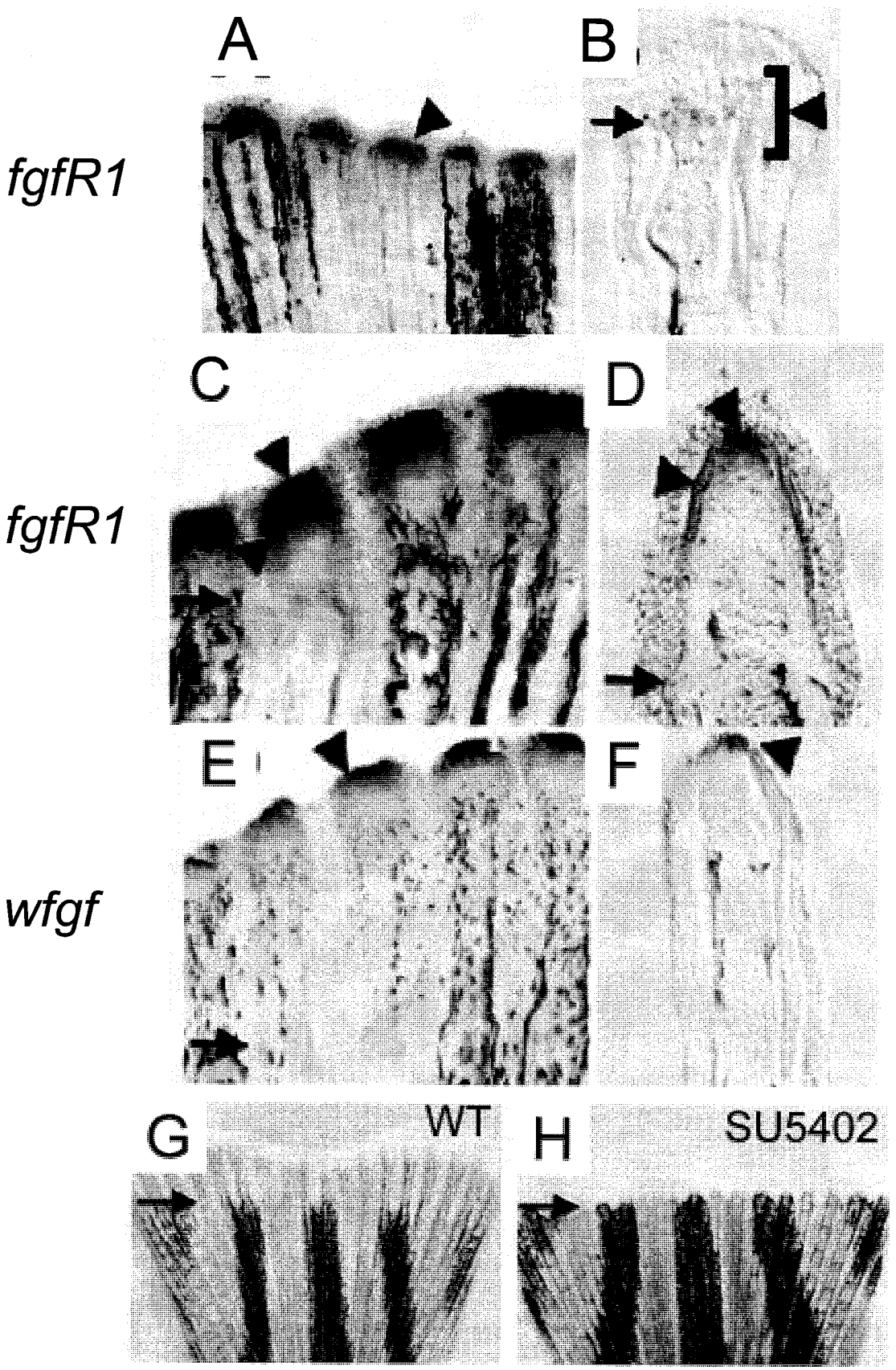


Figure 1.6

Figure 1.6: Expression of members of the Fgf signaling pathway during fin regeneration and the effect of inhibiting Fgf signaling on the regeneration process.

A-D: 12hpa *fgfr1* expression is detected, by *in situ* hybridization, in a group of cells on top of each regenerating ray (A), upon longitudinal sectioning of an 18 hpa regenerate hybridized with *fgfr1,* faint expression is seen in mesenchymal cells located between and just distal to the hemirays (bracket) (B). *Fgfr1* expression at 48hpa is detected in the distal regenerate in whole-mount fins (C) and sections done at this stage reveals its expression in two domains: the mesenchymal cells of the distal blastema and bilaterally in the basal layer of the epidermis (D). Arrowheads indicate expression and arrows the level of amputation. Adapted from: (Poss *et al.* 2000b)

E-F: at 48hpa *wfgf* is expressed in the distal regenerate (E). Longitudinal sections reveal its expression is restricted to the distal most cells of the regeneration epidermis (F). Arrowheads indicate expression and arrows the level of amputation. Adapted from: (Poss *et al.* 2000b)

G-H: Inhibition of Fgfr1 signaling with the pharmalogical inhibitor SU5402 blocks fin regeneration. (G) Photograph of the caudal fin of an untreated fish at 96hpa shows normal growth of the regenerate compared to a fish treated with SU5402 for 96h starting immediately following amputation (H). Adapted from: (Poss *et al.* 2000b)

At the molecular level, Poss *et al.*, (2000b) observed that inhibition of Fgf signaling, using SU5402, also abolished *msxb* and *msxc* expression. These results combined with the lack of blastemal proliferation suggest that Fgf signaling may activate *msx* gene expression to induce blastema formation through the transformation of mesenchymal cells into undifferentiated, proliferative cells. The importance of Fgfr1 during fin regeneration was confirmed by Thummel *et al.,* (2006) who developed a method to knockdown genes within the fin regenerate during the outgrowth phase by electroporation of antisense oligonucleotide morpholinos (MO). They found that electroporation of a morpholino (MO) against *fgfr1* into the ray blastemas of the dorsal half of the fin phenocopied the results found when fish were treated for 24hrs with SU5402. The effects included inhibition of regenerate outgrowth, decreased level of blastema cell proliferation and reduced *msxc* expression.

In addition to blastema formation and growth, Fgf signaling is thought to play a role in patterning the fin regenerate during the outgrowth phase. In other organisms, Fgf signaling has been shown to maintain the expression of *Shh* during development (Abud *et al.* 1996; Lebeche *et al.* 1999; Moon *et al.* 2000). The overlapping expression pattern of *shh* and *fgfr1* in the basal layer of the epidermis indicates their potential interaction during patterning of the fin regenerate (Laforest *et al.* 1998; Poss *et al.* 2000b). Treatment with SU5402, during the outgrowth phase, resulted in a reduced expression of *shh* indicating that Fgfs positively regulate *shh* transcription (Poss *et al.* 2000b).

More recently, Whitehead *et al.,* (2005) set out to characterize genes that initiate regeneration by performing a chemical mutagenesis screen for regeneration temperature sensitive mutants. Since the majority of the genes that are involved in regeneration also

function during embryonic development, temperature sensitive mutations circumvents the problem of embryonic lethality by only inducing the expression of the mutant phenotype when the adult fish is placed at 33°C (Johnson *et al.* 1995). Unexpectedly, they found a mutant fish named *dob* (*devoid of blastema*) that was unable to regenerate its fins at both 28°C and also at 33°C and was therefore not a temperature sensitive mutant (Whitehead *et al.* 2005). In addition, the mutation had no effect on embryogenesis, indicating that the affected gene was regeneration specific. Following fin amputation, *dob* mutants were found to form an abnormal wound epidermis that was thickened and the level of proliferation in the epidermis was the same as compared to wild type, indicating that this thickened epithelium is the result of abnormal migration. The following steps of the regeneration process also did not occur in these mutants: they showed no sign of mesenchymal disorganization, no cell proliferation, no cell migration or blastema formation. The *dob* mutation was found to be in the *fgf20a* gene, a newly identified member of the Fgf family. Using *in situ* hybridization, *fgf20a* transcripts were detected during the regeneration initiation phase with expression localized to the mesenchyme at the epithelial-mesenchymal interface and then was expressed within the blastema during its formation (Whitehead *et al.* 2005). Based on the expression of *fgf20a* and the phenotype of the *dob* mutant, it was concluded that *fgf20a* is essential for initiating fin regeneration and formation of the basal layer of the epidermis and the blastema.

1.2.6.3 Msx transcription factors

The Msx homeodomain family has been shown to be important factors in epithelial-mesenchymal interactions during organogenesis, and noteably limb development

(Davidson 1995; Koshiba *et al.* 1998; Tucker *et al.* 1998; Alappat *et al.* 2003; Lallemand *et al.* 2005). Msx proteins act as transcriptional repressors and are associated with the maintenance of cells in an undifferentiated state, therefore making their roles in both development and regeneration vital. For example, mouse embryos express *Msx1* in the progress zone of their limb buds and this expression is linked with the undifferentiated state of these cells (Wang *et al.* 1995). It has only been linked with regeneration in the *Xenopus* tail and newt limb (described in section 1.1.2) (Koshiba *et al.* 1998; Beck *et al.* 2006). Interestingly, fetal mice have the ability to regenerate their digit tips and it was shown that this ability is correlated with the expression of *Msx1* (Reginelli *et al.* 1995). When this gene is absent, as in *Msx1* mutants, regeneration does not occur (Han *et al.* 2003).

In zebrafish, there are 5 known *msx* genes, 4 of which are upregulated during zebrafish fin regeneration. Akimenko *et al.*, (1995) first showed that the *msxb* and *msxc* genes are expressed in the mesenchymal cells of the blastema, whereas *msxa* and *msxd* transcripts are detected in the distal epidermis overlying the blastema (Fig. 1.7A-D). The epidermal expression of *msxa* and *msxd* has not gained as much attention as the overlapping blastemal expression of the *msxb* and *msxc* genes. During development of many organs, Msx proteins have been associated to many signaling pathways, such as the Bmps, Fgf and Shh signaling pathways (Wang *et al.* 1995; Abud *et al.* 1996; Monsoro-Burq *et al.* 1996). They have been shown either to be induced by many of these factors or to induce the expression of these molecules. An example of the first case is during tooth morphogenesis where *Bmp2*, *Bmp4*, *Fgf2* and *Fgf4* expression in the dental epithelia induces *Msx1* expression in the underlying mesenchyme (Vainio *et al.* 1993; Bei *et al.*

1998; Yamashiro *et al.* 2003). Therefore, due to their expression patterns during fin regeneration and their known roles in regeneration in other organisms, many studies that examined the function of signaling pathways in fin regeneration have also examined the effects on the expression of the *msxb* and *msxc* genes (Poss *et al.* 2000b, 2002; Nechiporuk *et al.* 2002, 2003; Whitehead *et al.* 2005; Lin *et al.* 2008). For example, inhibition of Fgf signaling prevents blastema formation and also inhibits the induction of *msxb* and *msxc* expression (Poss *et al.* 2000b). It has been shown that during blastema formation *msxb* is expressed in all proliferating cells and then becomes restricted to the distal blastema region (DMB), comprised of a small population of slow cycling cells, during the outgrowth phase (Nechiporuk *et al.* 2002). From this, it was proposed that *msxb* may keep these DMB cells in an undifferentiated state to promote outgrowth of the fin. In support of this hypothesis, Poss *et al.*, (2002) observed an expanded domain of *msxb* expression during outgrowth in a temperature sensitive mutant. These mutants, called the *nightcap* (*ncp*) mutants, have a blockage of regeneration during the outgrowth phase due to severe blastemal proliferation defects and this mutation was found to be in the *mps1* gene. *mps1* is a cell cycle regulator that is specifically upregulated in the proximal fin blastema during outgrowth and has an overlapping expression pattern with proliferating cells labeled with PCNA (Fig. 1.7E, F). It was also shown to be expressed in the cells of the proximal blastema that did not express *msxb* (Fig. 1.7G). *mps1* was found to be necessary for proper blastemal proliferation and, when it is absent, it was proposed that the blastema no longer forms distinct domains or compartments, for example *msxb* is no longer restricted to the DMB but is expressed in a much wider domain (Poss *et al.* 2002). In these studies, no direct evidence for the function of *msxb*

was ever shown, until Thummel *et al.*, (2006) found that electroporation of a morpholino against the *msxb* gene into the regenerating fin resulted in an inhibition of fin outgrowth and a reduced number of proliferating blastema cells (Fig. 1.7H).

1.2.6.4 Wnt signaling

Like many of the other signaling pathways involved in development, the Wnt/ß-catenin pathway regulates progenitor cell fate and proliferation during embryogenesis. It is also involved in adult tissue homeostasis and in the control of cell proliferation during regeneration of muscle, liver and bone in mammals (Caubit *et al.* 1997; Poss *et al.* 2000a; Logan *et al.* 2004). Poss *et al.*, (2000a) showed that members of the Wnt signaling pathway are expressed during fin regeneration. The transcription factor *lef1* is expressed in the basal layer of the epidermis in a pattern similar to that of *shh*.

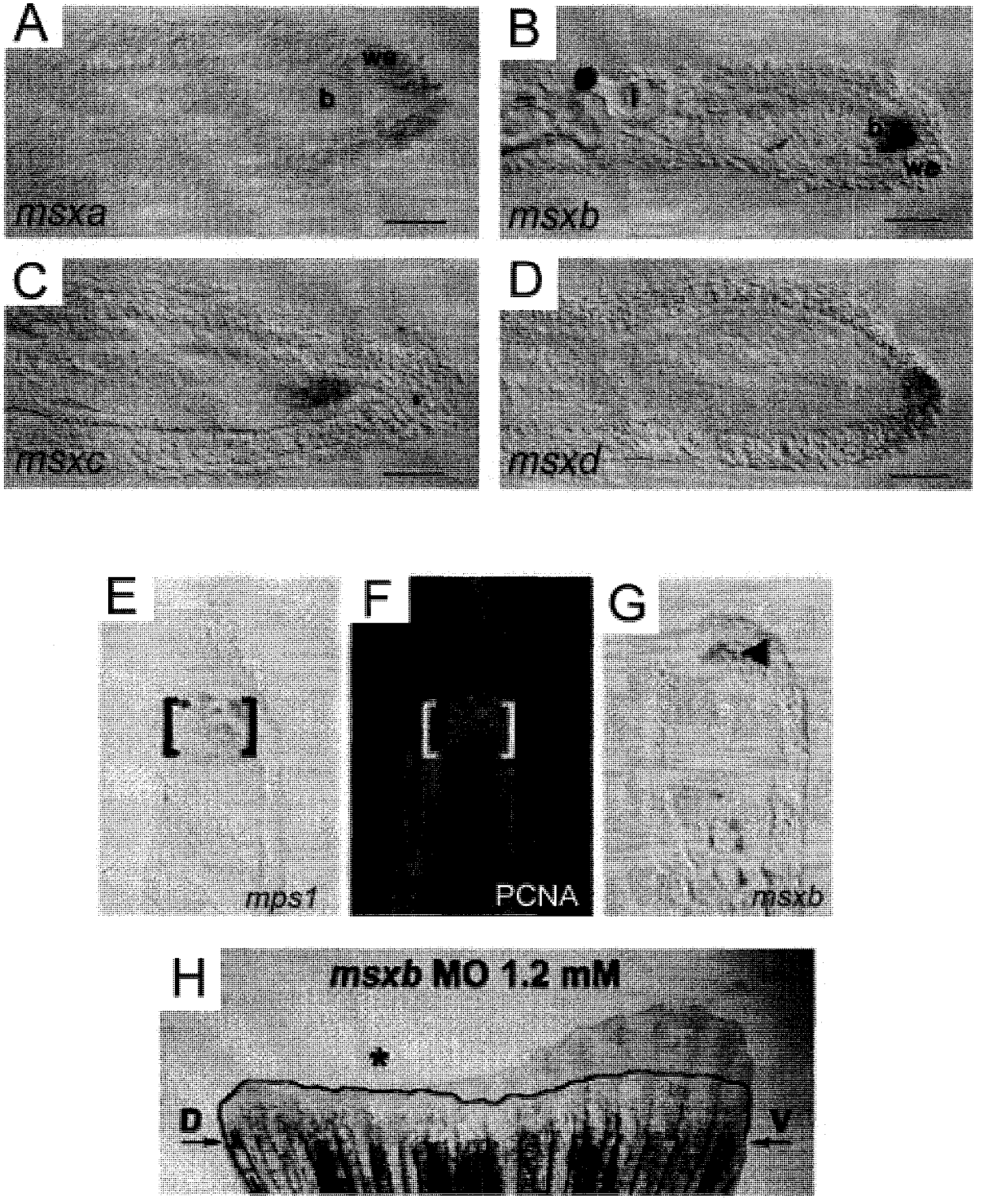


Figure 1.7

Figure 1.7: Expression of the *msx* and the *mps1* genes during fin regeneration and the effects of loss of function of these genes on the regeneration process.

A-D: Four msx transcription factors are expressed during fin regeneration. Longitudinal sections of 4dpa regenerates that were hybridized with the *msx* genes revealed *msxa* and *msxd* (A, D, respectively) are both expressed within the distal epidermis of the regenerate whereas *msxb* and *msxc* (B, C, respectively) are expressed in the distal mesenchymal cells of the blastema. b, blastema; we, wound epidermis. Adapted from: (Akimenko *et al.* 1995)

E-G: *mps1* expression in 3dpa regenerates is detected in the proliferative cells during outgrowth (brackets) and is absent in the distal blastema cells (E) that express *msxb* (G). *mps1* expression co-localizes with the PCNA antibody which labels proliferative cells (F). Adapted from: (Poss *et al.* 2002)

H: Electroporation of 1.2mM of msxb morpholino in the dorsal fin rays (D) leads to the inhibition of fin outgrowth (*) when compared to control ventral rays (V). black arrows indicate level of amputation. Adapted from: (Thummel *et al.* 2006).

In contrast to *lef1* expression, *wnt5* transcripts are detected throughout regeneration but only in the distal basal layer of the epidermis during regenerative outgrowth. *wnt3a* is undetectable during blastema formation and is faintly detected in the epidermis during outgrowth (Poss *et al.* 2000a). Recently, Stoick-Cooper *et al.,* (2007) found the expression of another member of the signaling family, wnt10a, in the distal tip of the blastema and also early in regeneration making it an excellent candidate for the molecule responsible for the activation of the Wnt/ ß-catenin pathway. They have confirmed the function of Wnt/ ß-catenin signaling in fin regeneration by creating a transgenic line of zebrafish with heat-shock inducible expression of an inhibitor of the Wnt/ ß-catenin pathway, *dickoppf1* (*hsDkk1:GFP*). Upon heat shocking at different stages in the regeneration process, they found that Wnt/ ß-catenin signaling is required for blastema formation, maintenance and/or proliferation. This inhibition through induction of *dkk1* expression results in a downregulation of *fgf20a* expression indicating that the Wnt/ ß-catenin signaling pathway regulates Fgf signaling during fin regeneration (Stoick-Cooper *et al.* 2007). Interestingly, they also found that an increase in signaling results in a faster regeneration time. This was done by making use of a heterozygous mutant fish that has only one copy of the *axin1* gene, which is an inhibitor of the Wnt/ ß-catenin pathway. They found that these mutant fish regenerated their fins more quickly than controls providing direct genetic evidence for the importance of Wnt signaling in the regeneration process (Stoick-Cooper *et al.* 2007).

1.2.6.5 Bone and extracellular matrix genes

The bone matrix of the rays in the zebrafish fin is made of glycoaminoglycans and type II collagen (Montes *et al.* 1982; Santamaria *et al.* 1991, 1992). The importance of collagen during teleost fin regeneration was demonstrated by treating fish with many drugs known to disrupt collagen metabolism and mammals (Bechara *et al.* 2000). Treatment with these drugs disturbed the deposition and organization of collagen fibrils which led to abnormally thin or absent lepidotrichia and actinotrichia, and also to disorganized fibrous connective tissue. Many *collagen* genes have been shown to be expressed during zebrafish fin regeneration, including *col1a1*, *col1a2*, *col2a1* and *col10a1* (Johnson *et al.* 1995; Padhi *et al.* 2004; Avaron *et al.* 2006). The *collagen type II* gene, *col2a1*, is one of the first *collagen* genes characterized in zebrafish fin regeneration. It is first detected in blastema cells close to the bony stump and later in scleroblasts on both sides of the regenerating lepidotrichia and in the basal layer of the epidermis (Johnson *et al.* 1995). A zebrafish *collagen type X alpha 1 chain gene* (*col10a1*) was recently isolated in our laboratory using a screen for genes differentially expressed during regeneration of the zebrafish caudal fin (Padhi *et al.* 2004). Surprisingly, zebrafish *col10a1* expression has been observed in the cleithrum, which is a dermal bone of the pectoral girdle of the developing zebrafish larvae (Padhi *et al.* 2004). It was also found to be expressed during the regeneration of the lepidotrichia, also of dermal origin, in the scleroblasts and in the basal layer of the epidermis (Padhi *et al.* 2004; Avaron *et al.* 2006). But recently, it has also been shown to be expressed in the cartilage cells within the craniofacial region of the zebrafish larvae (Avaron *et al.* 2006). These results indicate a possible dual function of *col10a1* in zebrafish development playing a new role in dermal bone formation likely in

the calcification of the dermal bone matrix and also in its more known role in chondrocyte hypertrophy and cartilage formation.

1.3 Bone and cartilage development

Skeletal elements are made via two different mechanisms: intramembranous ossification or endochondral ossification (Fang *et al.* 1997; Olsen *et al.* 2000; Buxton *et al.* 2003; Eames *et al.* 2004a, 2004b). Intramembranous ossification takes place in several craniofacial bones and parts of the clavicle in many different species (Hall *et al.* 1992, 2001). In the zebrafish several bones of the craniofacial skeleton including: the dentary, opercular and maxillary bones and parts of the pectoral appendage including the cleithrum are formed through intramembranous ossification (Cubbage, 1996). Also, the lepidotrichia or bony rays found in all the 5 types of fins are of dermal origin which is thought to be equivalent to intramembranous ossification (Haas 1962; Geraudie *et al.* 1982). In contrast, most of the axial and appendicular skeletons of all species including zebrafish (except above mentioned structures) are formed through endochondral ossification. For example in humans the bones of the limbs and ribs develop through endochondral ossification. Both types of skeletogenesis begin during development when a subset of mesenchymal cells receives signals to form either bone or cartilage. During endochondral bone formation cartilage replacement bones are formed when chondrogenic condensations form within this mesenchyme and differentiate into chondrocytes that eventually form a cartilage template that will be replaced by bone (Hall *et al.* 2000; Olsen *et al.* 2000). On the other hand, intramembranous bone formation is when mesenchymal cells differentiate directly into osteoblasts that will secrete the bone matrix (Fang *et al.*

1997; Olsen *et al.* 2000; Buxton *et al.* 2003; Eames *et al.* 2004a, 2004b). These are reviewed below.

1.3.1 Intramembranous ossification

This mechanism of bone formation does not involve the formation of a cartilage template. It does however involve the condensation of mesenchymal cells as is seen during endochondral bone formation but these mesenchymal cells differentiate directly into osteoblast cells that secrete the bone matrix (reviewed in: (Caplan 1988; Fang *et al.* 1997; Olsen *et al.* 2000; Buxton *et al.* 2003; Eames *et al.* 2004a). As the mesenchymal cells condense the surrounding tissue becomes vascularized as is seen in the later stage of endochondral ossification. These osteoblast cells then lay down the bone matrix rich in type I collagen which eventually mineralizes to form such bones as the flat bones of the skull and parts of the clavicle (Eames *et al.* 2004a, 2004b). The simple process of intramembranous bone formation is very similar to the steps following apoptosis of the hypertrophic chondrocytes seen in endochondral bone formation, which involves osteoblast and blood vessel invasion that results in the eventual replacement of cartilage with bone (Chung *et al.* 2001). Although endochondral and intramembranous bone formation have many similar features they appear to differ in the genes that regulate their development.

1.3.2 Endochondral ossification

The first skeleton to develop and be detectable in the embryo is a cartilage template which will eventually be replaced by bone through a complicated process called

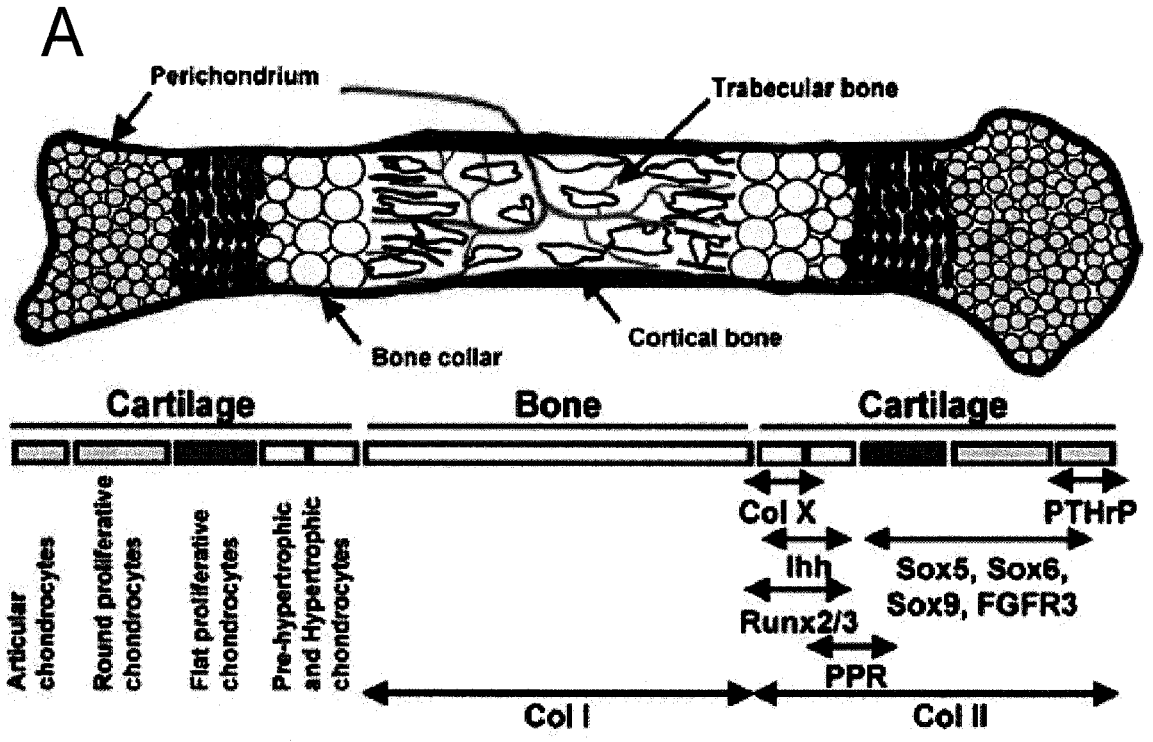
endochondral ossification (Erlebacher *et al.* 1995). The most studied example of endochondral bone formation is in the long bones of the limbs (reviewed in: (Kronenberg 2003). The process begins when mesenchymal cells from the mesoderm aggregate through the expression of adhesion molecules and form condensations, called prechondrogenic condensations (Hall *et al.* 2000). One of the factors that determine the size of each cartilage template is how many mesenchymal precursors adopt a chondrocyte fate. At the onset of skeletogenesis mesenchymal cells have been shown to receive a signal that activates transcription factors that commit these cells to chondrogenesis and to begin a complex differentiation process (reviewed in: (Goldring *et al.* 2006; Mackie *et al.* 2008). The mesenchymal cells first differentiate into prechondrogenic cells called chondroblasts and eventually into chondrocytes, which are the primary cell type of cartilage (reviewed in: (Kronenberg 2003; Goldring *et al.* 2006; Adams *et al.* 2007; Mackie *et al.* 2008). The cartilage template grows as chondrocytes rapidly multiply eventually forming columns of these highly proliferative cells. Proliferating chondrocytes have a characteristic shape, starting off very rounded and then flattening out and becoming columnar, and they secrete a matrix rich in type II collagen and the proteoglycan aggrecan (So *et al.* 2001; Kirn-Safran *et al.* 2004). As this is taking place, cells along the periphery of the forming cartilage element differentiate into a fibroblastic cell layer, called the perichondrium, which surrounds the cartilage rudiment (Chung *et al.* 1998, 2001, 2004; Kronenberg 2007). Following the rapid proliferation the chondrocytes become first pre-hypertrophic and then hypertrophic whereby they increase in size and change their genetic program to secrete a different type of matrix, rich in type X collagen and other elements of the matrix (Schmid *et al.* 1985a; Schmid *et al.* 1985b;

Jimenez *et al.* 1986). During the maturation process of a chondrocyte it increases its cell volume by almost 20 times its original size (Hunziker 1994). Based on this increase in size the hypertrophic chondrocyte is regarded as the master regulatory cell and the principal engine of bone growth (Noonan *et al.* 1998). Some of the roles of the hypertrophic chondrocyte in skeletal growth are directing the mineralization of the surrounding matrix, attracting blood vessels by producing Vascular endothelial growth factor (VEGF), and directing the cells of the perichondrium to become osteoblasts (Chung 2004). These osteoblasts will secrete a bone matrix mostly composed of type I collagen that will form a bone collar. Eventually, the hypertrophic chondrocytes will undergo apoptotic cell death leaving behind a matrix that will act as a scaffold for the invasion of blood vessels and osteoblasts that will secrete a true bone matrix and form primary ossification centers. As hypertrophic chondrocytes in the center of the cartilage mould are going through these multiple phases of maturation the mould continues to grow as chondrocytes on either side continue to proliferate and mature, which eventually leads to bone lengthening (summarized in Fig. 1.8A, B) (reviewed in: Kronenberg 2003; Goldring *et al.* 2006; Adams *et al.* 2007; Mackie *et al.* 2008).

1.4 Molecular specification during skeletogenesis

Upon first glance, there is no morphological difference between mesenchymal cells that will differentiate into cells involved in skeletogenesis and those that will not. Interestingly, there is also no noticeable morphological difference between mesenchymal cells that will differentiate into either cartilage or bone cells (Hall *et al.* 2000). However, it has been shown that, at both the histological and molecular level, it is possible to

distinguish bone cells from cartilage cells (MacCabe *et al.* 1973; Wezeman 1998; Eames *et al.* 2004a, 2004b). This is due to the fact that during the development of all organs, during embryogenesis, pluripotent cells receive signals specifying their lineage and they are not pre-programmed (Eames *et al.* 2003). This is also true during skeletogenesis, as the mesenchymal cells must receive signals to determine whether they will form bone or cartilage cells, and then from that point on will express molecular markers associated with their fate (Eames *et al.* 2003, 2004). It is has been shown that key regulatory genes control this decision which then activate the appropriate signaling and extracellular matrix molecules (Eames *et al.* 2004b). Although there are two major types of bone formation, endochondral and intramembranous, there is actually three principal skeletal tissues found in all vertebrates; bone, replacement cartilage and persistent cartilage (Smith *et al.* 1990; Eames *et al.* 2003, 2004). Therefore it has been proposed that skeletal tissue identity is determined by a unique pattern of expression of transcription factors during mesenchymal condensation and that a binary molecular code of these genes will determine which skeletal elements will form (Eames *et al.* 2004b). All three types of skeletal tissue arise from undifferentiated mesenchymal cells that receive signals that tell them which type of tissue to form and it is thought that it is the combination in expression of two genes, *Sox9* and *Runx2* that govern this process (Eames *et al.* 2004b).


A
Perichondrium
Trabecular bone
Bone collar
Cortical bone
Cartilage
Bone
Cartilage
Articular chondrocytes
Round proliferative chondrocytes
Flat proliferative chondrocytes
Pre-hypertrophic and Hypertrophic chondrocytes
Col X
PTHrP
Ihh
Sox5, Sox6, Sox9, FGFR3
Runx2/3
PPR
Col I
Col II

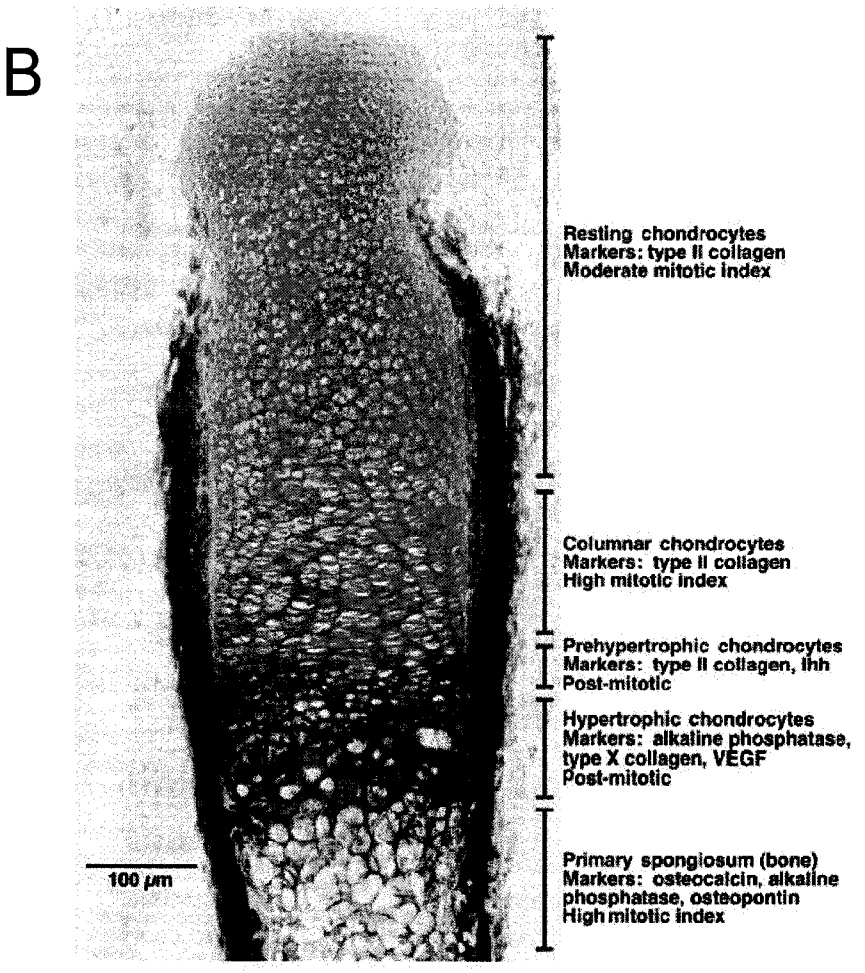

B
Resting chondrocytes
Markers: type II collagen
Moderate mitotic index
Columnar chondrocytes
Markers: type II collagen
High mitotic index
Prehypertrophic chondrocytes
Markers: type II collagen, Ihh
Post-mitotic
Hypertrophic chondrocytes
Markers: alkaline phosphatase, type X collagen, VEGF
Post-mitotic
Primary spongiosum (bone)
Markers: osteocalcin, alkaline phosphatase, osteopontin
High mitotic index
100 μm

Figure 1.8

Figure 1.8: Endochondral ossification

A: Schematic representation of endochondral bone formation in the mouse tibia at the later stages of fetal development. Each stage of chondrocyte maturation is illustrated detailing the different layers; first round, proliferative chondrocytes are found at the most distal end, these cells continue to proliferate and flatten as you go more into the center of the forming bone, then the chondrocytes begin to enlarge and become pre-hypertrophic and eventually hypertrophic. In the center of the growth plate, the hypertrophic chondrocytes die, blood vessels invade the area and finally the cartilage matrix secreted by the chondrocytes is replaced by trabecular bone. The specific genes expressed by each layer of cells are listed below the cell type. Adapted from: (Schipani *et al.* 2003)

B: Differential staining of a section through the growth plate of a fetal mouse metatarsal that is going through the process of endochondral ossification. The different stages of the chondrocyte lineage are evident. During their differentiation process, cells pass through several well-characterized intermediate cell types, including round and flattened proliferating chondrocytes (red), then drop out of the cell cycle and enter pre-hypertrophy and then begin to hypertrophy (blue). Blue staining indicates endogenous alkaline phosphatase activity in the hypertrophic chondrocytes, the perichondrium and the bone collar; whereas the red staining (Safarin O) labels the cartilage matrix. Adapted from: (Shum *et al.* 2002)

The Sox9 and Runx2 transcription factors are thought to be the master regulators of cartilage and bone formation, respectively (Bi *et al.* 1999; Ducy *et al.* 1999; Inohaya *et al.* 2000). Varying levels of expression of each of these transcription factors is then thought to activate a cascade of cell specific downstream targets to form the appropriate tissue (reviewed in: (Eames *et al.* 2003). A simplified model of this was proposed by Eames *et al.*, (2004) whereby chondrocytes of persistent cartilage are a result of high expression of Sox9 and low expression of Runx2 whereas osteoblast differentiation is the opposite with high levels of Runx2 and low levels of Sox9. Interestingly replacement cartilage, which is the most prevalent skeletal tissue, is an intermediate between persistent cartilage and bone, and is specified when both Sox9 and Runx2 are expressed (Fig. 1.9A). The chondrocytes that form both types of cartilage, and also the osteoblasts are said to express specific molecular markers that distinguish them from each other (Eames *et al.* 2004a and 2004b). These molecular markers for each skeletal tissue will be described in more detail below.

1.4.1 Replacement cartilage

The commitment of mesenchymal cells to chondrogenesis is dependent on the Sry-related HMG box domain transcription factor Sox9, which alone is sufficient to induce cartilage formation since, following its misexpression, ectopic cartilage is produced (Healy *et al.* 1999). The key role of Sox9 in cartilage formation is in the differentiation of mesenchymal progenitor cells into chondrocytes (Foster *et al.* 1994; Wagner *et al.* 1994; Kwok *et al.* 1995; Bi *et al.* 1999). *Sox9* expression begins in the condensing mesenchymal cells and remains high in prechondrocytes and proliferating chondrocytes

and then is turned off when these cells undergo hypertrophy (Wright *et al.* 1995; Ng *et al.* 1997; Zhao *et al.* 1997). The essential role of Sox9 in chondrogenesis was obvious when it was found that a heterozygous mutation in the gene results in a human skeletal syndrome called camptomelic dysplasia (CD), which results in a severe form of chondrodysplasia (Foster *et al.* 1994; Wagner *et al.* 1994; Kwok *et al.* 1995). The vital role for Sox9 in initiation of chondrogenesis was further demonstrated using both *in vivo* and *in vitro* experiments. Firstly, $Sox9^{-/-}$ cells do not form cartilage in vitro (Bi *et al.* 1999). Concrete evidence came from in vivo experiments where *Sox9* expression was specifically knocked down in the limb bud mesenchyme of mouse embryos allowing chondrogenesis to be examined at later stages (Akiyama *et al.* 2002). It was found that inactivation of Sox9 in limb buds before mesenchymal condensation resulted in complete absence of both replacement cartilage and persistent cartilage which includes the bones of the limbs and the joints, respectively (Akiyama *et al.* 2002). These data as well as other studies involving conditional knockout of Sox9 function at various time points during mouse limb development demonstrate that without Sox9 function during skeletal development in the limb, formation of cartilage is blocked. Crombrugghe *et al.,* (2003) performed similar knock out experiments by specifically deleting Sox9 in the cranial neural crest cells that give rise to the skeleton of the head (Mori-Akiyama *et al.* 2003). These experiments were especially interesting because they were able to examine the effect of deleting Sox9 on both endochondral and intramembranous bone formation since bones of the head skeleton can be formed by both processes. They found that all bones that use cartilage as a scaffold were absent whereas bones that do not involve a cartilage intermediate formed through intramembranous ossification were relatively unaffected

(Mori-Akiyama *et al.* 2003). Following its expression in cells of the mesenchymal condensation (chondroblasts) that are fated to become chondrocytes, *Sox9* is co-expressed with cartilage specific genes that encode for extracellular matrix molecules, such as *Col2a1* and *Aggrecan* in the differentiated chondrocytes (Sandell 1994; Lefebvre *et al.* 1997; Ng *et al.* 1997; Zhao *et al.* 1997). The importance of Sox9 in cartilage formation is in part due to its ability to directly upregulate some of these genes associated with cartilage formation. For example, Sox9 regulated Col2a1 expression by directly binding and activating the cartilage-specific regulatory elements of the gene (Bell *et al.* 1997; Zhou *et al.* 1998; Sekiya *et al.* 2000). It has also been shown in $Sox9^{-/-}$ mice that cells without Sox9 show a complete dowregulation in the expression of *Col2a1* and other chondrocyte-specific markers (Bi *et al.* 1999). Col2a1 is secreted by chondrocytes following differentiation at which time they embed themselves in this Col2a1 rich matrix. It is the most abundant ECM molecule during the proliferation phase of cartilage formation and then gradually declines as chondrocytes begin to hypertrophy, much like Sox9 (van der Eerden *et al.* 2003). The next phase of chondrogenesis is characterized by the pre-hypertrophy and hypertrophy of the proliferating chondrocytes. This transition is characterized by the expression of a new set of both signaling and ECM molecules. One example is another key regulator in endochondral ossification, the signaling molecule Indian Hedgehog (Ihh), which is expressed in pre-hypertrophic chondrocytes (Bitgood *et al.* 1995; Iwasaki *et al.* 1997; Vortkamp 1997). Mice deficient in Ihh signaling in skeletal elements display severe dwarfism and bone shortening due to reduced chondrocyte proliferation (St-Jacques *et al.* 1999; Long *et al.* 2004). They experience early chondrocyte maturation due to premature hypertrophy. Interestingly, there is also

an absence of osteoblasts in the bone collar (St-Jacques *et al.* 1999; Long *et al.* 2004). Through analysis of different mutants, Ihh has been shown to coordinate chondrocyte proliferation and differentiation during bone development through a feedback loop with a second secreted factor, the parathyroid hormone related peptide (PTHrP), which is expressed in distal chondrocytes (Lanske *et al.* 1996; St-Jacques *et al.* 1999). Through this feedback loop, Ihh negatively regulates the onset of hypertrophic differentiation by upregulating PTHrP which in turn signals to its receptor, PTH/PTHrP receptor (PPR), on proliferating and pre-hypertrophic chondrocytes, delaying these cells from differentiating into Ihh-expressing pre-hypertrophic chondrocyte (Vortkamp *et al.* 1996). This mechanism is important for controlling the length of the bones by keeping chondrocytes in a proliferative state and by controlling the rate and number of cells that enter the hypertrophic zone (St-Jacques *et al.* 1999). Vortkamp *et al.*, (1996) demonstrated the necessity of the feedback loop between Ihh and PTHrP by performing knockout studies in mice whereby the elimination of Ihh, PTHrP and PPR resulted in premature hypertrophy of chondrocytes and a decreased number of proliferating chondrocytes. Direct evidence linking this phenotype to the interaction between Ihh and PTHrP came when activation of Ihh in PTHrP and PPR mutants did not rescue the mutation. A role for Ihh in endochondral bone formation independent of PTHrP was discovered upon close examination of the mutant phenotypes since $Ihh^{-/-}$ mice have bones more severely reduced in size than $PTHrP^{-/-}$ mice (St-Jacques *et al.* 1999; Karp *et al.* 2000; Long *et al.* 2004). This reduction in bone size in $Ihh^{-/-}$ mice was thought to be a direct result of a reduction in chondrocyte proliferation based on the fact that the receptor of Ihh, *Ptc1*, and other downstream targets of the pathway are expressed in proliferating chondrocytes (St-

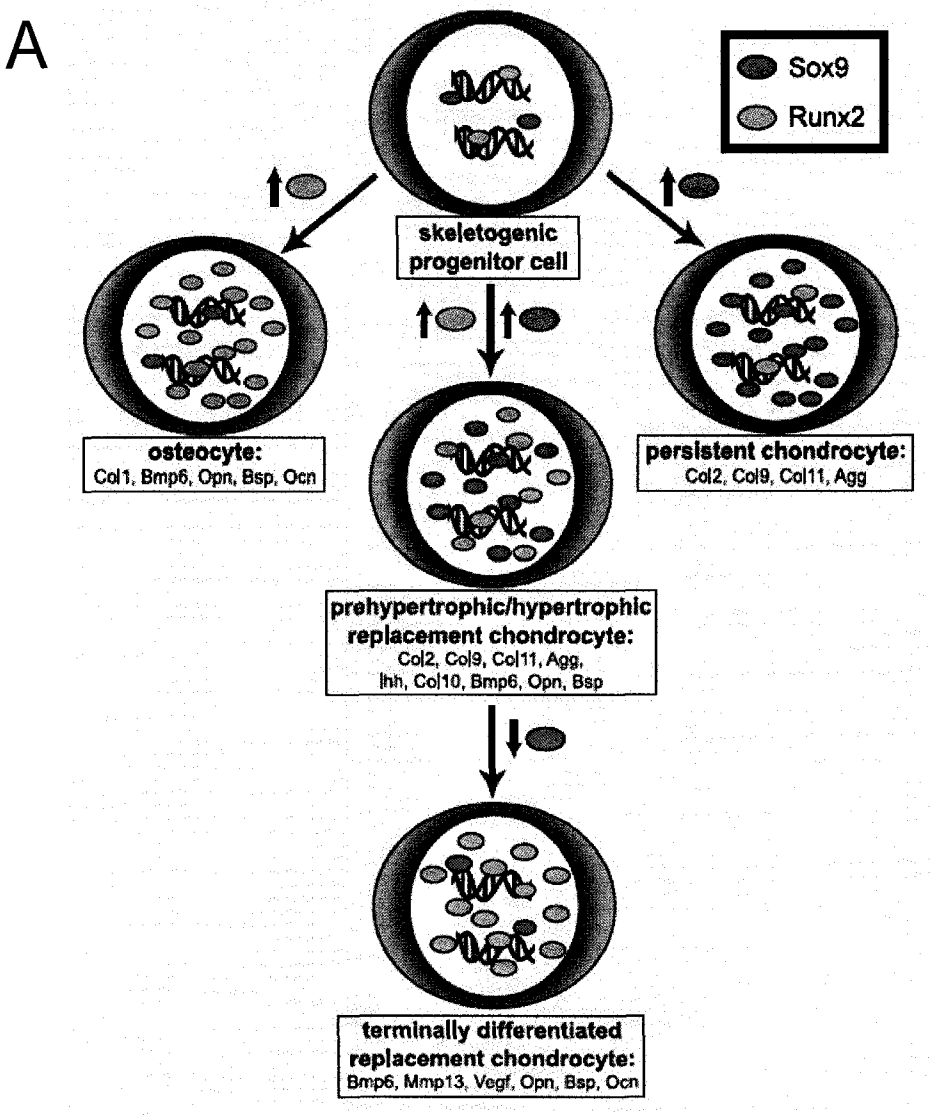
Jacques *et al.* 1999). The third role of Ihh signaling in bone formation is its ability to trigger osteoblast differentiation in the perichondrium. This function was discovered when $Ihh^{-/-}$ mutant mice were found to have a thinner perichondrium, which may be the reason for the cortical bone defects seen in these mutants (St-Jacques *et al.* 1999; Long *et al.* 2004). In conclusion, Ihh signaling plays many roles in cartilage and bone formation in replacement cartilage and is a well known marker for pre-hypertrophic chondrocytes in the growing long bones. The next stage in the formation of replacement cartilage involves the transition of chondrocyte from the pre-hypertrophic to the hypertrophic phase. This morphological change is also characterized by the synthesis of the ECM molecule Collagen X (ColX or Col10a1) (Schmid *et al.* 1985; Jimenez *et al.* 1986). Collagen X has been said to be the only known protein to be expressed and produced by hypertrophic chondrocytes, being expressed at very high levels until the eventual replacement of the cartilage by bone. Much like mutations in Sox9 and Ihh, interference with Collagen X signaling in both mice and humans results in the compression of the cells in the growth plate and affects trabecular bone formation which results in shortened bones and stature (Chan *et al.* 1998; Jacenko *et al.* 2002). In humans a mutation in the *Collagen X* gene leads to disproportionate dwarfism, a condition known as Schmid metaphyseal chondrodysplasia (Warman *et al.* 1993). Interestingly, many studies and reviews focusing on *Collagen X* expression as a marker specific to hypertrophic chondrocytes have gone so far as to say that its expression is only found in hypertrophic chondrocytes and no other tissue (Chan *et al.* 1998; Jacenko *et al.* 2002; Adams *et al.* 2007).

The Runx2 transcription factor has been extensively linked to bone formation often being referred to as the earliest transcriptional regulator of osteoblast differentiation; without it no osteoblasts are formed (Ducy *et al.* 1997; Komori *et al.* 1997, 1998; Otto *et al.* 1997). Komori *et al.,* (1997) first revealed the necessity for Runx2 in bone formation when they inactivated it in mice and found that embryos completely lacked any bone. Closer examination of $Runx2^{-/-}$ mice revealed the importance of this gene not only in bone formation but also in chondrocyte hypertrophy during endochondral ossification since, in these mice, many skeletal elements are devoid of hypertrophic chondrocytes or the transition of chondrocytes into hypertrophic chondrocytes is defective (Komori *et al.* 1997; Otto *et al.* 1997; Inada *et al.* 1999; Kim *et al.* 1999). Takeda *et al.,* (2001) examined the role of Runx2 more closely in cartilage development and found Runx2 to be expressed in mouse chondrocytes in the prehypertrophic and hypertrophic zones of developing limb long bones indicating its possible role in the induction of chondrocyte hypertrophy. This role was confirmed when they created a transgenic mouse which overexpresses Runx2 in non-hypertrophic chondrocytes under the control of a fragment of the mouse type II collagen promoter and its chondrocyte-specific enhancer (Takeda *et al.* 2001). This continuous expression of *Runx2* in non-hypertrophic chondrocytes induced and accelerated chondrocyte hypertrophy and endochondral ossification. Also, when they made use of the transgene to restore Runx2 signaling in knockout mice, they were able to restore chondrocyte hypertrophy and vascularization (Takeda *et al.* 2001). These results confirm the dual and distinct roles of Runx2 in both chondrocyte maturation and bone formation, therefore indicating that Runx2 is necessary for both intramembrnous ossification and also for

cartilage formation during endochondral bone formation. Therefore, the role of Runx2 as an osteoblast-specific transcription factor has now been expanded and its overlapping role in skeletogenesis has also raised the possibility of the existence of common precursor cells, osteo-chondroprogenitor, which may be examined more closely in the near future. When examining the role of Runx2 in hypertrophic chondrocyte differentiation the next question is how this transcription factor induces chondrocyte hypertrophy. It was hypothesized that Runx2 directly activates hypertrophic chondrocyte markers such as Collagen X. This is in fact the case since ectopic Runx2 expression in immature chick chondrocytes induced premature collagen X expression (Enomoto *et al.* 2000). Also, Runx2 binding sites have been identified in the promoter regions of both mouse and chick collagen X genes confirming that it is a direct transcriptional target of Runx2 (Drissi *et al.* 2003; Zheng *et al.* 2003).

Therefore, not only are there specific markers for each type of skeletal tissue but, during endochondral ossification, chondrocytes passing through each stage of differentiation express specific markers as well (summarized in Fig 1.8A and 1.9B).

The next steps of endochondral bone formation involve the invasion of the cartilage template by blood vessels. This process involves the expression of VEGF by


A
Sox9
Runx2
skeletogenic progenitor cell
osteocyte:
Col1, Bmp6, Opn, Bsp, Ocn
persistent chondrocyte:
Col2, Col9, Col11, Agg
prehypertrophic/hypertrophic replacement chondrocyte:
Col2, Col9, Col11, Agg,
Ihh, Col10, Bmp6, Opn, Bsp
terminally differentiated replacement chondrocyte:
Bmp6, Mmp13, Vegf, Opn, Bsp, Ocn

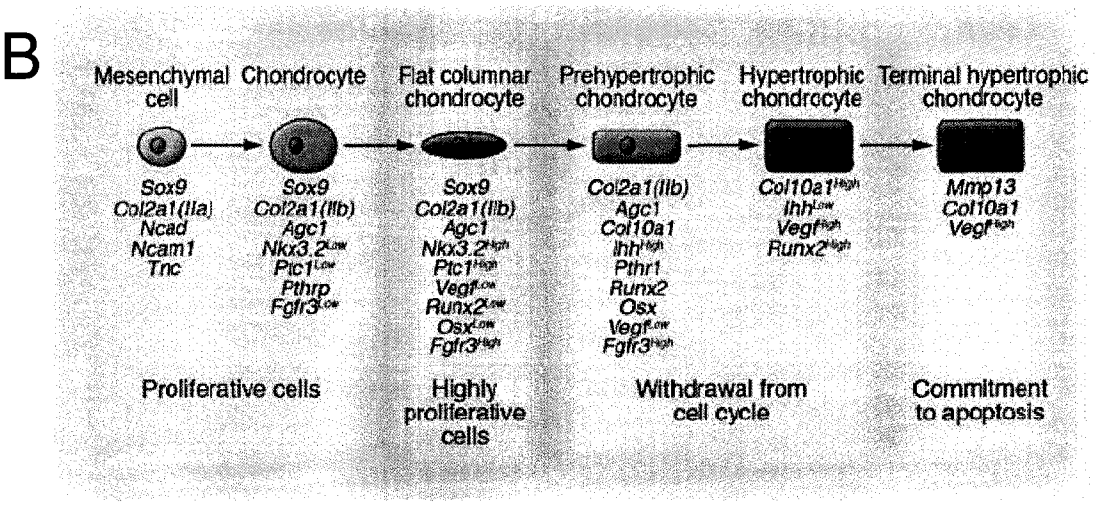

B
Mesenchymal cell
Chondrocyte
Flat columnar chondrocyte
Prehypertrophic chondrocyte
Hypertrophic chondrocyte
Terminal hypertrophic chondrocyte
Sox9
Col2a1(IIa)
Ncad
Ncam1
Tnc
Sox9
Col2a1(IIb)
Agc1
Nkx3.2Low
Ptc1Low
Pthrp
Fgfr3Low
Sox9
Col2a1(IIb)
Agc1
Nkx3.2High
Ptc1High
Vegfl.ow
Runx2Low
OsxLow
Fgfr3High
Col2a1(IIb)
Agc1
Col10a1
IhhHigh
Pthr1
Runx2
Osx
Vegfl.ow
Fgfr3High
Col10a1High
IhhLow
VegfHigh
Runx2High
Mmp13
Col10a1
VegfHigh
Proliferative cells
Highly proliferative cells
Withdrawal from cell cycle
Commitment to apoptosis

Figure 1.9

Figure 1.9: Model of skeletal fate decision and important molecular markers that define the different stages of chondrocyte and osteoblast differentiation.

A: A model detailing skeletal fate determination by the actions of two transcription factors, Runx2 and Sox9. The combination in expression of these transcription factors by skeletogenic precursor cells is thought to determine whether these precursor cells will differentiate into: osteoblasts and form bone, persistent chondrocytes and form persistent cartilage or replacement chondrocytes and form replacement cartilage. Adapted from: (Eames *et al.* 2004b)

B: A schematic outlining the process of chondrogenesis and the different stages of chondrocyte differentiation. Important molecular markers and which genes are expressed in each phase of chondrocyte differentiation are listed below the cell type. Adapted from: (Zuscik *et al.* 2008)

hypertrophic chondrocytes, which is believed to attract these blood vessels and begin angiogenesis (Gerber *et al.* 1999; Haigh *et al.* 2000). The other role of hypertrophic chondrocytes is to secrete signals that begin osteoblast differentiation for the eventual replacement of the cartilage template by bone. Ihh is one of the key regulators of this process; knockout studies have shown that there is a loss of endochondral bone formation in Ihh mutant mice indicating its role in controlling osteoblast differentiation in the perichondrium/periosteum (St-Jacques *et al.* 1999). Other evidence comes from the fact that ectopic expression of Ihh promotes the differentiation of osteoblasts (Long *et al.* 2004). Although Ihh seems to play a role in osteoblast differentiation, intramembranous bone formation seems to be unaffected in Ihh mutants (St-Jacques *et al.* 1999). Also absence of Ihh at later stages of osteoblast differentiation does not affect the differentiation process suggesting that Ihh signaling is likely an inductive signal that triggers other signaling molecules that govern this process rather than independently regulating the ossification process itself (Rodda *et al.* 2006). For example, certain Bmps involved in skeletogenesis are expressed in the perichondrium and many studies have shown that Bmps are a target of Hh signaling (Vortkamp *et al.* 1996; Pathi *et al.* 1999). Therefore it has been hypothesized that Bmps expressed in perichondrium are acting downstream of Ihh (Methot *et al.* 1999). Direct evidence for this came from experiments in chick embryos where ectopic Ihh expression turned on Bmp2 and 4 in the perichondrium (Pathi *et al.* 1999). Bmp signaling and its role in osteoblast differentiation will be discussed in the last section in greater detail.

1.4.2 Persistent cartilage

The beginning steps of chondrogenesis are shared for all cartilage elements but unlike those that are eventually replaced by bone, there are some parts of the skeleton that remain cartilaginous throughout the lifetime of the animal, and are referred to as persistent cartilage. Examples of these permanent cartilage structures are the Meckel's cartilage of the mandible (not in mammals), the trachea, nasal and ear cartilage, and intervertebral cartilages (reviewed in: (Eames *et al.* 2003).

There are 3 major differences that distinguish persistent cartilage from replacement cartilage; firstly the chondrocytes that will eventually give rise to permanent cartilage do not undergo pre-hypertrophy or hypertrophy and secondly the cartilage template itself does not undergo vascular invasion. As is expected from these characteristics of persistent cartilage, the molecular markers of these stages of chondrogenesis are not expressed, including both the pre-hypertrophic marker, *Ihh*, and hypertrophic marker, *Col10* (Eames *et al.* 2003, 2004). The third difference has been referred to as the most important. Interestingly, chondrocytes involved in the formation of replacement cartilage, not persistent cartilage, have been shown to express molecular markers that have been referred to as 'osteoblast-specific' at some point during there differentiation process. Such as *Runx2*, *Bone sialoprotein* (*Bsp*), *Osteocalcin*, *Osteopontin* and *Osteonectin* (Neugebauer *et al.* 1995; Sommer *et al.* 1996; Pines *et al.* 1998; Takeda *et al.* 2001). Therefore chondrocytes involved in the formation of replacement cartilage, at some point, express both hypertrophic chondrocyte and bone markers but chondrocytes that form persistent cartilage do not. To date little is known about what causes chondrocytes of persistent cartilage to halt their differentiation process before the hypertrophic phase.

1.4.3 Bone formation

Direct bone formation is a much simpler process than cartilage formation, both persistent and replacement, with the bone matrix being secreted by the cells specific to bone formation, osteoblasts (reviewed in: Hall *et al.* 1992; Fang *et al.* 1997; Chung *et al.* 2004). Osteoblasts play a major role in production of a characteristic ECM and mineralization of bone matrix during bone formation (both intramembranous and endochondral). Runx2 has been described as the master regulator of osteogenesis and is the key factor necessary for osteoblast differentiation. The realization of its importance stems from the examination of both human and mouse Runx2 mutants (Mundlos *et al.* 1995, 1997; Komori *et al.* 1997; Otto *et al.* 1997). Humans that lack one copy of the gene have a disease called cleidocranial dysplasia. Patients present with such symptoms as hypoplastic clavicles, large open spaces between the frontal and parietal bones of the skull and other skeletal dysplasias, mostly involving bones produced by intramembranous ossification (Mundlos *et al.* 1995, 1997). In mice, inactivation of Runx2 produces embryos that completely lack bone that are produced by both intramembranous and endochondral ossification (Komori *et al.* 1997; Otto *et al.* 1997). The importance of Runx2 in bone formation was also demonstrated using gain of function experiments whereby it was shown that ectopic Runx2 expression equals ectopic bone formation. In order for a gene to be classified as a major transcriptional activator of osteoblast differentiation, it has to meet 3 criteria and Runx2 has been shown to fulfill all of these (Karsenty 2001). Firstly, it is expressed in osteoblast progenitor cells during embryogenesis and secondly, as seen by mutant analysis, it is essential for osteoblast differentiation *in vivo* and also *in vitro* (Ducy *et al.* 1997; Komori *et al.* 1997; Otto *et al.*

1997). The third criterion is that the transcriptional activator should directly turn on the expression of many genes expressed in osteoblasts that are necessary for bone formation. Interestingly, it has been demonstrated that, to date, all genes expressed in osteoblasts that contribute to the ECM of the bone have binding sites for Runx2 such as Type I collagen, Bsp, Osteocalcin and Osteopontin (Ducy *et al.* 1997, 1999; Kern *et al.* 2001). Also, transfection of Runx2 into mouse cell lines leads to induction of the expression of these bone-specific genes also referred to as molecular markers of the osteoblast lineage (Ducy *et al.* 1997). For example, Collagen type I expression is a marker for early osteoblast differentiation and osteoblast cells primarily secrete type I collagen and then embed themselves in the fibers (Eames *et al.* 2003). Therefore, in summary, Runx2 is essential for osteoblast differentiation and for turning on the genes responsible for production of the extracellular matrix of the bone itself.

It is becoming more widely accepted that Sox9 and Runx2 are the key regulators of determining which type of the three skeletal fates will be produced from the initial mesenchymal condensation, making the decision whether cells make the choice to differentiate into chondrocytes or osteoblasts. They will also regulate the expression of genes that distinguish which type of skeletal tissue will be formed; replacement cartilage, persistent cartilage or bone. Besides their expression patterns, the most striking functional evidence for this was provided by Eames *et al.,* (2004) whereby they were able to not only produce ectopic bone or cartilage but also transform skeletal fate by misexpressing either Runx2 or Sox9 using viral vectors. For example, Sox9 misexpression in chick embryos not only results in ectopic cartilage but is able to

transform bone into replacement cartilage and replacement cartilage into persistent cartilage (Eames *et al.* 2004b). In contrast, Runx2 misexpression was able to produce ectopic bone but also convert persistent cartilage to replacement cartilage. From these experiments, they also concluded that chondrogenic fate determinants are dominant to osteogenic ones (Eames *et al.* 2004b).

Therefore, based on the expression of these master regulatory transcription factors and their downstream targets, it is thought that there is a molecular code to distinguish cartilage from bone. Simply, replacement cartilage can be identified by the expression of *Sox9* within the proliferating chondrocytes which triggers the expression of genes that encode ECM molecules or cartilage matrix genes such as *Col2a1*, then followed by expression of pre-hypertrophic (*Ihh*, *Pth*) and hypertrophic (*Col10a1*) markers.

Therefore molecular markers of replacement cartilage include genes encoding transcription factors, Sox9 and Runx2, extracellular matrix molecules Col2a1 and Col10a1 and signaling molecules Bmp6 and Ihh. In contrast, osteoblasts express the transcription factor *Runx2*, ECM molecule *ColI* and the signaling molecules *Bmp4* and *Bmp6*. To date, more and more genes are being shown to be expressed in these cells but the above mentioned genes are the most common (reviewed in: Eames *et al.* 2003; Eames *et al.* 2004a; Eames *et al.* 2004b). Interestingly, Eames *et al.*, (2000a) have stated that it is known that chondrocytes express both cartilage and bone-specific markers but it has never been shown that osteoblasts express cartilage-specific genes.

1.5 The expression and function of genes involved in skeletal development in zebrafish

More recently many of the genes involved in bone and cartilage development are being isolated and characterized in zebrafish. The usefulness of zebrafish as a model organism to study skeletal development is becoming clear (reviewed in: Yelick *et al.* 2002). Firstly, zebrafishes have an essentially conserved program of skeletogenesis with that of higher vertebrates. Secondly, zebrafish embryonic development is much more rapid than other higher vertebrates and embryos develop externally from the mother allowing for an easier accessibility to observing the development of the skeleton. For example, cartilage development in craniofacial elements from mesenchymal condensations commences at 2days post fertilization (dpf) and by 5dpf most of cartilages of pharyngeal arches are formed, then the process of bone replacement begins by 5-6dpf (Du *et al.* 2001; Flores *et al.* 2006). In the following section what is known about the expression and function of some of the key regulatory genes involved in zebrafish skeletal development will be reviewed.

1.5.1 Sox9

In 2001, Chiang *et al.*, first cloned and characterized the zebrafish orthologue of the Sox9 gene. Like many other zebrafish genes, it was found that there were duplicated Sox9 genes in zebrafish and they were called, *sox9a* and *sox9b*. During embryogenesis, the expression of the *sox9a* and *sox9b* genes was found to be in some regions of the brain and also in the head skeleton and fins (Chiang *et al.* 2001). Yan *et al.*, (2005) four years later, examined the expression and function of these sox9 co-orthologs in the development of the craniofacial skeleton and the pectoral fins of the zebrafish by examining both *sox9a* and *sox9b* mutants. Thorough examination of the expression of

the two genes showed that they are expressed in the same organs but the cell type tends to vary. For example, both *sox9a* and *sox9b* are expressed in the pharyngeal arches that will give rise to the craniofacial skeleton but are expressed in different domains and cell types. *sox9a* is expressed at high levels in the cells of the central mesenchyme of the arch which will give rise to the cartilage of the head and is also expressed in the surrounding perichondrium which gives rise to the osteoblasts that form the bone collar around the cartilage (Fig. 1.10A,B). *sox9b* on the other hand, is expressed in the epithelial sheath that surrounds the *sox9a*-expressing perichondrium and cartilage (Fig. 1.10C, D) (Yan *et al.* 2005). Interestingly, the expression of the co-orthologs tends to sum to the expression of *Sox9* in mouse. To examine the role of zebrafish sox9 in both bone and cartilage development, Yan *et al.*, (2005) then observed these tissues in *sox9a*, *sox9b* and double mutant embryos. Cartilage morphology was studied using alcian blue staining and it was found that *sox9b* homozygous mutants have reduced first and second pharyngeal arches whereas *sox9a* are missing all, except for a few cells of the ceratohyal indicating that *sox9a* plays a more vital role than *sox9b* in pharyngeal cartilage development. Double mutants, on the other hand, lack all pharyngeal cartilage (Yan *et al.* 2005). In conclusion, Sox9a and Sox9b have both redundant and gene-specific function in pharyngeal cartilage development. Similar effects were seen in cartilage of the neurocranium and pectoral fins suggesting the wide spread importance of the *sox9* genes in cartilage development in the zebrafish embryo (Yan *et al.* 2005). Next, they examined *sox9* mutations on bone development by staining mutant larvae with Alizarin red at a stage when many bones begin to differentiate. *sox9a* mutants were found to be missing a limited number of dermal bones of the craniofacial skeleton, others were smaller than normal and some

were completely unaffected, whereas the majority of cartilage replacement bones were completely missing and the rest were smaller than normal. On the other hand, in *sox9b* mutants, all cartilage replacement bones and some dermal bones were missing (Yan *et al.* 2005). Based on mutant analysis, they concluded that sox9a is vital for the development of cartilage-replacement bones and sox9b is important for the formation of both cartilage-replacement and dermal bones. To look deeper into the cause of these bone defects in *sox9a* and *sox9b* zebrafish mutants and to investigate the role of the genes in cartilage and bone morphogenesis, this group examined chondrocyte stacking and number in the pharyngeal arches of living embryos at 48-72hpf using confocal microscopy. They found that at 72hpf in *sox9a* mutants, chondrocytes failed to stack in rows as is seen in wild-types. *sox9b* mutants, on the other hand, had a different problem whereby they had fewer chondrocytes than wild-type but these cells did stack in orderly rows (Yan *et al.* 2005). Therefore from this study, it was concluded that the *sox9* genes play different roles during cartilage development with *sox9a* being essential for chondrocyte stacking, and sox9b for reaching the proper number of chondrocytes (Yan *et al.* 2005). Based on both the expression and function of *sox9a* and *sox9b* in zebrafish it is likely that the function of the mammalian Sox9 is shared by the two orthologs.

1.5.2 Runx2

Like the zebrafish *sox9* genes, when Flores *et al.*, (2004) sought to isolate the zebrafish orthologue of the *Runx2* mammalian gene, they found duplicate genes with a partitioned pattern of gene expression and designated them, *runx2a* and *runx2b*. Due to the known role of the runx2 transcription factor in skeletogenesis, they examined the early

expression of *runx2a* and *runx2b* in precursors of skeletal structures during zebrafish development (Flores *et al.* 2004, 2006). They found that within the first 24hrs of development, *runx2b* expression begins in the cleithrum and persists up until at least 3 dpf (Fig. 1.10E-G, I) (Flores *et al.* 2004, 2006). *runx2a* expression in the cleithrum does not begin until 2dpf, in lateral subset of cleithrum cells, and also persists until at least 3dpf (Fig. 1.10H, J). The opposite occurs in the opercle whereby *runx2a* expression in this structure (Fig. 1.10H, J) is detected before *runx2b* (Flores *et al.* 2004, 2006). The cleithrum along with the opercula are the earliest intramembranous bones formed in zebrafish (Flores *et al.* 2006).

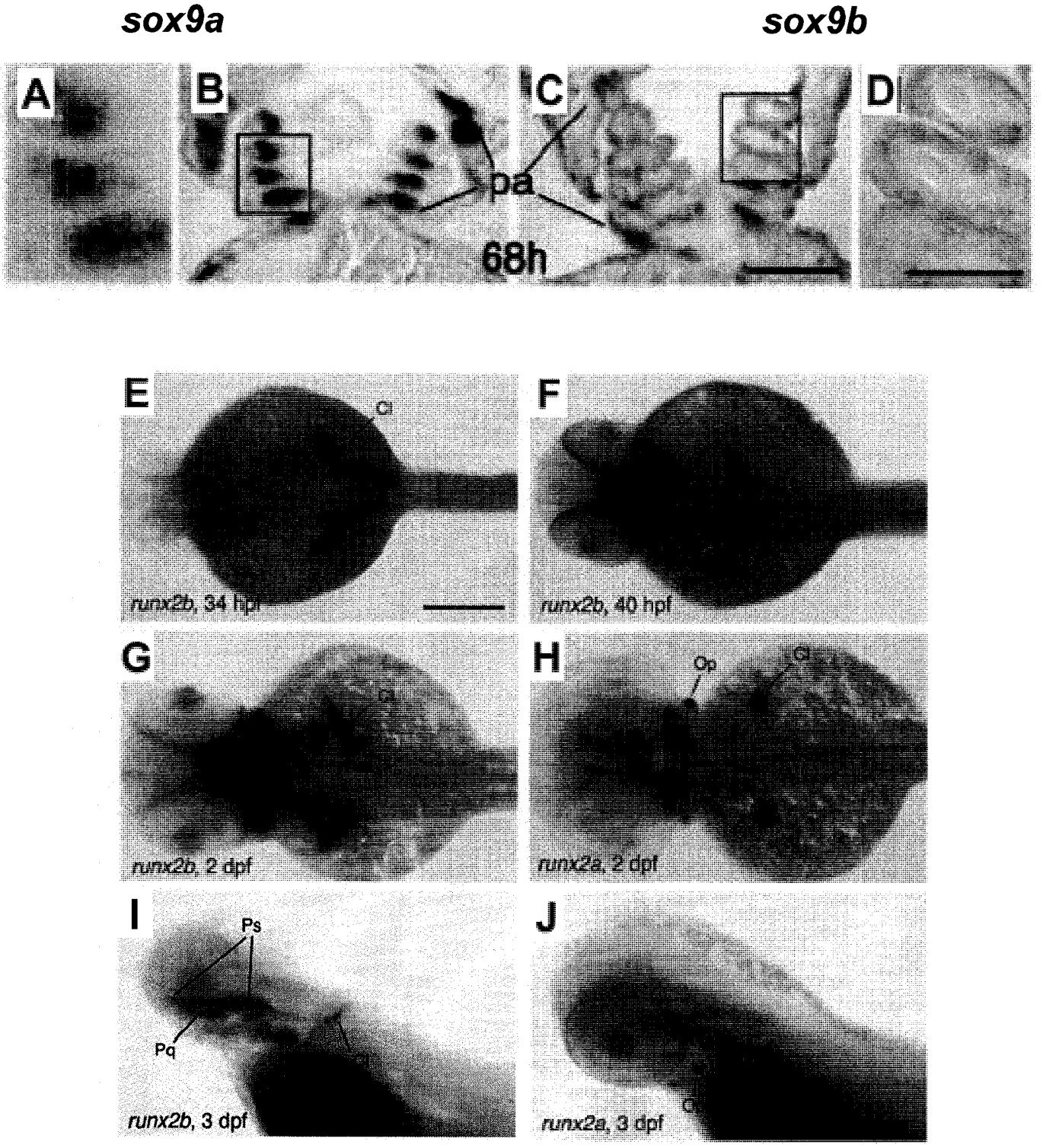

sox9a
sox9b
A
B
C
D
pa
68h
E
Cl
runx2b, 34 hpf
F
runx2b, 40 hpf
G
runx2b, 2 dpf
H
Op
runx2a, 2 dpf
I
Ps
Pq
runx2b, 3 dpf
J
runx2a, 3 dpf

Figure 1.10

Figure 1.10: Expression of the duplicate zebrafish *sox9* and *runx2* genes within the developing zebrafish embryo.

A-D: *sox9a* and *sox9b* expression within the branchial arches of a zebrafish larva at 68hpf. *sox9a* is expressed in the mesenchyme and perichondrium of branchial arches (A is the magnified inset of B). In contrast, *sox9b* is expressed in the epithelium that surrounds each arch (D is a magnification of the inset in C). Therefore, the co-orthologs *sox9a* and *sox9b* have distinct, complementary and non-overlapping expression patterns within the arches. pa, pharyngeal arches. Ventral view of zebrafish larvae anterior to the top. Adapted from: (Yan *et al.* 2005). 'Reproduced with permission of the Company of Biologists'

E-J: *runx2a* and *runx2b* expression within the craniofacial skeletal elements of the developing embryo. The expression patterns of the two genes are in some cases overlapping but with different onsets of expression. For example, *runx2b* expression is first detected in the cleithrum (Cl) starting at 34 hpf (E-G, I) and is still expressed at 3 dpf (I). *runx2a* is not expressed within the cleithrum until about 2dpf and is only detected in a more lateral subset of cleithrum cells (H, J). In contrast *runx2a* (H) is expressed before *runx2b* in the opercle. In the case of the ceratobranchial (Cb), expression of the two genes does not overlap since only *runx2b* is expressed in Cb1-4. cb, ceratobranchial; cl, cleithrum; op, opercle; ps, parasphenoid; pq, palatoquadrate. E-H: dorsal views of embryos and larva anterior to the left. I-J: lateral views of embryos and larva anterior to the left. Adapted from: (Flores *et al.* 2004)

In the days following, *runx2a* and *runx2b* transcripts can be detected in other parts of the craniofacial skeleton (Flores *et al.* 2004, 2006). Depending on the region of the craniofacial skeleton, it was observed that *runx2a* and *runxb* had either overlapping expression patterns with different onsets of expression, or their expression patterns do not overlap. A study done by the same group examined the function of Runx transcription factors in the initiation of cartilage formation in the zebrafish craniofacial skeleton. First, they performed a detailed analysis of the expression of the *runx2a* and *runx2b* transcription factors in the regions of formation of both dermal and cartilage bones (Flores *et al.* 2006). Secondly, they examined the function of Runx2a and Runx2b during the first 5 days of skeletal development, *in vivo* using antisense morpholino gene knock-down and determined their contributions to pharyngeal cartilage development (Flores *et al.* 2006). The resulting phenotypes reflect the patterning and timing of the expression of the two genes. During endochondral bone formation, *runx2b* is expressed before *runx2a.* Therefore it is not surprising that in *runx2b* morphants, cartilage formation is either reduced or completely abolished, whereas *runx2a* morphants have almost normal cartilage morphology (Flores *et al.* 2006). These results indicate that runx2b may compensate for the loss of runx2a. Interestingly, to date, no zebrafish study has focused on the role of the runx2 genes during intramembranous bone formation or osteoblast differentiation.

1.5.3 Ihh and Col10a1

Avaron *et al.,* in 2006, characterized for the first time the expression of the zebrafish ortholog of the *Ihh* gene, referred to as *ihha*, in the developing zebrafish and the

regenerating fin. The expression of *ihha* in fin regeneration has already been discussed above therefore this section will focus on its expression within the developing zebrafish cartilage elements, which is more closely related to its expression during development of higher vertebrates. Starting at 4dpf, both *ihha* and *col10a1* transcripts can be detected in pre-hypertrophic and hypertrophic chondrocytes, respectively, and in some bones of the developing craniofacial skeleton (Fig. 1.11A-C) (Avaron *et al.* 2006). These expressing cells possess the typical morphology of hypertrophic chondrocytes, since they are much larger than the neighboring cells and are surrounded by a thick acellular material consistent with active extracellular matrix secretion (Fig. 1.11 arrows) (Avaron *et al.* 2006). These results were expected based on the known expression and roles of these genes in other organisms, such as mouse and chick (Bitgood *et al.* 1995; Vortkamp *et al.* 1996; Iwasaki *et al.* 1997; St-Jacques *et al.* 1999). On the other hand, what was not expected was the expression of *col10a1* in bones of the head skeleton that are classified as intramembranous bones devoid of cartilage precursors (Avaron *et al.* 2006). In the following days *ihha* and *col10a1* expression become clearer in the developing larval cartilaginous skeleton but only *col10a1* expression is continuously detected in forming bones of the intramembranous skeleton (Fig. 1.11E black arrowheads). For example, at 3dpf, *col10a1* expression is detected in the developing cleithrum of the developing larvae (Fig. 1.11D) (Avaron *et al.* 2006). No *ihha* transcripts are detected in forming intramembranous bones of the craniofacial skeleton. These results coincide with what is seen in mice whereby eliminating Ihh signaling does not have an effect on the formation of intramembranous bones. Therefore, during craniofacial development *col10a1* transcripts can be detected in hypertrophic chondrocytes, in flattened cells which are

thought to represent osteoblasts that will give rise to the cranial intramembranous bones and the cells of the perichondrium/periosteum cells that play a role in the ossification process of many skeletal structures of endochondral origin. Interestingly, this expression of *col10a1* in zebrafish is not mirrored in mouse where *Col10a1* expression is absent from the perichondrium of developing mouse long bones (Chan *et al.* 1998; Jacenko *et al.* 2002).

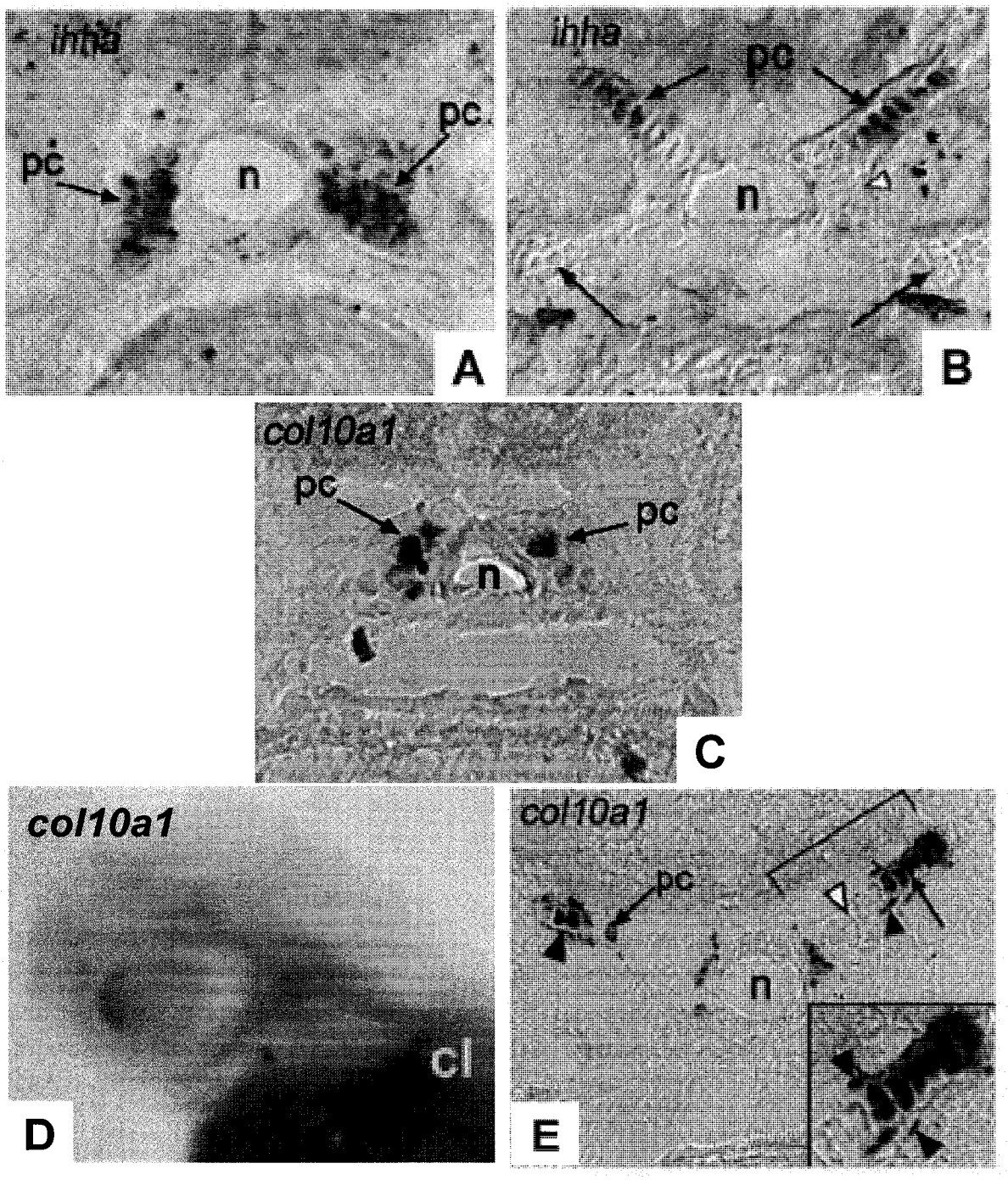

ihha
pc
pc
n
A
ihha
pc
n
B
col10a1
pc
pc
n
C
col10a1
cl
D
col10a1
pc
n
E

Figure 1.11

Figure 1.11: Expression of *ihh* and *col10a1* in skeletal elements during zebrafish development.

A-F: *in situ* hybridization performed on a whole-mount 3 dpf larva detecting *col10a1* expression (D) and on cross sections through the head region of 6dpf larvae detecting *ihha* (A, B) and *col10a1* (C, E) expression. Both genes are expressed in many cartilage elements including the parachordal plate (A, B, C, E) and are described as being expressed in hypertrophic chondrocytes of this structure (black arrows). In addition to *col10a1* expression in chondrocytes, transcripts are also detected in flat cells surrounding the cartilage, several of which will eventually form dermal bones (arrowheads) (E). Also at 3dpf, as detected by whole-mount *in situ* hybridization, *col10a1* is expressed in the cleithrum (cl) (D), which is the first dermal bone to develop in the larvae. bs, basibranchial; ch, ceratohyal; ect, ectopterygoid; n, notochord; pc, parachordal cartilage; ps, parasphenoid. D is a whole-mount lateral view of a 3 dpf larvae with anterior to the left.

(A, B, C, E) are adapted from: (Avaron *et al.* 2006)

(D) adapted from: (Padhi *et al.* 2004)

ihha expression was also analyzed in the endoskeleton of the fin in older larvae and was detected in several elements, radials and hyurals, in all the unpaired fins (Avaron *et al.* 2006). These endoskeletal elements are what attach and support the dermal fin rays and are formed through endochondral ossification. The expression of *ihha* is observed in two different populations of cells: small flat cells at the distal tip of the bones which are hypothesized to be less differentiated or proliferating chondrocytes, and in the more proximal region expression is detected in large round cells that are likely progressing towards ossification (Avaron *et al.* 2006). *ihha* expression in the above mentioned cells of the fin endoskeleton is similar to the expression of *Ihh* in the growth plate of mouse long bones. Interestingly, *ihha* transcripts were detected in the mesenchymal compartment underneath the epidermis of the forming scales of the fish (Avaron *et al.* 2006). This result is significant since the scales of a zebrafish are dermal bones and therefore form without a cartilaginous precursor.

1.6 The Bone morphogenetic protein (BMP) family

A researcher named Urist first discovered Bmps in 1960. He identified these proteins for their capability of inducing ectopic cartilage and bone formation when implanted in muscle pouches or subcutaneously (Wozney 1989; Reddi 1994). It was shown that the sequence of events that leads to this ectopic bone and cartilage formation is identical to what occurs during endochondral bone formation during limb development. Since their initial discovery, much more work has focused on the isolation and characterization of the now 20 family members and also on their function in the early stages of embryogenesis and their critical roles in skeletal development (reviewed in: (Xiao *et al.*

2007). Even though they were first identified and named for their ability to induce endochondral bone formation, they have been shown to be essential for patterning the embryo and also regulating neurogenesis, hematopoiesis and somite formation during early embryogenesis (Kishigami *et al.* 2005). Numerous Bmp knockout experiments revealed the diverse biological activities of Bmps during development highlighting their ability to regulate cell proliferation, differentiation and apoptosis during early embryonic patterning and organogenesis (Zhao 2003). Examining the role of Bmps in skeletal formation has been a little more difficult using knockout models, due to their importance in early development. Indeed, most Bmp mutations are fatal before any skeletal elements begin to form. In the following sections the structure of the Bmp proteins and also their signaling pathways will be described in detail. As well as what is known to date on their functions in skeletal development.

1.6.1 Structure

Bmps are members of the TGFß superfamily and can be distinguished from those of the TGFß and activin family due to their two extra conserved cysteines. All Bmps contain seven cysteines, six of which build a cystin knot and the seventh is used for dimerization with a second monomer (Schlunegger *et al.* 1992). Bmps are translated as large preproproteins which contain 3 regions prior to secretion: a signal peptide, a prodomain, and mature domain (Wang *et al.* 1990). Once secreted, the signal peptide is cleaved and the proproteins undergo dimerization. Then specific proteolytic enzymes cleave this dimerized proprotein to generate a biologically active mature protein. These functionally

active Bmp dimers are then able to bind to receptors on the surface of the target cells and activate a cascade of downstream signals (Wang *et al.* 1990).

1.6.2 Bmp signaling pathways

Like other TGFß family members, secreted Bmps exert their biological effects by binding to 2 types of serine/threonine kinase transmembrane receptors referred to as Bmp receptor type I (BMPR-I) and type II (BMPR-II) (Massague 1998; Zwijsen *et al.* 2003; Wan *et al.* 2005). BMPR-I receptors are further subdivided into BMPR-IA (ALK3), BMPR-IB (ALK6) and more recently Alk8 (Yamashita *et al.* 1995; Nishitoh *et al.* 1996; Natsume *et al.* 1997; Gilboa *et al.* 2000; Aoki *et al.* 2001; Bauer *et al.* 2001). Upon binding of the Bmps to their receptors, there is activation of their cytoplasmic serine/threonine kinase activities which then phosphorylates a set of intracellular substrate signaling proteins known as Smads (Massague 1998; Zwijsen *et al.* 2003). These proteins are important to intracellular signaling by most known TGFß superfamily members. There are three groups of Smad proteins; receptor regulated (R-Smads), a common partner Smad (Co-Smad) and inhibitory Smads (I-Smads) (Heldin *et al.* 1997). The R-Smads, Smad1, 5 and 8, are Bmp-specific and directly interact with activated BMPR-I receptors which phosphorylate them at their C-termini. Upon phosphorylation these R-Smads then form heteromeric complexes with the Co-Smad, Smad4. This activated complex is then able to translocate into the nucleus where it acts to regulate the transcription of various Bmp target genes through its ability to bind directly to DNA (Heldin *et al.* 1997). The third group of Smad proteins is the inhibitory Smads which are one of the bodys natural mechanisms for inhibiting Bmp signaling. The cytoplasmic

inhibitory Smad 6 and 7 inhibit Bmp-mediated Smad signaling by interfering with the phosphorylation of R-Smads and preventing their interaction with Smad4 (Heldin *et al.* 1997; Kawabata *et al.* 1998). Therefore, no complex is formed to activate the transcription of target genes. As was mentioned earlier, Bmps are growth and differentiation factors that mediate a wide range of biological processes ranging from early development to postnatal tissue homeostasis. Therefore the target genes turned on by the Bmp-mediated Smad complex vary depending on the organ, tissue or cell types being targeted.

1.6.3 Bmp function in bone and cartilage development

As mentioned above, there are now approximately 20 identified Bmp family members and the ones with the greatest osteogenic potential are Bmp 2, 4, 5, 6, 7 and 9. It has been shown that Bmp2, 4 and 6 are the most readily detectable of the family in osteoblast cultures and are potent osteoblast differentiation factors in vitro (Yamaguchi *et al.* 1996, 2000; Balint *et al.* 2003; Canalis *et al.* 2003; Wan *et al.* 2005). Bmp2, 4 and 7 have also been shown to induce mesenchymal cell chondrogenesis and regulate differentiation of isolated chondrocytes (Pathi *et al.* 1999). In summary, Bmps through a cascade of downstream signals have been shown to regulate many different stages of cartilage and bone formation, one of the most important roles being regulating the differentiation of both osteoblasts and chondrocytes from mesenchymal cells. Compelling evidence for the role of Bmp signaling in skeletogenesis firstly came from examining the expression patterns of individual Bmps within skeletal elements during development. Expression of Bmp2 was detected in cells that contribute to both bone and cartilage formation. During

bone formation, *Bmp2* is expressed in all osteogenic zones and in the periosteal region and during cartilage formation; transcripts are found in areas surrounding the initial cartilage condensation and also later on in hypertrophic chondrocytes in the sternum and humerus of embryonic mice. *Bmp4* is expressed in the perichondrium, while *Bmp6* is expressed in pre-hypertrophic and hypertrophic chondrocytes, for example in the mouse sternum and humerus (reviewed in: (Kronenberg 2003; van der Eerden *et al.* 2003; Xiao *et al.* 2007). These expression patterns suggested the role of Bmp in skeletogenesis, which was confirmed by functional analysis. A human mutation in a BMP type I receptor renders the receptor constitutively active and results in a disease called fibrodysplasia ossificans progressive, characterized by progressive extraskeletal ossification, providing one example of the role of Bmp in bone formation (Shore *et al.* 2006). Bmp2 and Bmp4 are indispensable for early development therefore traditional knockout methods cannot be used to evaluate their role in skeletogenesis due to the early death of the embryo (Winnier *et al.* 1995; Ying *et al.* 2001). In contrast, mice homozygous for loss of function mutations of Bmp5, 6 and 7 are viable and exhibit skeletal malformations. Bmp7 mutants have skeletal alterations in the rib cage, hind limbs and skull, and Bmp6 mutants have delayed sternum ossification (Katagiri *et al.* 1998; Solloway *et al.* 1998). One study examined the roles of Bmp2/4 in bone and cartilage development and demonstrated that overexpression of Bmp2 and 4 results in expansion of limb cartilage which is likely to due to an increase in chondrocyte cell number and also in cartilage matrix (Duprez *et al.* 1996). The injection of recombinant human Bmp2 alone into muscle tissue was sufficient for the induction of ectopic bone formation (Olmsted-Davis *et al.* 2002). Bmp2 has also been shown to induce *Runx2*

expression in osteoblast and chondrocyte cultures which, in turn, regulates transcriptions of many genes involved in both bone and cartilage formation (Lee *et al.* 2000; Leboy *et al.* 2001). The downstream targets of Bmp signaling, Smad 1 and 5, have been shown to be major signaling molecules responsible for regulating differentiation of C2C12 cells in osteoblasts during osteogenesis (Fujii *et al.* 1999; ten Dijke *et al.* 2003). This effect is mediated through the preferential interaction of SmadI and Smad5 with Runx2 activating bone-specific gene expression (Leboy *et al.* 2001). In summary, when BMP induces osteogenesis, it is the coordinated action of Smads and Runx2 that leads to induction of osteoblast differentiation and function. During cartilage development Bmp signaling plays a dual role: first in chondrocyte differentiation and, and then later on during chondrocyte hypertrophy it activates *Collagen X* expression through Runx2 (Enomoto *et al.* 2000; Drissi *et al.* 2003; Zheng *et al.* 2003). It has been demonstrated that treatment of pre-hypertrophic chondrocytes with Bmps induces *ColX* mRNA and expression of a dominant-negative Runx2 in proliferating chondrocytes prevents Bmp-mediated stimulation of *ColX* gene expression (Drissi *et al.* 2003). Also treatment of chondrocytes with Bmp6 or expression of constitutively active Bmp receptors leads to stronger expression of *ColX* (Grimsrud *et al.* 1999; Yoon *et al.* 2006). The Bmp signaling-induced expression of *Runx2* and subsequently *ColX* suggests the importance of this pathway in mediating chondrocyte hypertrophy which requires the interactions Smad1/5 and the transcription factor Runx2. Overexpression of Bmp2 and 4 in developing limbs results in an increase in chondrocyte cell number indicating their role in controlling chondrocyte proliferation and/or differentiation. This effect of Bmp signaling during the initial stages of chondrogenesis appears to be mediated by the induction of Sox9.

Treatment of mesenchymal cells with Bmp2 and 4 has been shown to induce *Sox9* expression (Healy *et al.* 1999).

Therefore Bmp signaling has been shown to induce differentiation of mesenchymal cells in osteoblasts and also endochondral ossification and chondrogenesis by activation of the two key transcription factors that govern these processes, Runx2 and Sox9, respectively.

1.7 Research Objectives

It became clear when researching bone formation in different organisms that very little is known about the process of intramembranous ossification, especially when compared to the large amount of research that has focused on endochondral ossification. The usefulness of the zebrafish caudal fin to study the process of regeneration has become highly regarded by the scientific community, but until recently its use in examining the molecular mechanisms behind ontogeny and regeneration of dermal bone has not gained a lot of attention. Work in our laboratory has focused on dissecting the molecular mechanisms behind dermal bone regeneration in attempts to elucidate some of the processes involved in intramembranous bone formation/regeneration in zebrafish and other organisms. We specifically examined the functions of the Shh and Bmp signaling pathways in dermal bone formation:

1) In the first manuscript we performed a functional analysis of Shh and Bmp signaling in zebrafish fin regeneration, by misexpressing and inhibiting each pathway to observe the effect on the regeneration process and specifically on dermal bone formation.

2) In the second manuscript we examined more closely the role of the Bmp signaling pathway in regenerate growth and bone formation. We inhibited endogenous Bmp signaling within the regenerate by misexpressing a natural inhibitor of the pathway and observed the consequences on the morphology of the bone secreting cells and the lepidotrichia, and the expression of downstream targets involved in bone formation.

3) In the third manuscript we compared the expression patterns of genes involved in fin regeneration using two *in situ* hybridization techniques: whole-mount followed by cryo-sectioning, and *in situ* on sections technique, which involves detection of mRNA transcripts for specific genes on fin regenerates that have been cryo-sectioned before being hybridized.

Chapter 2: Manuscript 1

Summary:

In order to understand tissue regeneration, in organisms capable of doing so, it is important to dissect the molecular mechanisms that mediate this process. In many instances regeneration of an organ or tissue involves re-expression of genes involved in the development of the lost or damaged structure. The zebrafish is capable of regenerating many structures including its bony fin rays, providing an excellent model to study the regeneration of dermal bone. A previous study done in our lab showed the re-expression of Sonic hedgehog (shh) signaling pathway genes within the fin regenerate in a similar pattern to what is seen during the development of the fin rays. The expression of *shh*, its receptor *ptc1* and downstream target *bmp2b* within the basal layer of the epidermis, and the expression of *ptc1* and *bmp2b* within the bone secreting scleroblast, suggested that this pathway may play a role in the formation of the regenerating dermal bone in the fin. Also, the changing expression pattern of *shh* before a ray bifurcation event suggested the pathway may also play a role in patterning of the regenerating rays. Based on the above mentioned expression patterns and the fact that Bmp factors act downstream of Shh in developing organs, we set out to demonstrate that the Shh signaling pathway plays a direct role in dermal bone formation within the regenerating zebrafish caudal fin. Therefore, to examine the role of Shh signaling in dermal bone regeneration we: 1) ectopically expressed *shh* and *bmp2b* in the blastema between two sister rays and 2) inhibited Shh and Bmp2b signaling using cyclopamine (an alkaloid known to block Shh signal transduction) and Chordin (a Bmp antagonist), respectively.

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Bone patterning is altered in the regenerating zebrafish caudal fin after ectopic expression of *sonic hedgehog* and *bmp2b,* or exposure to cyclopamine.

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Key words: Shh, BMP, chordin, fin regeneration, in vivo transfection, cyclopamine, zebrafish

Contribution of Authors

Elizabeth Quint: Responsible for performing a large percentage of ectopic shh and bmp2b injections and analyzing data, responsible for all experiments involving cyclopamine, completed large portion of writing of manuscript

Amanda Smith: I was responsible for the control experiments involving GFP injection shown in Fig1. C, and control fusion experiments shown in Fig1. E and D. I performed the *in situ* hybridization analysis on fins injected with both *shh* and *bmp2b* (Fig. 2). All of the experiments associated with *chordin* injection alone and also *shh-chordin* co-injections were performed by me.

Fabien Avaron: Compiling of figures and editing of manuscript

Lynda Laforest: Responsible for performing a percentage of ectopic shh injections.

Jennifer Miles: Provided technical assistance for entire project.

William Gaffield: Provided stock of cyclopamine and solanidine alkaloids

Marie-Andree Akimenko: Supervised project and participated in large portion of writing of manuscript

Abstract

Amputation of the zebrafish caudal fin stimulates regeneration of the dermal skeleton and reexpression of sonic hedgehog (shh) signaling pathway genes. Expression patterns suggest a role for shh signaling in the secretion and patterning of the regenerating dermal bone, but a direct role has not been demonstrated. We established an *in vivo* method of gene transfection to express ectopically genes in the blastema of regenerating fins. Ectopic expression of *shh* or *bmp2b* in the blastema-induced excess bone deposition and altered patterning of the regenerate. The effects of shh ectopic expression could be antagonized by ectopic expression of chordin, an inhibitor of bone morphogenetic protein (bmp) signaling. We disrupted shh signaling in the regenerating fin by exposure to cyclopamine and found a dose-dependent inhibition of fin outgrowth, accumulation of melanocytes in the distal region of each fin ray, loss of actinotrichia, and reduction in cell proliferation in the mesenchyme. Morphological changes were accompanied by an expansion, followed by a reduction, in domains of *shh* expression and a rapid abolition of *ptc1* expression. These results implicate shh and bmp2b signaling in the proliferation and/or differentiation of specialized bone-secreting cells in the blastema and suggest *shh* expression may be controlled by regulatory feedback mechanisms that define the region of bone secretion in the outgrowing fin.

Introduction

The extent of regenerative capacity varies between species and tissue types. Analysis of the regenerative events is not only informative in its own right but may also provide information pertaining to earlier morphogenetic events, because regeneration often recapitulates development. An example of this is the dermal skeleton component of the zebrafish (*Danio rerio*) caudal fin, which regenerates rapidly after amputation by processes reminiscent of those occurring during larval stages (for review see ref. Geraudie *et al.*, 1998), including reexpression of developmental genes (Akimenko *et al.* 1995; Laforest *et al.* 1998; Poss *et al.* 2000a, 2000b; Rawls *et al.* 2000; Borday *et al.* 2001).

The dermal skeleton of the zebrafish fin comprises mineralized lepidotrichia (fin rays) and more distal collagenous actinotrichia (Fig. 2.1*A*). The lepidotrichia are composed of two segmented hemirays that bifurcate periodically along their proximal-distal axis forming sister-ray branches. After amputation, epithelial cells migrate from the stump to cover the wound region (Santamaria *et al.* 1996; Poleo *et al.* 2001), beneath which a blastema containing undifferentiated proliferative mesenchymal cells forms. Scleroblasts then differentiate within the blastema at the epithelial/mesenchymal interface and begin to secrete the matrix that will form the new dermal bone.

During regeneration, the signaling molecule *sonic hedgehog* (*shh*) involved in patterning of many structures (reviewed in: Ingham *et al.* 2001), its membrane-receptor *patched1* (*ptc1*) (Ingham *et al.* 2001) and bone morphogenetic protein 2b (*bmp2b*), a member of the transforming growth factor-B (TGFβ) growth factor family (Nikaido *et al.* 1997) are

all initially reexpressed in a single domain at the distal stump of the amputated ray. Expression is found in a subset of cells in the basal layer of the epidermis, and *ptc1* and *bmp2b* transcripts are also found in the bone matrix-secreting scleroblasts within the mesenchyme, beneath the epithelial cells (Laforest *et al.* 1998). By 1-2 days after initiation of expression, an imminent bifurcation is signaled in all except the outermost (lateral) rays (which never bifurcate) by the duplication of the single domain of *shh* and *ptc1* expression into two domains (Fig. 2.1*B*).

The re-expression of *shh* and *bmp2b* in the region of cell proliferation and new bone deposition, along with their changing expression domains before ray bifurcation events, implicates them in the formation and patterning of new dermal bone and possibly cell proliferation during fin regeneration. In developmental systems that depend on epithelial-mesenchymal interactions, shh and bone morphogenetic proteins, such as bmp2 and bmp4, are often coexpressed or expressed in adjacent cell populations (Bitgood *et al.* 1995). In a few of these developing organs, such as the feather bud (Jung *et al.* 1998), the gut (Roberts *et al.* 1995) and the genital tubercle (Haraguchi *et al.* 2001), the bmp factors have acted as downstream targets of shh signaling. However, in other cases, such as the tooth (Dassule *et al.* 2000) which, like the fin ray, is a dermoskeletal structure, misexpression of shh does not seem to affect expression of bmp factors suggesting that, in these organs, shh and bmp signaling are acting in parallel and independent pathways.

To test shh function and the relationship between shh and bmp2b signaling in dermal bone formation directly, we established an *in vivo* transfection method to express *shh* and *bmp2b* ectopically in localized regions of regenerating fin and investigated the effects of

impaired shh-signaling by using cyclopamine, a *Veratrum californicum* alkaloid known to block shh signal transduction (Cooper *et al.* 1998; Incardona *et al.* 1998, 2000; Neumann *et al.* 1999).

Materials and Methods

Animals

Zebrafish were purchased at a local pet store and maintained at 28.5°C using standard methods.

Fin amputation

Adult zebrafish were anaesthetized in 0.6 mM Tricaine ® and their caudal fins amputated either one segment below (proximal to) the level of the first ray bifurcation (low cut; see Fig. 2.1*A*) or in the range four to six segments above (distal to) the first bifurcation (high cut; see Fig. 2.1*A*).

DNA Injections

DNA injections were performed at 2 days after a high cut. Tissue separating the blastema of regenerating branches of rays was microinjected with 0.2-0.6 nl of 100 ng/µl circular plasmid DNA. Co-injection of *shh* and *chordin* plasmids were performed with 250 ng/µl of each DNA construct. For ectopic expression of *shh* and *bmp2b*, DNA was injected in each ray of the ventral lobe. Dorsal rays were injected with a control plasmid encoding the *enhanced green fluorescent protein* gene ($_{pCMV5}$EGFP).

To analyze the effects of *shh* or *bmp2b* injection on bone deposition, fins were allowed to regenerate for 1 week, fixed in 4% paraformaldehyde in phosphate buffered saline (PBS)

for 2 h at room temperature. High-cut fins were stained with 0.01% Alcian blue in 70% ethanol and 30% acetic acid, rinsed in PBS, stained in 0.1% Sirius red in saturated picric acid for 1 h, washed in 0.01N HCl, and rinsed in distilled water. Selected rays were cut into 16-μm sections by standard cryosectioning procedures. To analyze the effects of DNA-construct injections on endogenous gene expression, fins were allowed to regenerate for 2 days after injection, cut into 16-μm sections, and processed for *in situ* hybridization.

In situ hybridization

In situ hybridization on whole-mount fins and cryosections was performed as described (Laforest *et al.* 1998). The *shh* digoxigenin-labeled riboprobe corresponded to a 1,440-bp C-terminal fragment that excludes a region conserved between different *hedgehog* genes (Krauss *et al.* 1993). The *ptc1* probe consisted of a 1,150 bp cDNA fragment encoding amino acids 400-780 (Concordet *et al.* 1996).

Construct Subcloning

$_{pCMV5}$EGFP, the enhanced *GFP* gene (EGFP) was excised from plasmid pEGFP-C1 (CLONTECH) and ligated into the multiple cloning site of pCMV5 (CMV, cytomegalovirus) (Department of Molecular and General Biochemistry, University of Texas, Southwestern Medical Center). $_{pCMV5}$shh-NH_2; a 632-bp, N-terminal fragment of the shh cDNA (Roelink *et al.* 1994), was obtained by PCR by using the following primers: forward, 5' –ATTAAGCTTGCGGCAAAATGCGG-3'; reverse, 5'-GAAGATCTCCCCCAGATTTCGCAGC-3'. It was cloned into the pCMV5 vector at the *Hin*dIII and *Bgl*II sites. Position 632 of the *shh* sequence corresponds to the cleavage

site of the shh precursor. $_{pCMV5/pEGFP}$bmp2b, a modified version of pCMV5 vector, was made to create convenient restriction sites (*Eco*RI and *Kpn*I sites) to place the *bmp2b* cDNA under the control of the CMV promoter. $_{pCMV}$chordin (kindly provided by Dr. M. Halpern, Carnegie Institute of Washington, Baltimore); a 2.5-kb fragment encoding the zebrafish chordin cDNA (Miller-Bertoglio *et al.* 1997), was inserted into the pCS2+ expression vector containing the CMV promoter.

Cyclopamine Preparation and Treatment

Water-soluble complexes of the alkaloids cyclopamine and solanidine were prepared in PBS and evaporated under nitrogen. Complexes consisted of 10 mg cyclopamine, 5 mg citric acid and 125 mg cyclodextrin or 10 mg solanidine, 5 mg citric acid and 250 mg of cyclodextrin. Stocks of 10 mM alkaloid complex were made with distilled water and stored in the dark at 4°C.

Zebrafish were placed in beakers containing water with 1, 5, or 10 μM alkaloid (cyclopamine or the control, solanidine) for 2 days after a low-cut amputation. Fish were maintained in the alkaloid solution for 5 days in the dark at 28.5°C with a solution change on the third day. Each day, the fish were anaesthetized and the length of the regenerated fin measured (same rays each day). After treatment, fins were recut and fixed in 4% paraformaldehyde/PBS, then either stained for histological examination by using Alcian blue and Sirius red or processed for *in situ* hybridization.

BrdU Incorporation

Proliferating cells were labeled with BrdU according to ref: (Poleo *et al.* 2001) with some modifications (Santamaria *et al.* 1996), except that after incubation with a mouse anti-

BrdU monoclonal antibody (Sigma), BrdU incorporation was visualized with an ABC alkaline-phosphatase detection kit (Serotec).

Results

Ectopic Expression of shh and bmp2b Disrupts the Normal Patterning of Regenerating Rays

To investigate the role of shh and bmp2b in bone deposition and patterning, we developed a method of local gene transfection (based upon gene-therapy studies, e.g., (Nishi *et al.* 1996; Chang *et al.* 1999) to express ectopically *shh* and *bmp2b* in specific regions of the regenerating fin.

Pilot experiments showed that 10-60 mesenchymal cells could be transfected with plasmid DNA containing the *EGFP* reporter gene, placed under the control of the CMV promoter, when injected at concentrations between 100 and 250 ng/μl into the tissue separating the blastemas of regenerating branching rays (Fig. 2.1*C*). The normal morphology of rays 24 h after injection (assessed with the light microscope) indicated that the procedure did not interrupt normal regenerative patterning. GFP fluorescence continued for at least 1 week after injection. Transfection efficiency was low in unamputated rays, indicating that this technique is suited to either traumatized tissue and/or undifferentiated, highly proliferative tissue.

To investigate shh signaling in ray bifurcation, *shh* and *bmp2b* constructs were misexpressed between two sister ray branches, where *shh* and *bmp2b* are not normally expressed. Rays were amputated at a "high-cut" level (Fig 2.1*A*) where normal transient

fusion of the lepidotrichial branches rarely occurs (Laforest *et al.* 1998). Fins were injected 2 days after amputation (dpa), with plasmids containing either the amino-terminal coding portion of *shh* [*N-shh*; the portion responsible for its biological activity (Roelink *et al.* 1994)], the *bmp2b* gene (Nikaido *et al.* 1997), or the control *EGFP* reporter.

When N-*shh* or *bmp2b* gene constructs were transfected between the blastemas of the two sister rays, an increase in the incidence of fusion was observed, most noticeable at four to five segments above the origin of bifurcation. At this level, 51% of rays injected with N-*shh* (n = 159/300) were transiently fused, compared with 15% of fins injected with *EGFP* (n = 48/320), and 40% of rays injected with *bmp2b* (n = 18/45) showed fusions compared with 13% (n = 4/30) of *EGFP* controls.

Chordin Can Antagonize the Formation of Bone Fusion Induced by shh Ectopic Expression

Chordin, a secreted protein that directly binds to bmp factors and prevents BMP-BMP-receptor interactions (Piccolo *et al.* 1996), was coinjected with N-*shh* after a high-cut. Coinjection resulted in fewer (3.4%; n = 1/29) transient fusions of the rays at four to five segments above the origin of bifurcation compared with N-*shh* injected alone (51%; see above). Coinjection also reduced the occurrence of natural ray fusion at 1-2.5 segments above the origin of bifurcation from 100% to 52% of injected fins (n = 10/19). The inhibition of bone fusion by chordin indicates that ectopic bone formation requires a bmp factor. Furthermore, our observation that both natural and shh-induced fusions are

inhibited suggests that ectopic shh expression leading to bone fusion is mediated through activation of bmp signaling.

Dermal Bone is Deposited in the Interray Region During Fusion

To determine where the additional dermal bone matrix was deposited after both N-*shh* (Fig. 2.1*F*) and *bmp2b*injection (Fig. 2.1*H*), control (Fig. 2.1*D*) and injected (Fig. 2.1*F* and *H*) rays (at least four per construct) were stained with Alcian blue and cut into horizontal sections in the region of the fusion (Fig. 2.1*E*, *G*, and *I*). In each of the fused fins examined, ectopic matrix was found only in the space between the basal layer of the epithelium and the mesenchymal cells of the inter-ray (Fig. 2.1*G* and *I*). No dermal bone deposits were found in the mesenchymal interior of the lepidotrichia where exogenous N-*shh* and *bmp2b* were expressed by transfected cells, which suggests that only cells competent to form dermal bone [i.e., scleroblasts adjacent to the basement membrane (Mari-Beffa *et al.* 1996; Santamaria *et al.* 1996)] were able to do so in response to ectopic gene expression.

Ectopic Expression of shh but Not bmp2b Alters the Patterns of *ptc1* Expression

In the developing limb bud, shh signaling is thought to be regulated by feedback loops with other proteins such as fibroblast growth factors (Laufer *et al.* 1994; Niswander *et al.* 1994). We observed that chordin, an inhibitor of bmp signaling, can antagonize the formation of bone fusion induced by shh, suggesting an interaction between shh and bmp signaling. To investigate a possible reciprocal interaction between bmp2b and shh during bone formation, we examined whether *ptc1* expression was affected by the ectopic

expression of N-*shh* and *bmp2b*. Two days after injection of N-*shh*, *ptc1* expression was induced in the mesenchymal cells and in cells of the basal epithelial layer located between the two ray branches (Fig. 2.*2D*), where *ptc1* and also *shh* and *bmp2b* are normally not expressed (Fig. 2.*2A-C*). In contrast, 2 days after *bmp2b* injection, no change occurred in *ptc1* expression compared to noninjected fins (Fig. 2.2*E*). This result suggests that the Shh signaling pathway is not activated in response to ectopic expression of *bmp2b*.

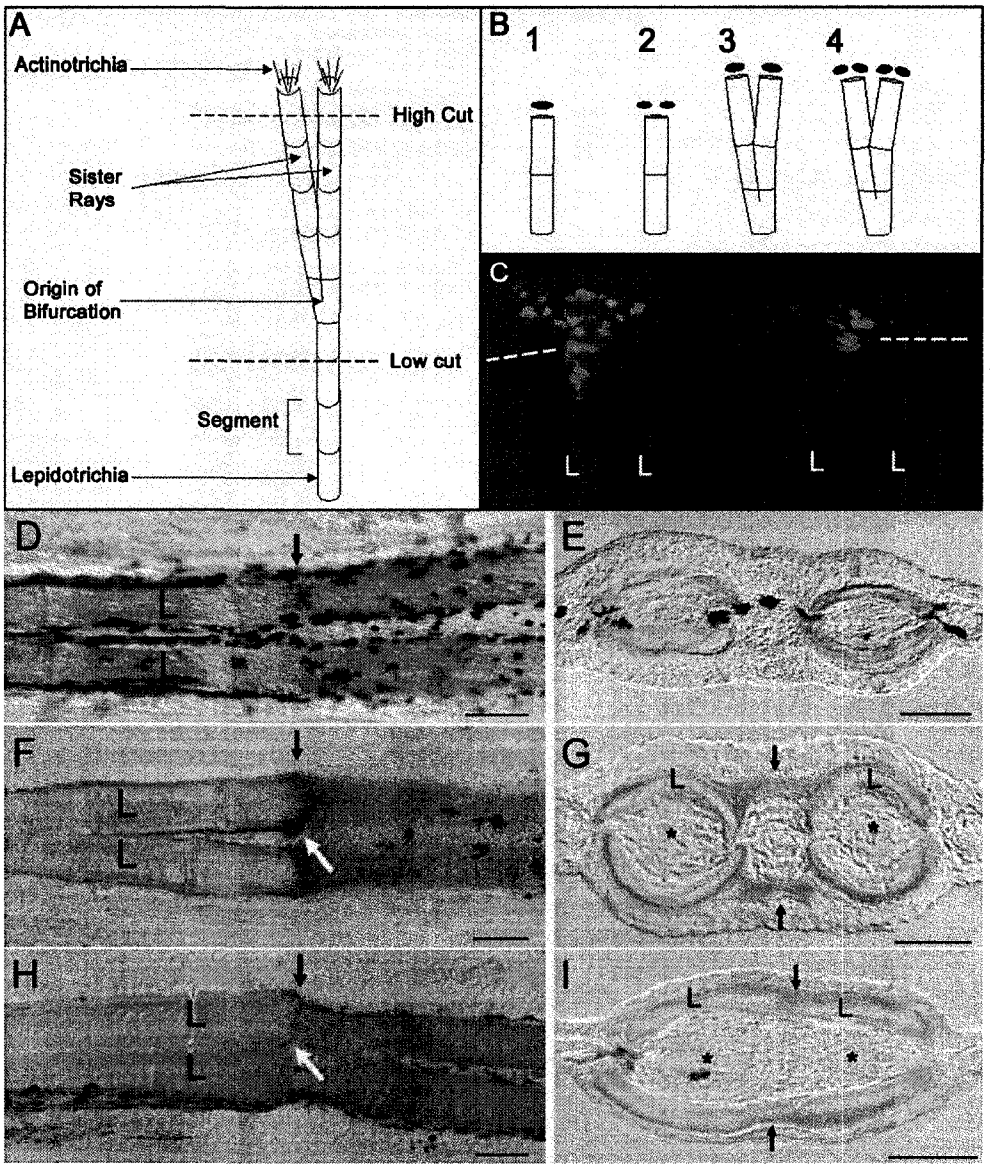
A
Actinotrichia
High Cut
Sister Rays
Origin of Bifurcation
Low cut
Segment
Lepidotrichia
B
1
2
3
4
C
L
L
L
L
D
E
F
G
H
I
control
N-shh injected
bmp2b injected

Figure 2.1

Figure 2.1: Ectopic expression of shh and bmp2b disrupts the normal patterning of the regenerating rays.

(A) Schematic representation of the skeleton of a ray in the zebrafish fin. Dermal bone forms two segmented, concave and opposing hemirays. The diagram illustrates the position of amputations, relative to the bifurcation of the lepidotrichia; 'low cut' and 'high cut' are performed one to two segments proximal and four to six segments distal to the origin of bifurcation, respectively. (B) Sequence of *shh* expression patterns after a low cut, leading to ray bifurcations: (B1) 2 dpa, an unbifurcated ray expresses one *shh* domain in the distal blastema; (B2) by 4-5 dpa *shh* is expressed in two domains, preceding the formation of a bifurcation; (B3) two newly formed sister rays each exhibit one *shh* expression domain; (B4) the process repeats itself to form a second round of bifurcations (not to scale). (C) Ectopic expression of EGFP 24 h after injection of the $_{pcmv5}$EGFP reporter construct in the tissue separating the branches of lepidotrichia (L) of two adjacent rays. Injections were performed at 2 dpa (high cut shown by the dotted line). (D-I) Normal bifurcating (D,E) and N-shh (F,G) or bmp2b (H,L) injected fused rays stained with Alcian blue. Constructs were injected in the region indicated by white arrows (F,H). (D,F,H) Whole-mount control and fused rays; proximal part of the fin is to the left. (E,G,I) cryo-sections (16 μm) in the region of normal bifurcation (E) or fusion (G,I). Arrows in G and I indicate the region of ectopic bone matrix deposition. No ectopic bone deposition occurs in the mesenchyme interior of the lepidotrichia (L) as marked with asterisks. (Bar = 50 μm).

Cyclopamine Inhibits Fin Regeneration and Alters *shh* and *ptc1* Gene Expression.

We investigated the effects of shh-signaling downregulation on bone patterning by exposing regenerating fins to cyclopamine, a steroidal alkaloid that blocks hedgehog (hh) signal transduction (Cooper *et al.* 1998; Incardona *et al.* 1998, 2000; Neumann *et al.* 1999). Caudal fins were amputated at a low-cut level (Fig. 2.1*A*), then treated with 1, 5, or 10 μM alkaloid, starting at 2 dpa, when *shh* expression is already established (Laforest *et al.* 1998). The morphology and length of the regenerated portion was assessed daily. As a control, an equal number of fish were treated at the same dosage with solanidine, a steroidal alkaloid of similar structure, but without known effects on hh signaling. Fin growth in the presence of solanidine is similar to the growth in untreated fins (data not shown).

Treatments with 10 μM (Fig. 2.3*A*) and 5 μM (Fig. 2.3*B*) cyclopamine significantly inhibited fin outgrowth by day 2 ($P < 0.05$) and 4 ($P < 0.05$), respectively. By day 5, both doses caused an accumulation of melanocytes in the distal blastema (Fig. 2.3*D* and 4*F*) where newly deposited bone seemed thicker and terminated abruptly (Fig. 2.3*D* and *F*) compared with controls (Fig. 2.3*C* and *E*). Also, at 10 μM, the actinotrichia found in the distal ray (Fig. 2.3*E*) were absent (Fig. 2.3*F*). Inhibition of fin outgrowth correlated with significant reductions in the number of regenerated segments ($P < 0.001$, Table 1). However, the length of each segment did not seem to be affected (not shown). Unlike solanidine-treated fins, rays exposed to cyclopamine did not bifurcate.

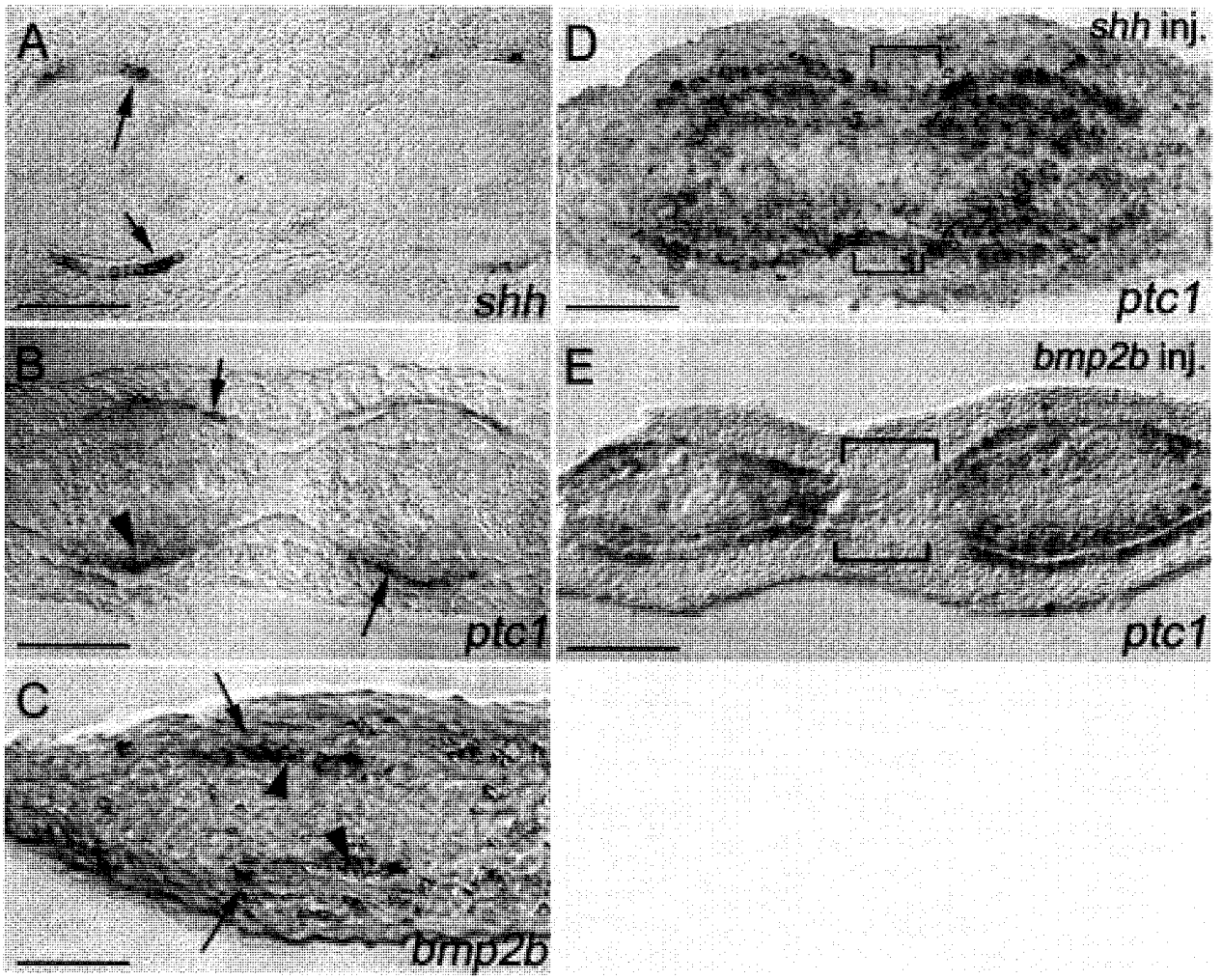

Figure 2.2

Figure 2.2: Ectopic expression of shh but not bmp2b alters the patterns of *ptc1* expression

Transverse sections of regenerating fins showing the normal expression patterns of *shh* (A), *ptc1* (B) in two adjacent rays; *bmp2b* (C) in one ray at 4 dpa; *ptc1* expression 2 days after injection of *N-shh* (D) or *bmp2b* (E) constructs. Note the ectopic expression of *ptc1* in mesenchyme and basal epithelial cells separating the branching rays (indicated by the brackets) after ectopic expression of N-*shh* (D) but not *bmp2b* (E). Arrows in (A-C) indicate *shh*, *ptc1*, and *bmp2b* expression in basal epithelial cells. Arrowheads in (B and C) indicate *ptc1* and *bmp2b* expression in scleroblasts. (Bar = 50 μm).

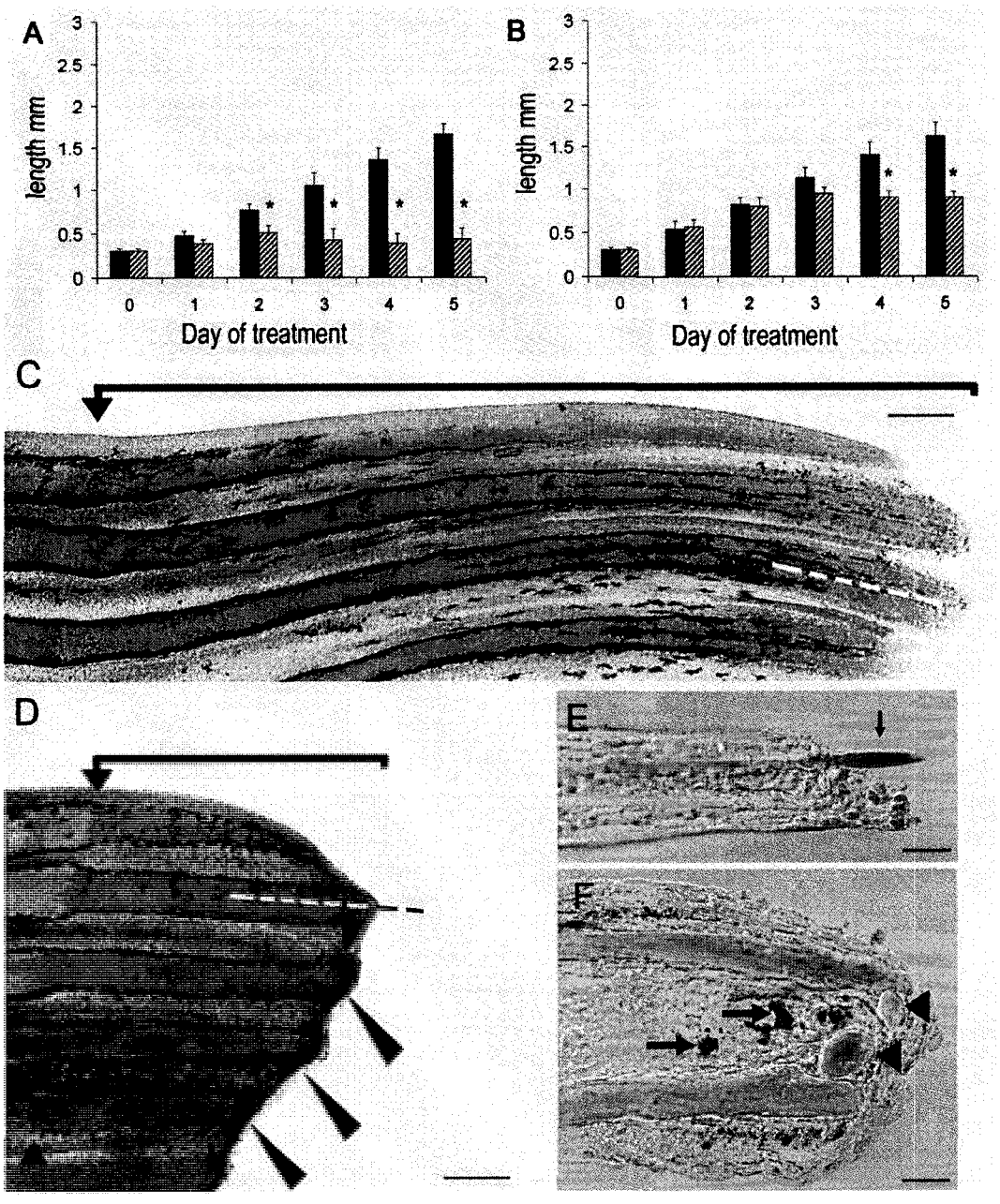

Figure 2.3

Figure 2.3: Blocking Shh signaling using cyclopamine inhibits fin regeneration.

(A,B) Growth curves of fins treated with 10 μM (A) or 5 μM (B) solanidine (black bars) or cyclopamine (diagonal hatched bars). The ordinate indicates the length of the fins in millimeters and the abscissa the day of treatment, where day 0 is the day fish were first put into alkaloid solution. At 10 μM, fin outgrowth is significantly inhibited by day 2 of treatment (asterisks, $P < 0.05$) and by day 4 of treatment at 5 μM (asterisks, $P < 0.05$). *P* values were derived using Student's t test, error bars = SEM (C-F) Effects of cyclopamine on caudal fin regeneration, proximal part of the fin is to the left; (C,D) whole mount fins stained with Alcian blue. Exposure to 5 μM cyclopamine (D) for 5 days results in the inhibition of fin outgrowth compared with 5 μM solanidine (C). Melanocytes accumulate in the distal stumps of the rays (large arrowheads) and the blastema is clearly absent. Small arrowhead in D indicates the level of fin amputation (low cut). The dotted line indicates the plane of sections shown in (E and F). Cryo-sections (16 μm) along the longitudinal axis of fins treated with 10 μM cyclopamine (F) or solanidine (E) and stained with Alcian blue and Sirius red. Note pigment accumulation and extra bone depositon in the distal-most portion of the section (arrows) and the lack of actinotrichia as shown in E (arrow). [Bar (C,D) = 100 μm; (E,F) = 25 μm.]

Table 1: Mean number of segments ± s.e.m. in alkaloid-treated fins after 5 days of treatment

	Solanidine	Cyclopamine
5 μM	4.6 ± 0.4 n = 69	1.8 ± 0.7 n = 69
10 μM	4.6 ± 0.4 n = 77	1.26 ± 0.19 n = 77

n, number of rays counted; s.e.m, standard error of the mean.

We examined the expression patterns of *shh* and *ptc1* after 1, 3, and 5 days of treatment with 5 μM alkaloid (n = 4 per group). *ptc1* expression, usually present in the distal regenerate (Fig. 2.4*A*), was absent at all time points in cyclopamine-treated fins (Fig. 2.4*B*). In contrast, *shh*, which in controls is expressed in two domains in each blastema (Fig. 2.4*C* and *E*), was expressed in one expanded domain at 1 day (Fig. 2.4*D*) and a smaller diffused domain at 3 (Fig. 2.4*F*) and 5 (not shown) days. Exposure to 1 μM cyclopamine (n = 14) had no obvious effects on morphology or *shh/ptc1* gene expression (not shown).

Cyclopamine Affects Cell Proliferation and the Morphology of the Blastema/Epithelial Interface

Cessation of fin outgrowth and altered expression of *shh* and *ptc1* suggest that cyclopamine can affect blastema cells, which are the sites of cell proliferation, scleroblast differentiation, and bone matrix secretion. It is likely that cyclopamine somehow affects the competence of the blastema to extend distally. To determine whether this was caused by decreased proliferation of cells contributing to the regenerate, we examined BrdU incorporation after 1 and 3 days of treatment with 10 μM cyclopamine or solanidine (n = 4 per group), beginning at 2 dpa. At 24 and 72 h after the onset of treatment, fish were injected with BrdU and fins harvested 7 h later (Poleo *et al.* 2001).

One day after treatment onset, no significant difference existed in the amount of cell proliferation between solanidine (Fig. 2.5*A*) and cyclopamine (Fig. 2.5*B*) groups (P > 0.05; Table 2). BrdU-positive cells were visible in the epithelial layer along the length of the fin (arrows) and in the mesenchyme of the blastema (Fig. 2.5*A* and *B*, arrowheads).

By day 3, cyclopamine treatment resulted in a significant reduction in cell proliferation in the regenerate, both in the blastema ($P < 0.005$; Table 2, Fig. 2.5*C* and *D*) and epithelial cells ($P < 0.005$; Table 2, Fig. 2.5*C* and *D*, arrows) and significantly more BrdU-positive epithelial cells in the stump ($P < 0.005$; Table 2). Although it is possible that the absence of shh signaling affects epithelial cell in addition to blastema cell proliferation, it seems that it may rather affect the distribution of epithelial cells because these cells are known to migrate from the lateral side of the stump to the regenerate (Poleo *et al.* 2001). Epithelial cell migration may be impaired in cyclopamine-treated fish as a result of regenerate growth arrest.

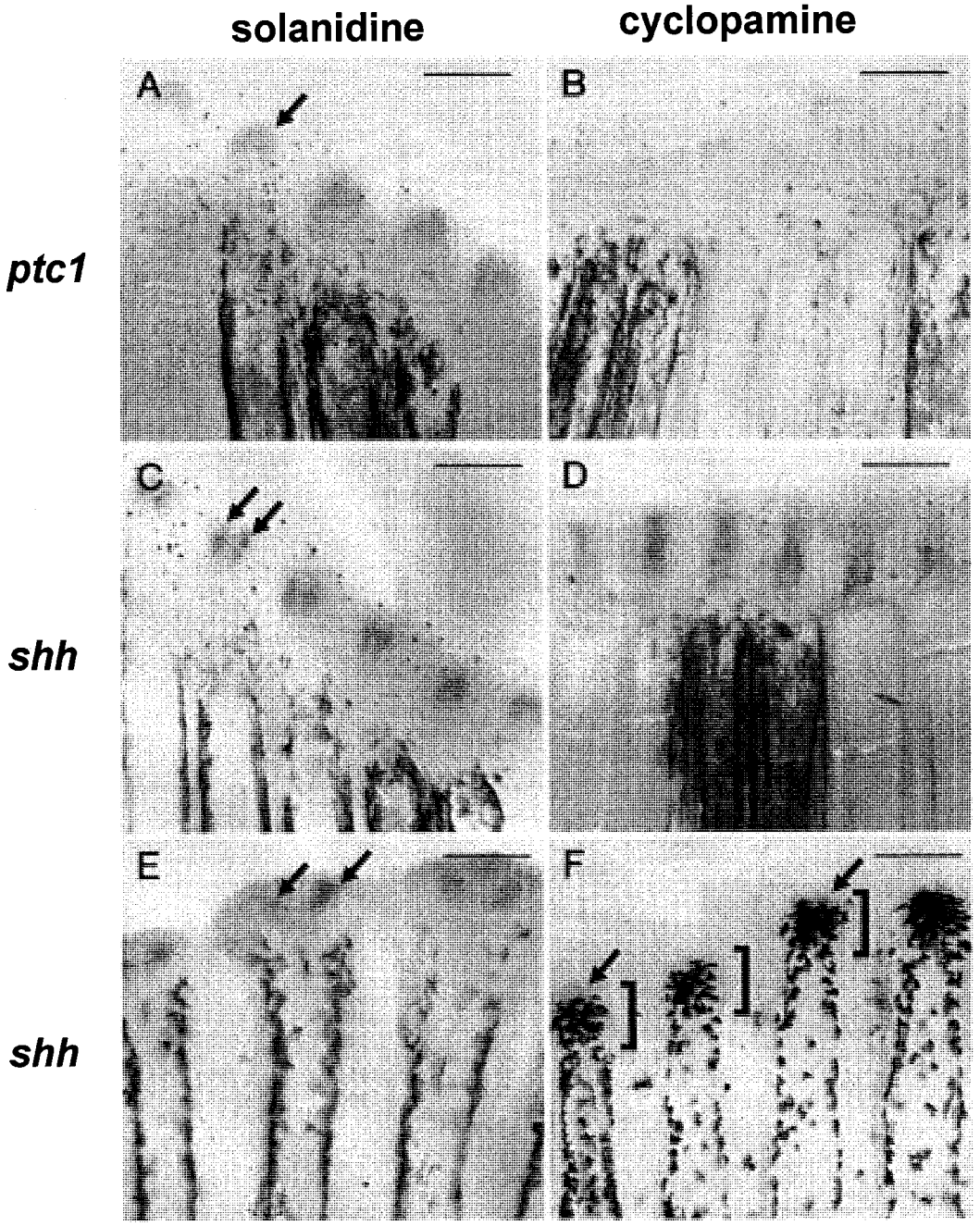

Figure 2.4

Figure 2.4: Treatment of regenerating fins with cyclopamine alters *shh* and *ptc1* genes expression

Gene expression in regenerating caudal fins treated with 5 μM solanidine (A,C,E) or cyclopamine (B,D,F). After 1 day of cyclopamine, *ptc1* expression, normally expressed in a single domain (A), is absent (B) and *shh,* normally expressed in 2 domains (C,E, arrows) is present as a single and expanded domain along the proximo-distal axis (D). By day 3 of treatment, cyclopamine-treated fins show only one weak and diffuse domain of *shh* expression (F, arrows) in the region of pigment accumulation and lepidotrichia termination (square brackets). (Bar = 150 μm.)

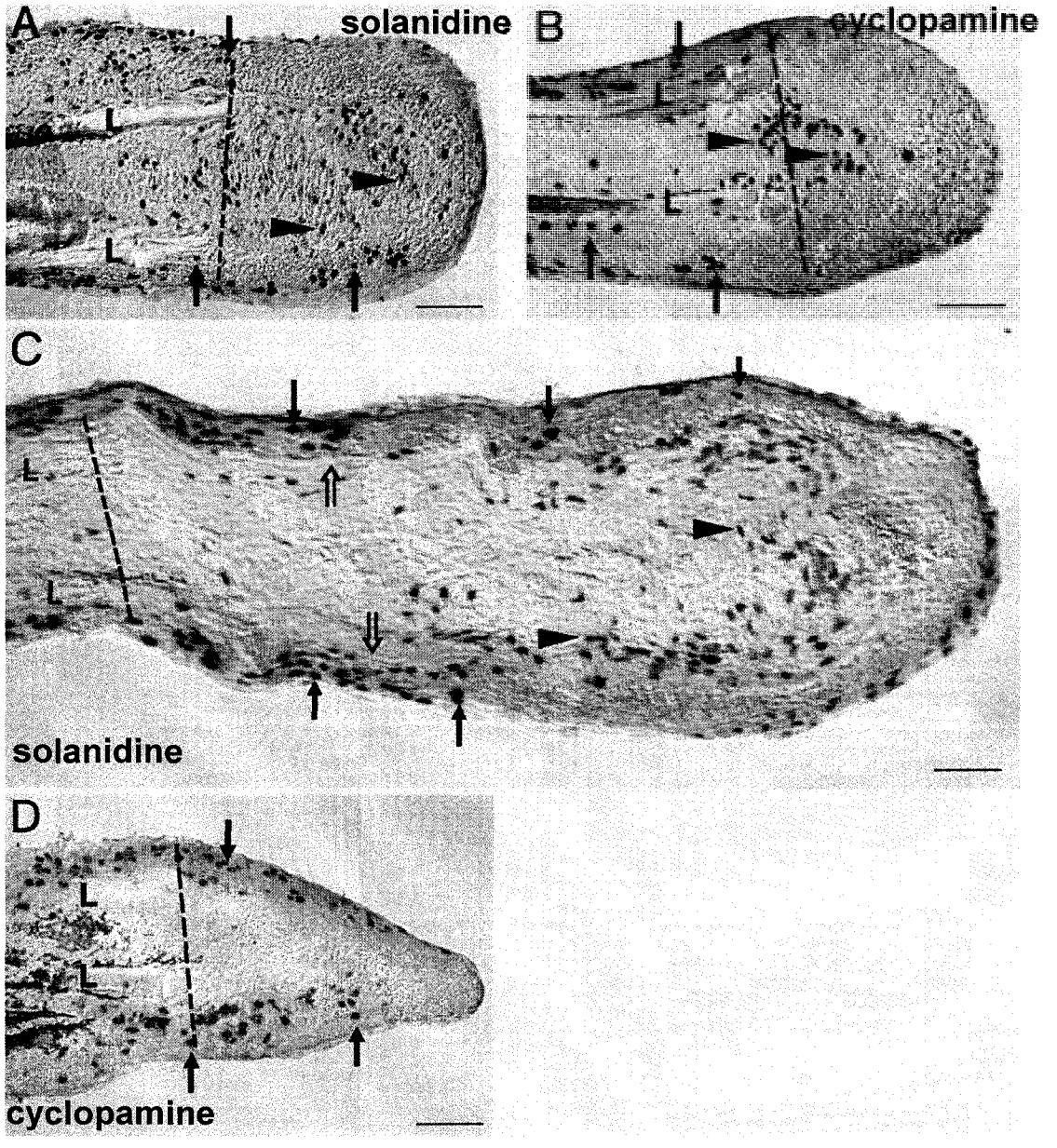

Figure 2.5

Figure 2.5: Cyclopamine treatment affects cells proliferation and the morphology of the blastema/epithelial interface.

BrdU-incorporation in fins treated with cyclopamine or solanidine. After 1 day of treatment (initiated at 2 dpa) with either solanidine (A) or cyclopamine (B), proliferating cells were identified in the epidermis (solid arrows) and mesenchyme (arrowheads) in the region of new bone matrix secretion. By day 3, BrdU-labeled cells were still found in the epidermal layers of both control and cyclopamine-treated fins (C,D, solid arrows) and in the mesenchyme of solanidine-treated fins (C, arrowheads), but not in the mesenchyme of cyclopamine-treated fins (D). Dotted lines mark the level of amputation. (Bar = 50 μm.)

Discussion

Ectopic Bone Deposition After N-*shh* and *bmp2b* Ectopic Expression

By using *in vivo* transfection of DNA, we were able to target expression of N-*shh* and *bmp2b* in the amputated fin, between the branches of already bifurcated rays where endogenous *shh* and *bmp2b* are not usually expressed. Injection of reporter-gene DNA in the blastema of amputated rays had no adverse effects on regeneration. Ectopic expression of N-*shh* and *bmp2b* resulted in additional bone deposition in the interray normally devoid of bone. However, excess bone was found only between the basal layer of the epidermis and the mesenchyme where the bone matrix-secreting scleroblasts are found (Mari-Beffa *et al.* 1996; Santamaria *et al.* 1996). This effect depended on the distance between the amputation site (injection location) and origin of bifurcation, being maximal four to five segments above the origin of bifurcation and diminishing when DNA was injected further away. This finding suggests that N-*shh* and *bmp2b* can stimulate the secretion of bone matrix, possibly by influencing the differentiation of bone-secreting scleroblasts, but only in mesenchymal regions competent to do so.

Table 2: Mean number of BrdU-positive cells ± s.e.m. in alkaloid-treated fins after 1 and 3 days of treatment

			n	Stump*		Regenerate	
Day 1	Epithelium	Cyclopamine	9	39.89 ± 2.6	p=0.468	23.9 ± 5.5	p=0.909
		Solanidine	7	51.7 ± 15		23.14 ± 3.2	
	Mesenchyme	Cyclopamine	9	24.78 ± 2.4	p=0.075	49.2 ± 5.5	p=0.2
		Solanidine	7	33.43 ± 3.7		62.4 ± 8	
Day 3	Epithelium	Cyclopamine	10	53.7 ± 5.4	p<0.001	29.3 ± 3.8	p=0.002
		Solanidine	8	9.75 ± 3		118 ± 18	
	Mesenchyme	Cyclopamine	10	3.7 ± 1.5	p=0.49	6.7 ± 1.7	p=0.001
		Solanidine	8	5 ± 1.1		108.3 ± 17	

n = number of sections from 4 (cyclopamine) or 3 (solanidine) fins.

p value is derived from a two-sample student's t-test

*BrdU-positive cells in the stump were counted over the length of one segment.

No bone was found in the vicinity of N-*shh*–expressing cells located in the mesenchymal interior of the lepidotrichia, where scleroblasts and their precursors are not usually found. *shh* expression in epithelial cells is known to play a role in interactions with adjacent mesenchymal tissue in other systems. For example, *shh* expression in the chick feather-bud epithelium is thought to induce mesenchymal condensations that form the feather placode (Ting-Berreth *et al.* 1996) and *shh* expression in the skin is required for the morphogenesis of the hair follicle (St-Jacques *et al.* 1998). *shh* is also important in the differentiation of mesenchymal cells in the developing lung (Bellusci *et al.* 1997) and in the normal growth and morphogenesis of the tooth, but it does not seem to be required for the differentiation of ameloblasts and odontoblasts (Dassule *et al.* 2000). It is not yet clear, however, whether the ectopic secretion of bone matrix described here is due to the differentiation of new scleroblasts from existing precursor cells in the inter-ray region or to the stimulation of bone secretion by existing scleroblasts.

We showed that coinjection of *chordin*, an inhibitor of *bmp* signaling, can antagonize the formation of ectopic bone by shh, suggesting an interaction between the shh and bmp pathways in fin dermal bone formation and that a bmp factor acts as a downstream target of shh. Conversely, we observed that, in contrast to shh, ectopic expression of bmp2 does not affect endogenous *ptc1* expression, a target of shh signaling, questioning existence of a positive feedback mechanism between the bmp2 and shh pathways in dermal bone formation.

Inhibition of Fin Outgrowth by Cyclopamine

Exposure of regenerating fins to cyclopamine initially reduced, then inhibited fin outgrowth and resulted in fewer or no actinotrichia and a distal accumulation of pigment cells. These effects were accompanied by a reduction in cell proliferation within the blastema and a diminution in blastema size.

Inhibition of the hh signaling pathway by cyclopamine is consistent with an interference of blastema maintenance, including proliferation and differentiation of specialized cell types within the mesenchyme, possibly including the bone-secreting scleroblasts and/or the cells synthesizing the actinotrichia, which are thought to help new tissue migrate distally (Mari-Beffa *et al.* 1989). Although cyclopamine may affect signaling by all hedgehog-related proteins, the effects we observed are likely mediated by shh, rather than other *hedgehog*-related genes such as *tiggy-winkle hedgehog* (Akimenko *et al.* 1995; Ekker *et al.* 1995) and *echidna hedgehog* (Currie *et al.* 1996), because these family members are not expressed in the regenerating fin during the course of these experiments (unpublished observations). We cannot exclude the possibility that as-yet undescribed *hedgehog*-like genes are expressed in the regenerating fin that may be affected by cyclopamine treatment.

Our studies implicate shh signaling in the proliferation and/or differentiation of the specialized bone-secreting cells in the blastema during regeneration, which may be by epithelial-mesenchymal interactions involving shh/ptc1 and bmp2b, with a subset of basal epithelial cells expressing *shh*, and the same cells plus adjacent mesenchymal cells expressing *ptc1* and *bmp2b*. In regenerating zebrafish fins, inhibition of fibroblast

growth factor (fgf) function leads to a down-regulation of epithelial *shh* expression and in turn to an inhibition of cell proliferation and blastema outgrowth (Poss *et al.* 2000b). In developing limb buds a relay between fgfs in the apical ectodermal ridge and shh in the zone of polarizing activity has been well documented (Laufer *et al.* 1994; Niswander *et al.* 1994; Zuniga *et al.* 1999; Kraus *et al.* 2001). Expression analysis of fgfs after treatment with cyclopamine may help determine the importance of epithelial-mesenchymal interactions during fin regeneration.

Changes in *shh* and *ptc1* Expression Patterns After Exposure to Cyclopamine

We found that rays treated with cyclopamine rarely bifurcated, which may be due to the disruption of *shh* expression domains, which are clearly linked to the bifurcation process. A difference in gene expression was noticeable after 1 day of cyclopamine treatment where fins treated with 5 μM cyclopamine showed an expansion of *shh* expression along the proximal-distal axis and a loss of *ptc1* expression. After 3 days of treatment, when fin outgrowth had virtually stopped, only one weak domain of *shh* expression existed, and still no *ptc1* transcripts. The rapid up-regulation of *shh* expression after cyclopamine treatment suggests that a feedback mechanism is involved in the definition of *shh* expression domains and further supports the hypothesis that such mechanisms lead to the confinement of *shh* expression in the distal-most portion of the regenerate. The eventual down-regulation of *shh* that accompanies cessation of fin outgrowth suggests that shh signaling may be important in either the proliferation and differentiation of bone-secreting cells in the blastema or bone deposition itself. However, a direct effect on bone deposition appears unlikely as bone is still deposited during the first 3 days of cyclopamine treatment, even in the absence of the shh receptor, ptc1.

We reported that *shh* expression in regenerating fins correlated with the region of new bone matrix deposition (Laforest *et al.* 1998). In addition, we observed that division of *shh* expression into two domains correlated with the formation of a bone bifurcation, the two domains of *shh* expression corresponding to the bone-forming region of the new branches. These observations suggested a role for shh in dermal bone patterning (Laforest *et al.* 1998). However, it was not clear whether the absence of shh function was the consequence or the cause of absence of bone between branches. Here, we have presented evidence that ectopic expression of *shh* or *bmp2b* in the blastema of regenerating fins leads to excess bone deposition in regions competent to produce bone-secreting cells. Conversely, treatment of regenerating fins with cyclopamine results in a reduction in fin outgrowth and eventual cessation of cell proliferation and distal bone deposition in the blastema. These results suggest that shh signaling plays a role in the proliferation and/or differentiation of the bone-secreting cells within the blastema during fin outgrowth and is supported by the observation that treatment with cyclopamine results in an early expansion, then later reduction of *shh* expression once fin outgrowth has ceased. shh and bmp2b signaling may play a fundamental role in defining the region of bone deposition in the regenerating fin, possibly by influencing the differentiation of the bone-secreting scleroblasts. However, shh signaling is not required for the secretion of bone matrix by already differentiated scleroblasts.

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Chapter 3: Manuscript 2

Summary:

In the first study, we found that ectopic expression of *shh* or *bmp2b* in the blastema between bifurcating sister rays, where these genes are not normally expressed, resulted in ectopic bone deposition in an area that is normally devoid of bone. Interestingly, this ectopic bone was located in a region competent to produce scleroblasts. These results suggested a role for the Shh signaling pathway in scleroblast differentiation and/or function. We also found that co-injecting *shh* and *chordin*, a Bmp signaling inhibitor, blocked the formation of ectopic bone, suggesting that a Bmp factor does in fact act downstream of Shh signaling in dermal bone regeneration in the fin regenerate. Therefore to investigate further the function of the Bmp signaling pathway in dermal bone regeneration, we inhibited endogenous Bmp signaling within the regenerate and examined the effects on: blastemal proliferation, bone calcification in the regenerating fin rays, scleroblast differentiation and function, and bone morphology. In addition, based on the known targets of Bmp signaling during bone development in other organisms, we set out to characterize members of the Bmp signaling pathway that are active in dermal bone regeneration in the zebrafish fin rays.

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Inhibition of BMP signaling during zebrafish fin regeneration disrupts fin growth and scleroblast differentiation and function.

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Contribution of Authors

Amanda Smith: I was responsible for all of the experiments and the writing of the manuscript with technical assistance from Danielle Guay and writing assistance from Marie-Andree Akimenko.

Fabien Avaron: Isolated *ihha* gene, provided useful discussions and technical advice

Danielle Guay: Responsible for RT-PCR on larvae tails and fin regenerates (supplementary figure 3.2)

Bhaja Padhi: Isolated and characterized *col10a1* cDNA

Marie-Andree Akimenko: Supervised project and participated in large portion of writing of manuscript

Abstract

The zebrafish caudal fin provides a simple model to study molecular mechanisms of dermal bone regeneration. We previously showed that misexpression of Bone morphogenetic protein 2b (Bmp2b) induces ectopic bone formation within the regenerate. Here we show that in addition to *bmp2b* and *bmp4* another family member, *bmp6*, is involved in fin regeneration. We further investigated the function of BMP signaling by ectopically expressing the BMP signaling inhibitor Chordin which caused: (1) inhibition of regenerate outgrowth due to a decrease of blastema cell proliferation and downregulation of *msxb* and *msxC* expression and (2) reduced bone matrix deposition resulting from a defect in the maturation and function of bone-secreting cells. We then identified targets of BMP signaling involved in regeneration of the bone of the fin rays. *runx2a/b* and their target *col10a1* were downregulated following BMP signaling inhibition. Unexpectedly, the *sox9a/b* transcription factors responsible for chondrocyte differentiation were detected in the non-cartilaginous fin rays, *sox9a* and *sox9b* were not only differentially expressed but also differentially regulated since *sox9a*, but not *sox9b*, was downregulated in absence of BMP signaling. Finally, this analysis revealed the surprising finding of the expression, in the fin regenerate, of several factors which are normally the signatures of chondrogenic elements during endochondral bone formation although fin rays form through dermal ossification, without a cartilage intermediate.

Key words: BMP signaling; Zebrafish, Fin regeneration; Chordin; Runx2; Col10a1; Sox9; Col2a1; Intramembranous bone.

Introduction

Teleosts, including zebrafish, are among the very few vertebrate organisms that possess the ability to regenerate many organs and tissues following amputation or injury during adulthood (for reviews, see (Akimenko *et al.* 2003; Poss *et al.* 2003). The relative simplicity and the easy access of the caudal fin and of its skeletal elements make it an excellent system to dissect the molecular pathways involved in bone regeneration (Akimenko *et al.* 2003; Poss *et al.* 2003). The skeletal elements of the ray, called lepidotrichia, are acellular and mineralize directly from bone matrix in a manner that is equivalent to intramembranous bone formation (Geraudie *et al.* 1982; Santamaria *et al.* 1992). On the other hand, the fin endoskeleton, which is the support of the fin rays and is located at the base of the fin, forms by endochondral ossification and has a cartilaginous origin (Grandel *et al.* 1998; Crotwell *et al.* 2004). While resection of the fin at the level of the endoskeleton is not followed by regeneration, amputation of the dermal fin rays results in complete replacement of the lost structures within approximately 3 weeks (Mari-Beffa *et al.* 1996; Akimenko *et al.* 2003). Ray regeneration is possible because of the formation, shortly after amputation, of a mass of undifferentiated and proliferating cells called a blastema, distal to the stump of each ray (Becerra *et al.* 1996; Poleo *et al.* 2001; Akimenko *et al.* 2003; Poss *et al.* 2003). This blastema proliferates beneath the wound epidermis which is composed of a well defined basal layer of cuboidal epithelial cells covered by a multilayered epidermis (Becerra *et al.* 1996; Akimenko *et al.* 2003). As blastemal cells leave this area, they differentiate to give rise to all the cell types involved in the regeneration of connective and skeletal tissues. The differentiation program leading to bone regeneration begins with the differentiation into scleroblasts of

blastemal cells in contact with the epidermal layer. These cells then synthesize and release the dermal bone matrix in the subepidermal space. As the dermal bone matrix accumulates, it will progressively mineralize, beginning in the proximal (more mature) region of the regenerate (Geraudie *et al.* 1982; Becerra *et al.* 1996; Mari-Beffa *et al.* 1996; Akimenko *et al.* 2003). To date, little is known about the signals that govern bone regeneration within the fin but likely candidates are members of the bone morphogenetic family of proteins (BMP).

BMPs are secreted growth factors whose signaling regulates many aspects of skeletal development and play important roles in embryonic growth and patterning in both vertebrates and invertebrates (Hogan 1996; Graff 1997; Balemans *et al.* 2002; Wan *et al.* 2005). Bmp2, 4 and 6 are the members of the BMP family thought to play the most important roles in skeletogenesis. They are involved in differentiation of chondrocytes to form cartilage (Minina *et al.* 2001; Yoon *et al.* 2004), and both differentiation and maturation of cells of osteoblastic lineages which will give rise to bone (Yamaguchi *et al.* 1991; Thies *et al.* 1992; Rickard *et al.* 1994; Canalis *et al.* 2003; Wan *et al.* 2005). Bmp2 is considered one of the most potent extracellular signaling molecules that stimulates bone formation and osteoblast differentiation (Choi *et al.* 2005). Upon short exposure to Bmp2, mouse C2C12 cells become committed to the osteoblast lineage (de Jong *et al.* 2004). Bmp6 is abundantly expressed in hypertrophic chondrocytes (Iwasaki *et al.* 1997; Inada *et al.* 1999) and stimulates chondrogenic and osteogenic phenotypes in vitro (Ebisawa *et al.* 1999; Grimsrud *et al.* 1999; Boskey *et al.* 2002; Kugimiya *et al.* 2005). In vivo studies show that while $Bmp2^{+/-}$ mice and $Bmp6^{-/-}$ mice present no skeletal

abnormality, compound deficient mice ($Bmp2^{+/-}$; $Bmp6^{-/-}$) exhibit mild growth retardation and trabecular bone deficit due to impaired osteoblast differentiation or function. These results suggest that, in vivo, Bmp2 and Bmp6 cooperate to regulate bone formation (Ebisawa *et al.* 1999; Grimsrud *et al.* 1999; Boskey *et al.* 2002; Kugimiya *et al.* 2005). Besides the obvious role of BMPs in skeletal development, they also regulate a wide range of cellular processes including proliferation, differentiation, motility, adhesion, cell death and specification of developmental cell fate during embryogenesis (Hogan 1996; Massague 2000; Wozney 2002; Yamamoto *et al.* 2004). BMPs have recently been linked to regeneration in both the larval tail of *Xenopus laevis* (Koshiba *et al.* 1998; Slack *et al.* 2004; Sugiura *et al.* 2004; Satoh *et al.* 2005; Beck *et al.* 2006) and the digit tip of fetal mice (Han *et al.* 2003, 2005).

Previous analyses have shown that at least two members of the *bmp* gene family, *bmp2b* and *bmp4*, are expressed during regeneration of the zebrafish caudal fin (Laforest *et al.* 1998; Murciano *et al.* 2002). *bmp4* expression is restricted to the distal blastema indicating a possible role in controlling blastemal cell proliferation and regenerate outgrowth (Murciano *et al.* 2002). *bmp2b*, on the other hand, is expressed in the newly differentiated scleroblasts located at the level of the proximal blastema as well as in the adjacent cells of the basal epidermis, suggesting a role in bone formation (Laforest *et al.* 1998). This hypothesis was supported by the fact that ectopic expression of Bmp2b between regenerating fin rays can induce ectopic bone matrix deposition leading to fusion of the bones of sister rays (Quint *et al.* 2002). Furthermore, ectopic bone was only observed in the interface between the basal epidermal cells and the blastema, suggesting

that epithelial-mesenchymal interactions are probably involved in the differentiation of the blastema cells into scleroblasts and/or the correct patterning of the bone. Such interactions are likely to involve the BMP and Hedgehog signaling pathways as gene expression analyses first suggested. Two members of the Hedgehog family are expressed in complementary domains which overlap with the *bmp2b* expression domain: *sonic hedgehog* (*shh*) transcripts are found in the basal epidermal cells and one of the zebrafish orthologues of the *indian hedgehog* gene (*ihha*) is expressed in the scleroblasts while *patched 1* (*ptc-1*), the receptor of hedgehog signals, is expressed in a pattern very similar to that of *bmp2b* (Laforest *et al.* 1998; Avaron *et al.* 2006).

In this report, we further investigated the role of BMP signaling during fin regeneration. We isolated a cDNA fragment of the zebrafish *bmp6* gene, a yet uncharacterized member of the BMP family in zebrafish, and showed that its expression pattern suggests a role in fin growth and bone formation. We then inhibited BMP signaling by the ectopic expression of Chordin in the ray blastema. Chordin, through its binding to BMP factors including Bmp2/4/5/6/7, inhibits BMPs' interactions with their receptors and thus inhibits BMP signaling (Piccolo *et al.* 1996; Bachiller *et al.* 2003; Nakayama *et al.* 2004). We found that Chordin misexpression induces a transient arrest of fin outgrowth. This effect, which is correlated with a drastic decrease in blastema cell proliferation, is probably caused by the inhibition of Bmp4 and possibly Bmp6 signaling in the distal blastema cell population. In addition, Chordin misexpression induces a delay in bone matrix deposition and scleroblast function as indicated by a delay in both ray mineralization and bone thickening and is most likely due to the inhibition of Bmp2b and Bmp6 signaling.

The observed bone defects led us to examine the known downstream targets of BMP signaling involved in cartilage and bone formation in other species. We first demonstrated that many of these factors were expressed in cells involved in bone formation. We found that, in the fin regenerate, the major osteogenic transcription factors Runx2a and Runx2b are under the control of BMP signaling. More unexpectedly since fin rays are described as dermal bones, we found that the transcription factors associated to cartilage formation, *sox9a* and *sox9b,* are differentially expressed within the fin regenerate, and *sox9a*, but not *sox9b*, expression, depends on BMP signaling. The expression of *sox9* genes, and other chondrocyte specific factors in higher vertebrates, including the signaling molecule Ihha and the extracellular matrix molecules Col10a1 (Padhi *et al.* 2004; Avaron *et al.* 2006) and Col2a1 (this study and (Johnson *et al.* 1995), would not be a surprise in chondrocytes leading to endochondral bone formation but the fin rays are described as dermal bones. Altogether, this study provides evidence that within the fin regenerate BMP signaling regulates genes associated with both bone and cartilage formation.

Materials and Methods

Animals

Zebrafish were obtained from our animal colony and maintained at 28.5°C. All experiments were performed in adult fish (6 to 12 months old).

Fin Amputation

Adult fish were anaesthetized using 0.6M tricaine, and caudal fins were amputated one or two segments proximal to the origin of bifurcation.

Plasmid constructs

The Chordin-expressing construct (kindly provided by M. Halpern, Carnegie Institute of Washington, Baltimore) is a 2.5 kb fragment encoding the zebrafish *chordin* cDNA inserted into the pCS2+ expression vector containing the CMV promoter (Miller-Bertoglio *et al.* 1997). The pEGFP-N1 construct (Clontech) (from here referred to as enhanced green fluorescent protein, EGFP) was also under the control of the CMV promoter.

Construct injections

500 ng of DNA of the Chordin- and of the EGFP-expressing plasmid constructs was injected into each blastema of the regenerating rays of the ventral half and dorsal half of the caudal fin, respectively. Fins were injected at 3 days post amputation (dpa) and allowed to regenerate for 1-9 days depending on the experiment. For statistical analysis, the length of the regenerating rays was measured before injection (3 dpa) and the days following injection using Northern Eclipse software. Each individual ray was measured from the level of amputation to the distal end of the regenerate. The length of each ray was compared to the length of the corresponding ray on the opposite side. Rays on both sides were numbered from 0 to 6, ray 0 being the outermost non-bifurcating ray (Fig. 3.1E). The six central-most and smallest fin rays (rays number 7 to 9) were not included

in this analysis. Injection of *EGFP* was used as a control and allowed us to test the injection efficiency by visualizing the cells expressing the injected DNA (Fig. 3.1D).

Analysis of the effect of Chordin expression on ossification was performed on regenerates at 6 days post injection (dpi). The regenerates were fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4°C, then rinsed with PBS and stained with 0.01% Alcian blue in 70% ethanol and 30% acetic acid for 6 h at room temperature. Following staining, fins were dehydrated in an ethanol series and stored in 100% ethanol overnight. The fins were rehydrated, stained in 0.1% Alizarin red solution with 0.5% KOH for 4-5 h at room temperature and rinsed in water overnight.

Gene expression analyses on *chordin* and *EGFP*-injected fin rays were performed at 1 or 2 dpi. Fin regenerates were processed for whole mount *in situ* hybridization or sectioned (16 μm) using standard cryosectioning procedures (Quint *et al.* 2002) and processed for *in situ* hybridization or immunostaining.

In situ hybridization

In situ hybridization on whole-mount fins (Akimenko *et al.* 1995; Jowett 1997) and *in situ* on cryosections (Quint *et al.* 2002) were performed as previously described. Digoxigenin labeled antisense riboprobes were generated using the following cDNAs: *msxb* (1.12kb) (Akimenko *et al.* 1995), *chordin* (2.5kb) (Fisher *et al.* 1999), *shh* (2.5kb) (Roelink *et al.* 1994), *ihha* (890bp) (Avaron *et al.* 2006) and *col2a1* (2.5kb) (Johnson *et al.* 1995). *runx2a* (NM_212858) and *runx2b* coding sequences (NM_212862) were

obtained by RT-PCR from cDNA of 4 dpa fin regenerate using the following primers: *runx2a* forward 5'CCACGCCGAACTCCTTCAATC3', *runx2a* reverse 5'TCAATATGGCCGCCACACGGA3', *runx2b* forward 5'ATGCGCATTCCCGTAGATCC3' and *runx2b* reverse 5'TCAATACGGCCTCCAAACGCC3'. The probes synthesized from the *runx2a* and *runx2b* cDNAs were 700bp and 1.3 kb long, respectively. The *col10a1* probe (713 bp) was made from the zebrafish 1-A06 clone (Padhi *et al.* 2004). The *sox9a* (758bp) and *sox9b* (1.27kb) cDNAs were isolated from 4 dpa fin regenerate using previously published primers (Chiang *et al.* 2001). The *bmp6* cDNA fragment (705 bp) was obtained by RT-PCR from cDNA of 4 dpa fin regenerates using the following primers designed from the zebrafish EST fw719d09: *bmp6* forward 5'GCACCTGAAGGCTACGCAGCA3' and *bmp6* reverse 5'ACAACGCTAGCATCTGCTAGC3'.

BrdU analysis

Following injection at 3 dpa, fish were placed in fish system water containing 50 μg/ml BrdU (5-bromo-2'-deoxy-uridine, Roche 280879) for 16 h. Regenerates were collected and fixed in 4% PFA in PBS overnight at 4°C, rinsed in PBS and processed for cryosectioning (16 μm). Proliferating cells on cryosections were immunodetected as described in Poleo *et al.*, 2001 using a 1:250 dilution of the mouse anti-BrdU monoclonal antibody (Sigma), then processed as described below.

Immunodetection

Detection of differentiated scleroblasts on fin sections was performed using the Zns-5 monoclonal antibody (dilution 1:200; Zebrafish Resource Centre, Eugene Oregon). Binding was revealed using a biotinylated anti-mouse IgG secondary antibody (dilution 1:150). Zns-5 antibody detection was visualized with a Peroxidase ABC-Vectastain kit (Vector laboratories, PK4001).

Results

bmp6 expression suggests a role in growth of the regenerate and bone formation

Due to its known role in both osteoblast differentiation and function, we characterized a cDNA fragment of the zebrafish *bmp6* gene and examined its expression during fin regeneration. We identified an EST assembly (wz42215.1) of 762 bp from the Washington University zebrafish EST database **(http://fisher.wustl.edu/fish_lab/cgi-bin/search.cgi)** whose sequence presents a strong similarity with the mouse and human BMP6 proteins (98% / 93% similarity/identity with the corresponding mouse and human BMP6 fragments). We designed primers from the EST assembly wz42215.1 to amplify a 705 bp fragment by RT-PCR on RNA prepared from 4 days post amputation (dpa) fin regenerates. This fragment was used to synthesize an antisense RNA probe to compare *bmp2b*, *bmp4* and *bmp6* expression during the outgrowth phase of fin regeneration. At 4 dpa, *bmp6* transcripts are detected in the proliferating blastema, the basal epidermal layer, the newly differentiating scleroblasts adjacent to the basal epidermal layer and more medial cells surrounding the actinotrichia (Fig. 3.1A). The expression domain of *bmp6* is much broader than that of *bmp4* in the blastema (Fig. 3.1B and Murciano *et al.* 2002) and

extends more proximally in the differentiated scleroblasts and basal epidermal layer than does *bmp2b* (Fig. 3.1C and Laforest *et al.* 1998).

Misexpression of Chordin inhibits outgrowth of the regenerating fin rays

To test the effects of BMP signaling inhibition on fin regeneration, ray blastemas of the caudal fin were injected at 3 dpa with either *chordin* or *EGFP* plasmid constructs (Figs. 3.1D, E). *chordin* endogenous expression is undetectable by *in situ* hybridization in fin regenerates between 3 and 6 dpa (data not shown). We chose to perform injections at 3 dpa, a time point corresponding to the onset of *bmp2b* and *bmp6* expression in the differentiating scleroblasts and the basal layer of the epidermis. We previously showed that microinjection of plasmid constructs in the fin regenerate was efficient in transfecting blastema cells (Fig. 3.1D), and had no effect, by itself, on the normal regeneration process (Quint *et al.* 2002). Each ray was measured immediately before injection to ensure that there was no significant length difference between dorsal and ventral rays (Fig. 3.2A). Fin rays were then individually measured 1, 2 and 3 days following injection. Injection of the *chordin* construct in the blastemas of the ventral rays resulted in a drastic reduction in ray length compared to *EGF*-injected rays in the dorsal half of the same fin 1 dpi (Fig. 3.2B). The length difference was most noticeable in the outermost rays, likely because these rays grow at a faster rate than the inner rays. The percentage reduction in length of rays 1-6 (see Fig. 3.1E for ray numbering) was calculated comparing the *chordin* to the *EGFP*-injected halves. At 4 dpa there was a 33% reduction in length between ray 1 of the *chordin*-injected side and the corresponding

ray 1 on the *EGFP*-injected side ($P < 0.0001$, $n = 73$) (Fig. 3.2G). The percentage difference decreased

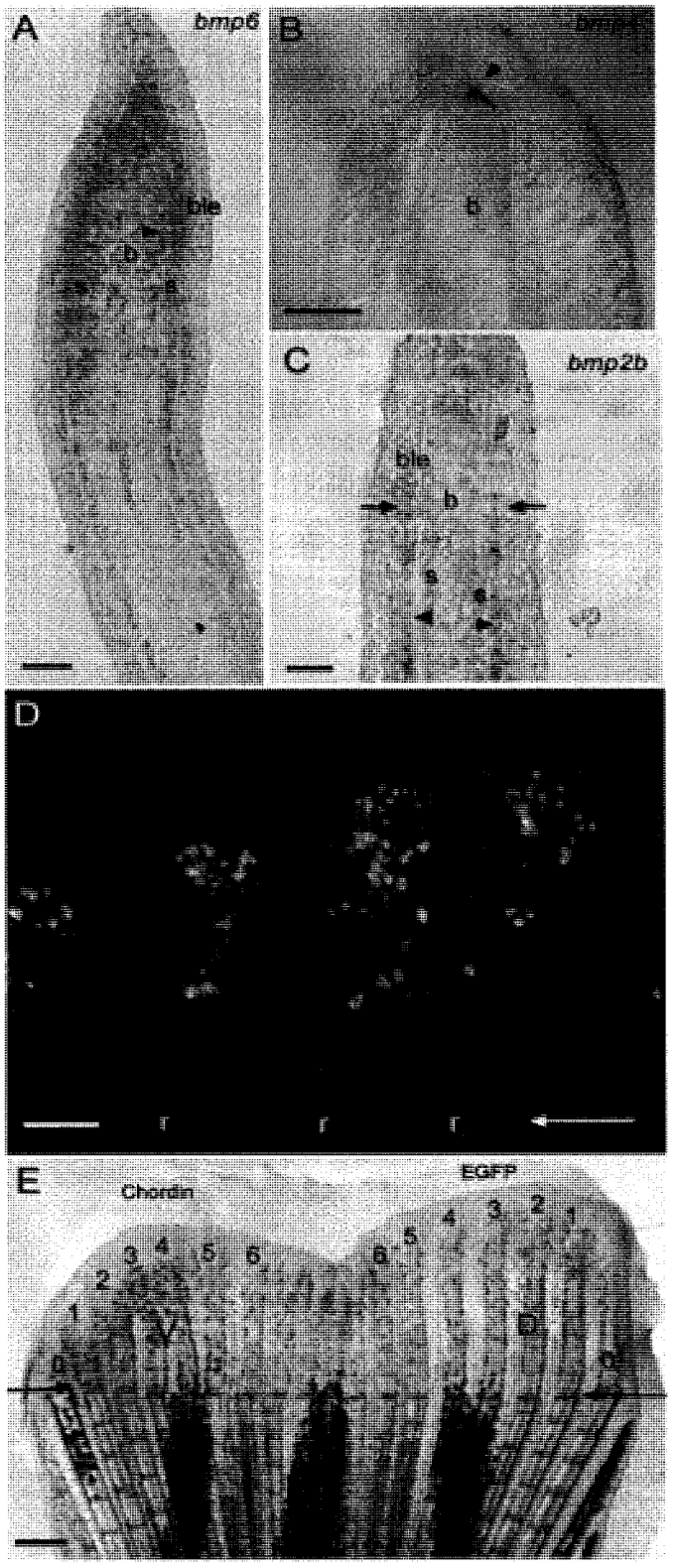


Figure 3.1

Figure 3.1: Expression of *bmp2, 4* and *6* on longitudinal sections of 4 dpa fin regenerates using *in situ* hybridization.

(A) *bmp6* is expressed in the scleroblasts, cells medial to the actinotrichia (arrowhead), cells of the basal layer of the epidermis in the distal part of the regenerate and the proliferating blastema. (B) *bmp4* expression is restricted to the distal blastema (arrowhead). (C) *bmp2b* expression is found in the newly differentiating scleroblasts and in the adjacent cells of the basal layer of the epidermis (arrow). (D) EGFP expression in 20 to 50 mesenchymal cells 1 day after in vivo transfection by microinjection of the CMV-EGFP reporter construct into the blastema of three fin rays at 3 dpa. (E) Zebrafish caudal fin 3 days following injection of the CMV-EGFP construct in the blastema of rays 1 to 6 of the dorsal half of the fin and of the CMV-*chordin* construct in the ventral (V) rays (1-6). Outermost non-bifurcating rays (rays 0) were not counted. Arrows in panels D and E indicate the level of amputation. D, dorsal; V, ventral. 1-6 represents ray number. r, fin ray; s, scleroblasts; b, blastema; ble, basal layer of the epidermis. Scale bars: (A-C) = 25 μm, (D) = 100 μm, (E) = 400 μm.

in more inner rays but was significant in rays 2-5 [ray 2: 22% reduction ($P < 0.0001$, $n = 75$), ray 3: 15% reduction ($P < 0.0001$, $n = 73$), ray 4: 11% reduction ($P < 0.0001$, $n = 76$), ray 5: 6% reduction ($P < 0.0001$, $n = 76$)], ray 6 showed no significant difference in length. Length difference remained significant at 5 and 6 dpa (Fig. 3.2H and data not shown). However, inhibition of BMP signaling following *chordin* injection was transient and by 7 and 8 dpa the length difference between dorsal and ventral rays became non-significant (data not shown). Following a double injection of *chordin* at 3dpa and 5dpa, a more pronounced reduction in ray length was observed 1, 2 and 3 days after the second injection (Figs. 3.2D-F). These results indicate that inhibition of BMP signaling at 3 dpa has a drastic effect on regenerate outgrowth. A control group of fish, injected with the *EGFP* construct in all rays, did not show any significant length difference between the dorsal and ventral rays at 1 dpi (Fig. 3.2C) and at 4, 5 and 6 dpi (red bars Figs. 3.2G, H, $n = 6$). Therefore, the delay in ray growth seen following *chordin* injection is a result of BMP signaling inhibition and is not due to a difference in the regeneration rate between the ventral and dorsal rays.

chordin injection results in a decrease in proliferation of blastema cells, correlating with a downregulation of _msxB_ expression

The lack of outgrowth observed following Chordin misexpression could be due to an inhibition of Bmp4 and Bmp6 signaling in the distal blastema. To examine cell proliferation, fish were treated with BrdU for 16 h starting at 3 dpa, immediately following plasmid injection. BrdU incorporation into the DNA of proliferating cells was then detected by immunostaining. Rays injected with *chordin* showed a significant

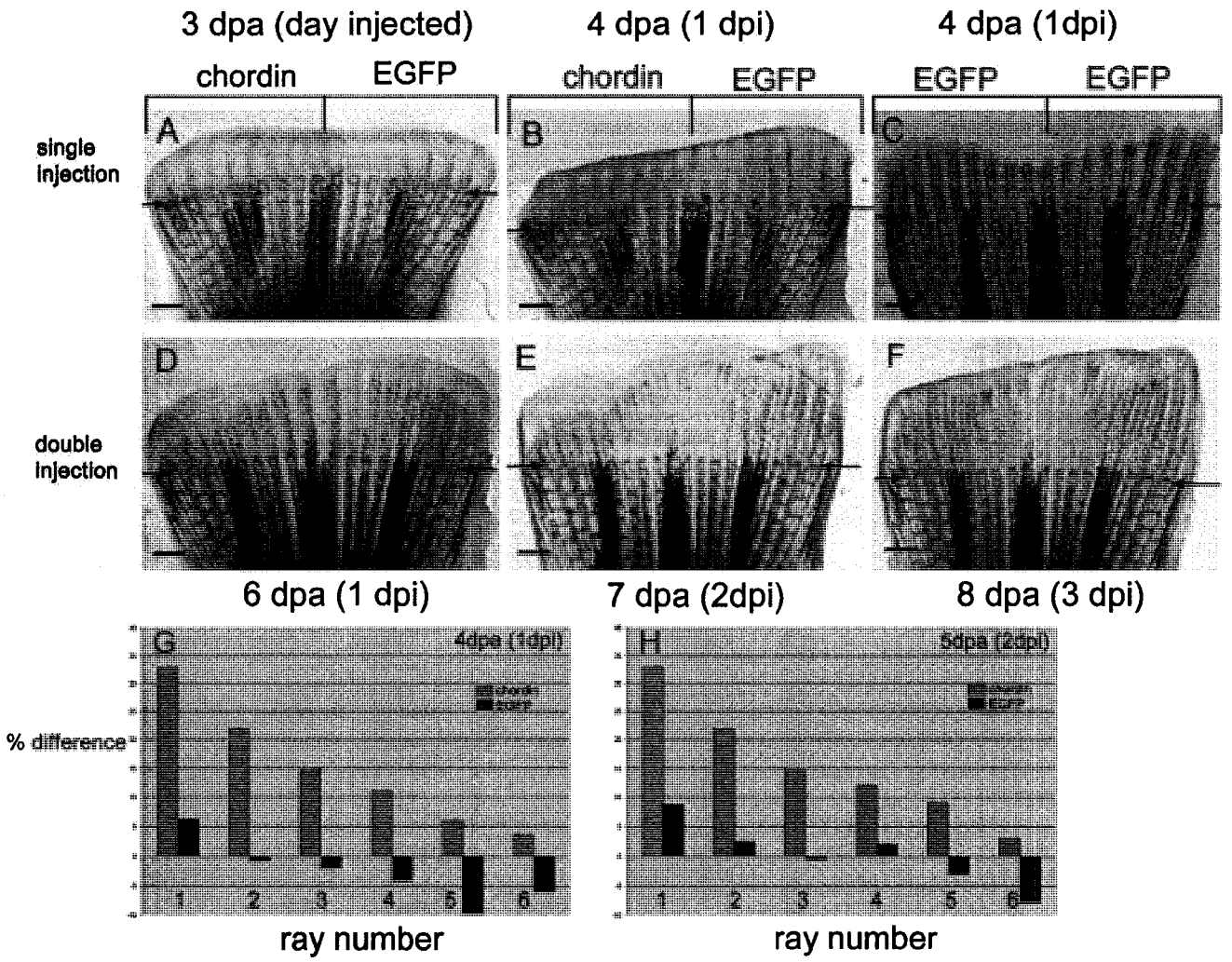

3 dpa (day injected)
4 dpa (1 dpi)
4 dpa (1dpi)
chordin
EGFP
chordin
EGFP
EGFP
EGFP
single injection
A
B
C
double injection
D
E
F
6 dpa (1 dpi)
7 dpa (2dpi)
8 dpa (3 dpi)
% difference
G
4dpa (1dpi)
H
5dpa (2dpi)
1
2
3
4
5
6
ray number
ray number

Figure 3.2

Figure 3.2: Effects of *chordin* injection on ray length.

(A) Regenerating fin at 3 dpa immediately before injection showing that the lengths of the regenerating rays on the dorsal and ventral sides are the same. (B) One day post injection (1 dpi) of *chordin* (ventral side, left side of the panel) and *EGFP* (dorsal side, right side of the panel) constructs at 4 dpa, a size difference between the two sides is noticeable. (C) Control fin 1 day following injection of *EGFP* construct in all the fin rays shows no size difference between the ventral and dorsal sides. (D-F) Pictures showing the same fin at different time points following injections at 3 dpa and 5 dpa (D: 6 dpa/1 dpi, E: 7 dpa/2 dpi and F: 8 dpa/3 dpi). A pronounced length difference of the ventral versus dorsal fin rays is observed. (G, H) Percentage length difference of dorsal *EGFP*-injected rays compared to ventral *chordin*-injected rays was calculated and plotted for rays 1-6 at 1 dpi (G) and 2 dpi (H) following a single injection of *chordin* (blue bars). The most noticeable differences were observed in the outer rays. A control group of fish was injected with *EGFP* in all dorsal and ventral rays and plotted on the same graph to show no significant difference in length between dorsal and ventral rays (red bars). Arrows indicate level of amputation. Scale bars: (A-F) = 400 μm.

decrease in cells labeled with BrdU within the blastema compared to *EGFP*-injected rays (Figs. 3.3A-C). The mean number of proliferating cells detected in sections of *EGFP*-injected rays (n = 2 rays, 9 sections) was 98.1 compared to the *chordin*-injected rays (n = 4 rays, 20 sections) which had a mean of 19.4 labeled cells within the blastema 2 days following injection (Fig. 3.3C, $P < 0.0001$). Bmp4 is known in other systems to induce or upregulate the expression of the Msx1 homeodomain transcriptional repressor, which is thought to maintain cells in an undifferentiated state (Song *et al.* 1992; Watanabe *et al.* 1996; Hu *et al.* 2001). In zebrafish, both *msxB* and *msxC* are expressed in the distal blastema of the fin regenerate (Akimenko *et al.* 1995) and it has been proposed that *msxb*-expressing cells specify the boundary of cell proliferation and provide direction for regenerative outgrowth (Nechiporuk *et al.* 2002). *msxB* and *msxC* expression was found to be significantly decreased or completely downregulated in *chordin*-injected rays compared to *EGFP*-injected rays (Figs. 3.3D, E and Supplemental Fig. 3.1, n = 9 rays). These results suggest that inhibition of BMP signaling causes a downregulation of *msx* gene expression which may lead to a corresponding decrease in blastema cell proliferation and fin outgrowth.

Inhibition of BMP signaling results in a delay in bone formation and scleroblast maturation or function.

Staining with Alizarin red 6 dpi showed that Chordin misexpression starting at 3 dpa resulted in the eventual reduction in bone matrix mineralization in all rays examined (1-6), with a most noticeable effect on the outermost three rays (Fig. 3.4A). The effects of Chordin misexpression on the differentiation or function of the scleroblasts were

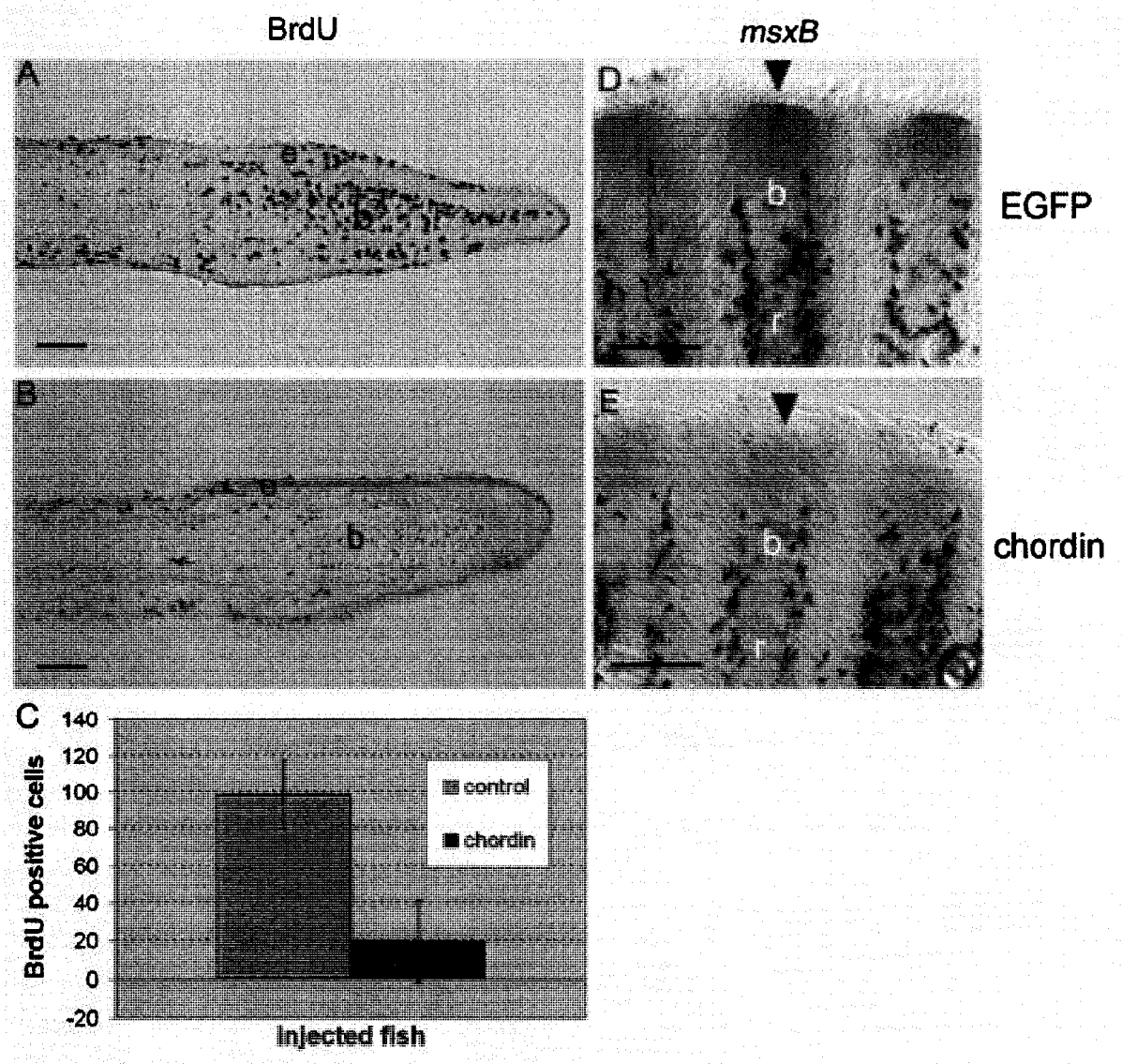
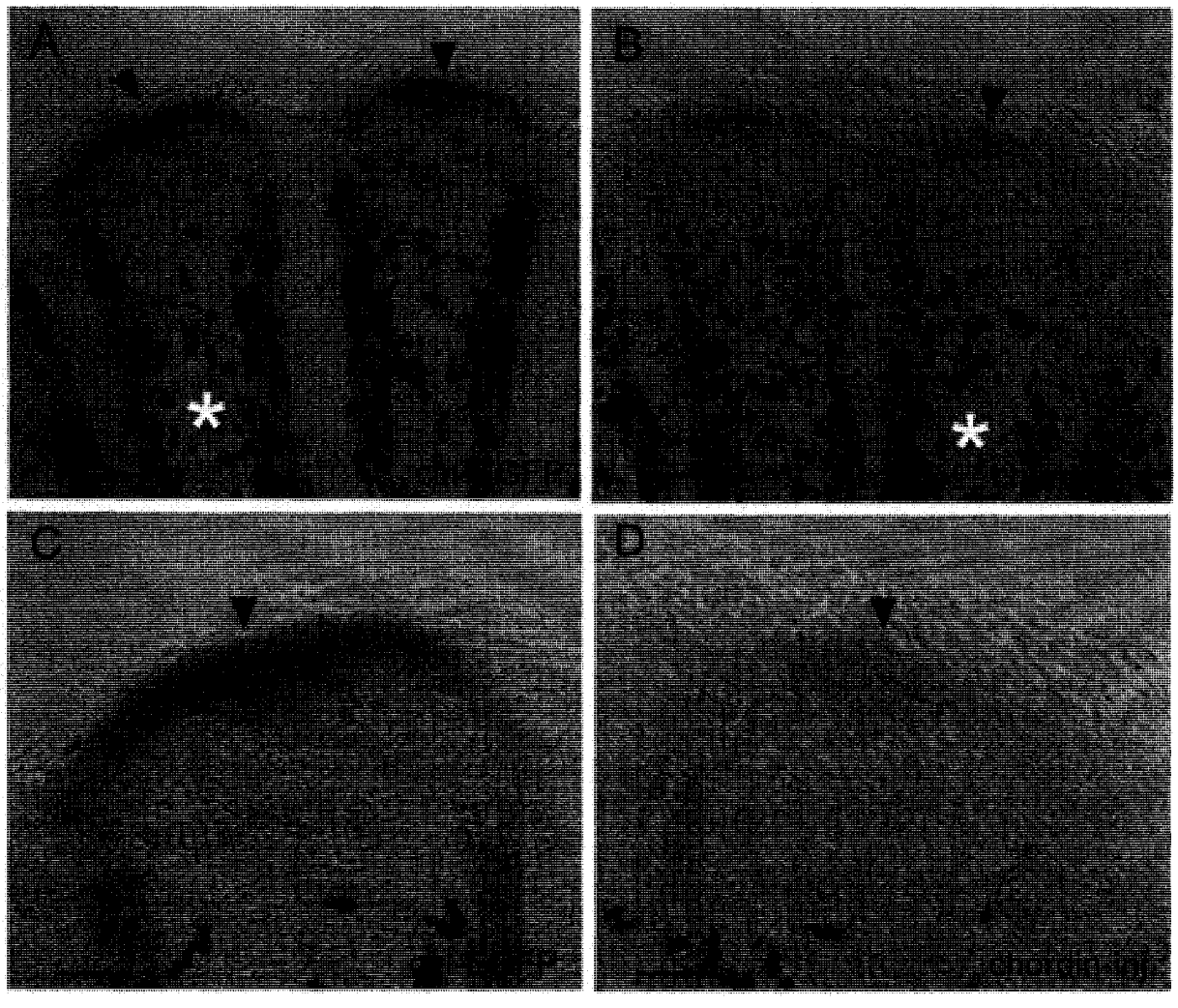

BrdU
msxB
A
D
EGFP
B
E
chordin
C
BrdU positive cells
140
120
100
80
60
40
20
0
-20
control
chordin
Injected fish

Figure 3.3

Figure 3.3: Reduced cell proliferation and *msxB* expression following *chordin* construct injection

(A-B) Immunodetection of BrdU incorporation using an anti-BrdU antibody on longitudinal sections of fin rays at 4 dpa, 1 day after *EGFP* (A) and *chordin* (B) injections. Fish were treated with BrdU for 16 h starting immediately after injection. (C) For statistical purposes BrdU labeled cells were counted on 20 sections of *chordin*-injected fin rays (n = 4 rays) and 9 sections of *EGFP* injected rays (n=2 rays) and the mean number of BrdU positive cells was calculated for both groups and plotted on a graph (Chordin = 19.4 cells, EGFP = 98.1 cells). Student's t-test results indicate that injection of *chordin* significantly decreased the number of proliferating cells within the blastema ($P < 0.0001$). (D-E) *msxB* gene expression on whole-mount fins at 4 dpa, 1 day after *EGFP* (D) and *chordin* (E) injections. *msxB* expression in the distal blastema (D, arrowhead) is strongly reduced in *chordin*-injected rays (E, arrowhead). b, blastema, e: epidermal layer, r: fin ray. Distal part of the regenerate is to the right (A, B) and to the top (D,E). Scale bars: (A, B) = 25 μm, (C, D) = 100 μm.



Supplementary Figure 3.1

Supplementary Figure 3.1: Reduced *msxc* expression following chordin construct injections

Expression of *msxC* at 4 dpa using *in situ* hybridization on whole mount fins one day after injection of *EGFP* (A, C) and *chordin* (B ,D). *msxC* expression seen in the distal blastema of 2 fin rays (A) and on a higher magnification image of the domain of expression in the blastema of one ray indicated by an asterisk in (C), one day following *EGFP* injection. The expression is strongly reduced in the distal blastema one day following *chordin* injection as seen on 2 fin rays (B) and at a higher magnification of the distal blastema of the ray indicated by the asterisk in (D). Scale bars: (A, B) = 20 μM; (C, D) = 40 μm.

examined using the Zns-5 antibody, a marker for all stages of scleroblast differentiation (Johnson *et al.* 1995). As shown by Zns-5 expression in *EGFP*-injected rays, newly differentiated scleroblasts are localized along the border of the blastema in the distal part of the regenerate (Figs. 3.4B, E, arrowheads in B). Bone matrix is deposited at the interface between these new scleroblasts and the epidermis. As the regenerate matures, i.e. in more proximal regions of the regenerate, more mature scleroblasts are found on both internal (Figs. 3.4B, D (red arrows)) and external (Figs. 3.4B, D (black arrows)) surfaces of the bone, due to the migration of scleroblasts around the initial bone template (Santamaria *et al.* 1992). Bone deposition by scleroblasts surrounding the bone template accelerates bone thickening. When compared to the *EGFP*-injected rays (Figs. 3.4B, D (black arrow), n = 4 rays) *chordin*-injected rays showed a decreased number of scleroblasts on the external surface of the bone in the proximal part of the regenerate (Figs. 3.4C, F (black arrow), n = 4 rays). As a consequence of this decreased number of scleroblasts, the bone in this region is less thick than in the *EGFP*-injected rays (Figs. 3.4D, F; n = 4 rays; (I) indicates bone thickness). In other organisms, osteoblast maturation is accompanied by a decrease in cell body size; it is estimated that osteoblasts lose approximately 70% of their cell body volume as they mature (Franz-Odendaal *et al.* 2006). Similarly, in the control *EGFP*-injected rays, scleroblasts in the more mature, proximal part of the regenerate have a more flattened appearance compared to the newly differentiating scleroblasts of the distal regenerate (Figs. 3.4D, E). In contrast, in the *chordin*-injected rays, the scleroblasts on the internal surface in the proximal part of the regenerate keep a round shape (Fig. 3.4F) similar to the newly differentiating scleroblasts located in the distal part of the regenerate (Fig. 3.4G). These results suggest that BMP

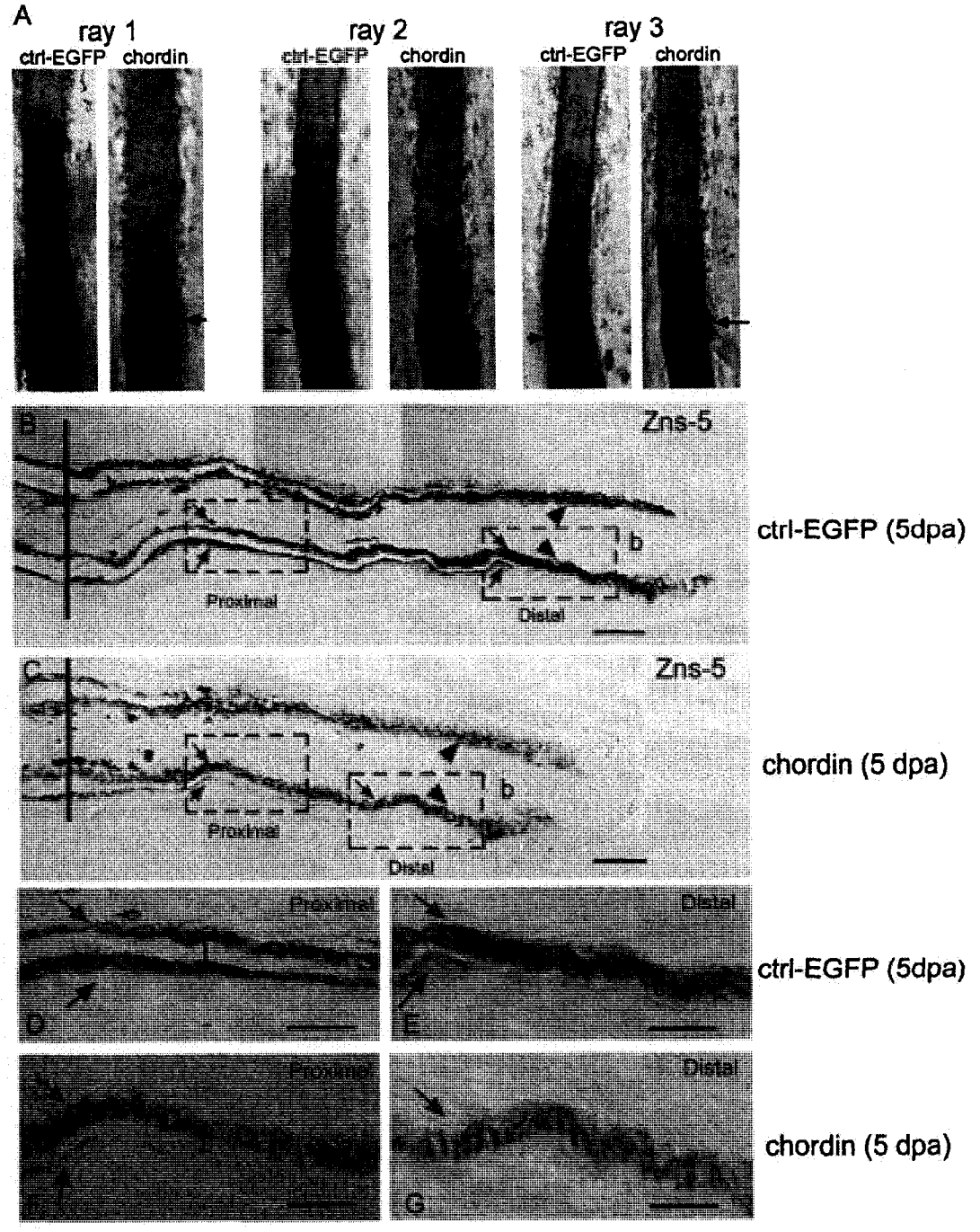

A
ray 1
ctrl-EGFP
chordin
ray 2
ctrl-EGFP
chordin
ray 3
ctrl-EGFP
chordin
B
Znc-5
Proximal
Distal
b
ctrl-EGFP (5dpa)
C
Znc-5
Proximal
Distal
b
chordin (5 dpa)
D
Proximal
E
Distal
ctrl-EGFP (5dpa)
F
Proximal
G
Distal
chordin (5 dpa)

Figure 3.4

Figure 3.4: Morphology of the fin rays following *chordin* injection.

(A) Comparison of the ossification progression using Alizarin red staining in rays 1, 2 and 3 of the *EGFP* and *chordin*-injected lobes at (9 dpa/6 dpi) shows a reduction of matrix mineralization in *chordin*-injected rays. (B-G) Immunodetection of the scleroblasts using the Zns-5 scleroblast-specific antibody on longitudinal sections of fin regenerates at 5 dpa, 2 days after injection of *EGFP* (B,D,E), and of *chordin* (C,F,G). (B) in the control rays at 5 dpa, newly differentiating scleroblasts (arrowheads) in the distal region (dashed box on the right side of the panel) are located on the internal surface of the bone matrix while more mature bone secreting cells are found on the internal (red arrow) and external (black arrows) surfaces of the proximal lepidotrichia (dashed box on the left side of the panel). (C) *chordin*-injected regenerate at 5 dpa shows fewer mature scleroblasts on the external surface of the proximal lepidotrichia (black arrow) and shorter in length compared to the control regenerate. (D-G) Higher magnification of the dashed boxes shown in panels B and C; (D, E) proximal and distal regions, respectively, of the control *EGFP*-injected rays and (F, G) proximal and distal regions, respectively, of the *chordin*-injected rays. Note the reduced number of mature scleroblasts on the external surface of the proximal part of the lepidotrichia (F) when compared to the control ray (D); the flattened shape of the scleroblasts on the internal and external surfaces of the lepidotrichia only observed in the proximal part of the regenerate of the control *EGFP*-injected rays; the reduced bone thickness observed in the proximal part of the regenerate of *chordin*-injected rays (F) when compared to the corresponding region of the control ray (D) as indicated by the bars [I]; and the absence of bone matrix in the distal part of the *chordin*-injected rays (G) compared to the corresponding region in the control ray (E). Arrows in panel A and solid lines in B and C indicate the level of amputation. Arrowheads in panels B and C indicate newly differentiating scleroblasts. L, lepidotrichia; s, scleroblasts; b: blastema. Distal part of the regenerate is to the top (A) and to the right (B-G). Scale bars: (A) = 100 μm, (B, C) = 25 μm, (D-G) = 50 μm.

signaling inhibition halts or slows down both outgrowth of the regenerating fin, deposition of new bone matrix, scleroblast maturation and migration of scleroblasts to the external side of the developing lepidotrichia.

Osteoblast specific transcription factors *runx2a* and *runx2b* expression in differentiating scleroblasts during caudal fin regeneration is downregulated in absence of BMP signaling, as well as the Runx2 downstream target Col10a1

We next examined the expression of known downstream targets of BMP signaling in osteogenesis, and how their expression is affected following Chordin misexpression. Runx2/CBFA1 is known as an essential transcriptional determinant of osteoblast differentiation and function, as well as of chondrocyte maturation (Ducy *et al.* 1997; Karsenty *et al.* 1999; Kim *et al.* 1999; Choi *et al.* 2002; Iwamoto *et al.* 2003). In other systems, *Runx2* is a direct downstream target of BMP signaling (Chen *et al.* 1998; Lee *et al.* 2000; Zhang *et al.* 2000; Zaidi *et al.* 2002; Canalis *et al.* 2003; Afzal *et al.* 2005; Wan *et al.* 2005; Phimphilai *et al.* 2006). Duplicated *runx2* genes, *runx2a* and *runx2b*, have recently been identified in zebrafish, and they are both expressed in developing skeletal elements (Flores *et al.* 2004). *In situ* hybridization on adjacent sections of regenerating fin rays show that *runx2a* and *runx2b* are expressed in the differentiating scleroblasts in overlapping expression patterns (Figs. 3.5A, B and Figs. 3.6A, C). These cells are located at the same proximal-distal level as the scleroblasts expressing *bmp6, bmp2b* and *ihha* (Figs. 3.5C-E). Following *chordin* injection, we observed that the expression of the *runx2a* and *runx2b* genes is downregulated (Figs. 3.6A- D, $n = 4$ rays).

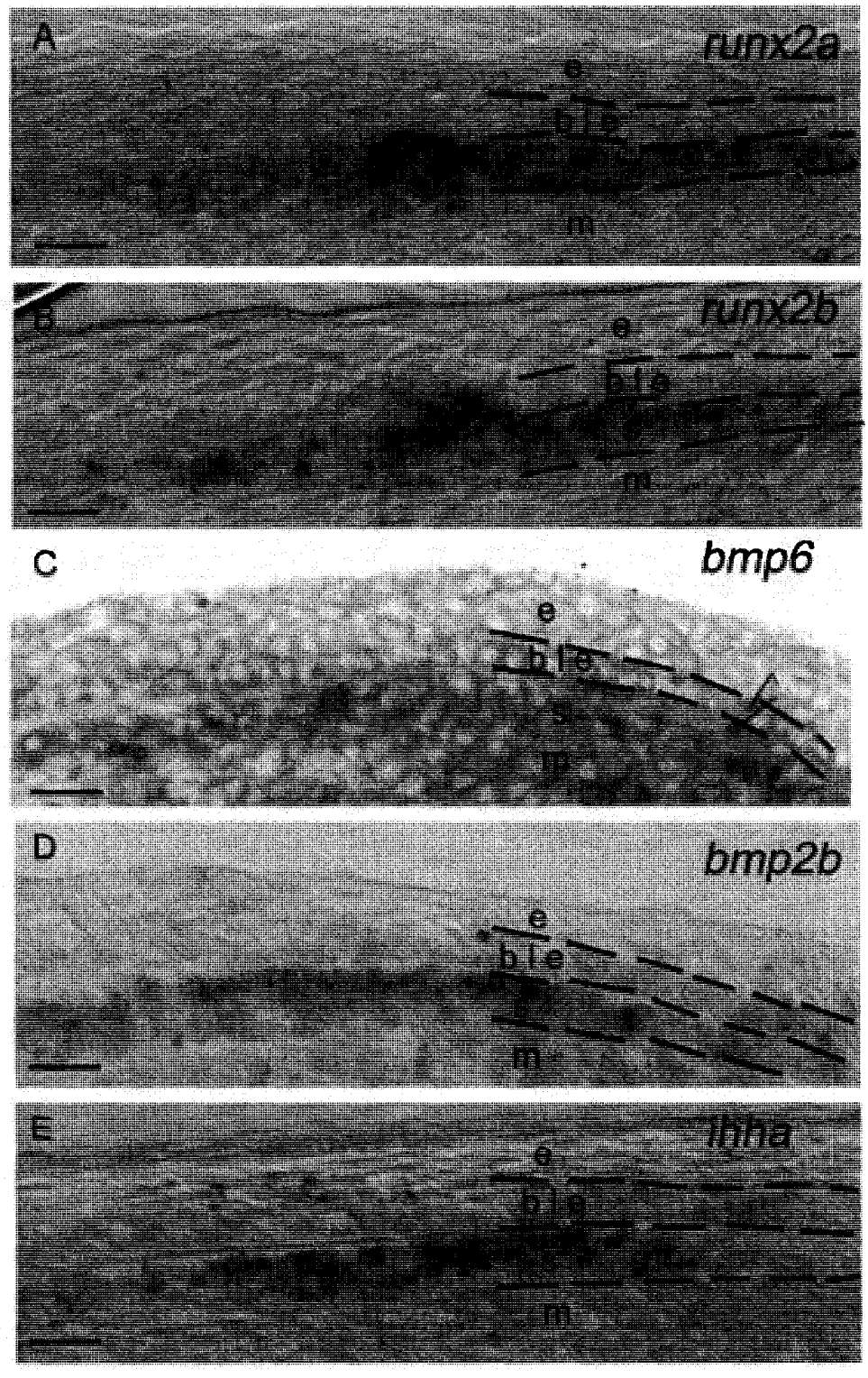

A
runx2a
e
b
f
m
B
runx2b
e
b
f
m
C
bmp6
e
b
f
m
D
bmp2b
e
b
f
m
E
lhha
e
b
f
m

Figure 3.5

Figure 3.5: Gene expression analysis using *in situ* hybridization on sections of 4 dpa regenerates.

The pictures are focusing on a portion of the distal part of the regenerate taken at the same proximal-distal level and highlighting the common expression of *runx2a, runx2b, bmp6, bmp2b*, and *ihha* in the newly differentiating scleroblasts. (A-B) the duplicate genes *runx2a* and *runx2b* are expressed in the differentiating scleroblasts in an overlapping expression pattern as seen on these adjacent sections (16 μM apart). (C) *bmp6* is expressed in the differentiating scleroblasts, the mesenchymal cells of the blastema and a subset of cells of the basal epidermal layer. (D, E) *bmp2b* and *ihha* are similarly expressed within differentiating scleroblasts. Dashed lines separate different cell types within the fin regenerate. b, blastema; ble, basal layer of the epidermis; e, epidermis; m, mesenchyme; s, scleroblasts. Scale bars: (A-E) 12.5 μm.

These results suggest that BMP factors are likely signaling through the Runx2 transcription factors to activate scleroblast differentiation or function.

One of the direct targets of Runx2 in mouse, chick and human is the Type X Collagen gene (*Col10a1*), which is expressed in hypertrophic chondrocytes and whose promoter contains multiple Runx2 binding sites (Ninomiya *et al.* 1986; Zheng *et al.* 2003; Shen 2005). We recently characterized the zebrafish orthologue of the mammalian *col10a1* gene and showed that, during fin regeneration, *col10a1* expression is detected in the scleroblasts lining both sides of the proximal lepidotrichia and also in the distal newly differentiating scleroblasts and basal layer of the epidermis (Padhi *et al.* 2004). Comparison of *col10a1* expression pattern between *chordin*- and *EGFP*-injected fin rays shows that 1 day following Chordin misexpression, *col10a1* expression is diminished and its domain of expression reduced (Figs. 3.6 F, G; $n = 9$). We did not observe a complete downregulation of *col10a1* probably because, at the time of injection (3 dpa), this gene is already highly and broadly expressed in the fin regenerate (Fig. 3.6E). These results indicate that *col10a1* is a downstream target of the BMP signaling pathway involved in bone regeneration and is possibly regulated by the Runx2 transcription factors.

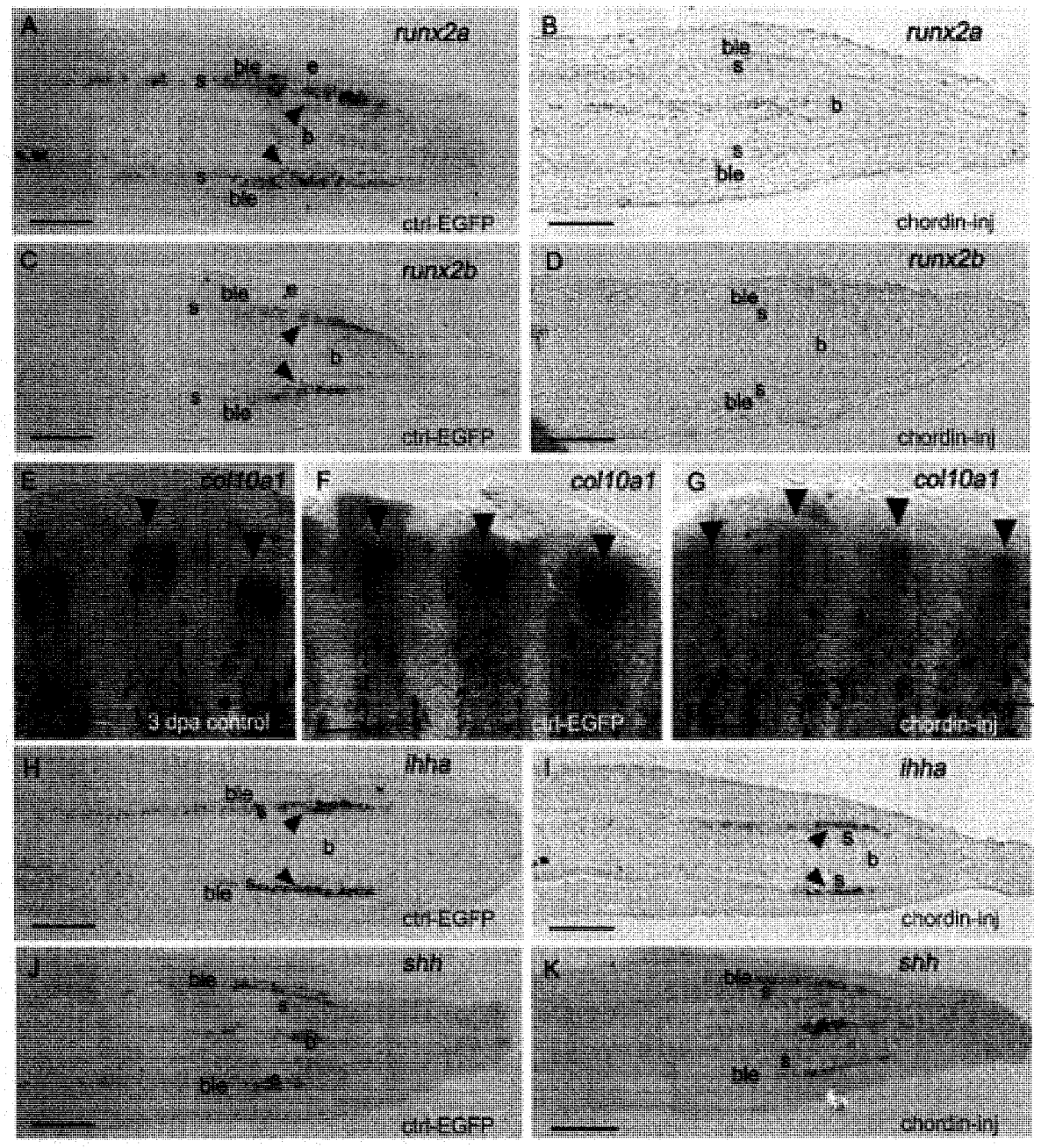
indian hedgehog a (ihha) and _sonic hedgehog_ (_shh_) expression are not affected by Chordin misexpression

Many studies have shown that Ihh regulates several aspects of endochondral ossification (St-Jacques *et al.* 1999; Long *et al.* 2004). To test a possible interaction between BMP and Ihh signaling, we examined the effects of Chordin misexpression on *ihha* expression

in newly differentiating scleroblasts in the fin regenerate. One day following *chordin* injection, *ihha* expression in the new scleroblasts remained unchanged when compared to control rays even though the *chordin*-injected rays had grown minimally since the day of injection (Figs. 3.6 H, I; $n = 4$ rays). These results suggest that *ihha* is not a downstream target of the Bmp2b signaling pathway during fin regeneration. We previously showed that *shh* is co-expressed with *bmp2b* in a subset of cells of the basal epidermal layer adjacent to the differentiating scleroblasts expressing *ihha* and *bmp2b* (Laforest *et al.* 1998; Avaron *et al.* 2006). We also showed that, as with Bmp2b, misexpression of Shh between fin rays leads to ectopic bone formation. However, misexpression of both Shh and Chordin, an antagonist of BMP signaling, fails to produce any bone fusion, therefore suggesting that Bmp2b signaling is acting downstream of Hedgehog signaling (Quint *et al.* 2002). Here we show that, as in the case of *ihha*, expression of *shh* (Figs. 3.6 G, H) and *ptc-1* (data not shown) following *chordin* injection is unchanged at 1 dpi, corroborating our previous findings that Shh signaling is acting upstream of the BMP pathway in the fin regenerate (Quint *et al.* 2002).

The cartilage markers Sox9a, Sox9b and Col2a1 are expressed in the regenerating fin and the *sox9* genes appear to be regulated by different pathways

We observed the expression in the fin regenerate of two genes, *ihha* and *col10a1*, previously described as only being involved in endochondral bone formation (Padhi *et al.* 2004; Avaron *et al.* 2006). We examined whether other genes associated to cartilage formation would also be expressed in the regenerate and regulated by BMP signaling. Sox9 is the earliest marker for cells committed to chondrogenesis and is a key


A
runx2a
ble
e
s
b
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ctrl-EGFP
B
runx2a
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chordin-inj
C
runx2b
ble
e
s
b
s
ble
ctrl-EGFP
D
runx2b
ble
s
b
ble
s
chordin-inj
E
col10a1
3 dpa control
F
col10a1
ctrl-EGFP
G
col10a1
chordin-inj
H
Ihha
ble
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Ihha
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s
chordin-inj
J
shh
ble
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shh
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Figure 3.6

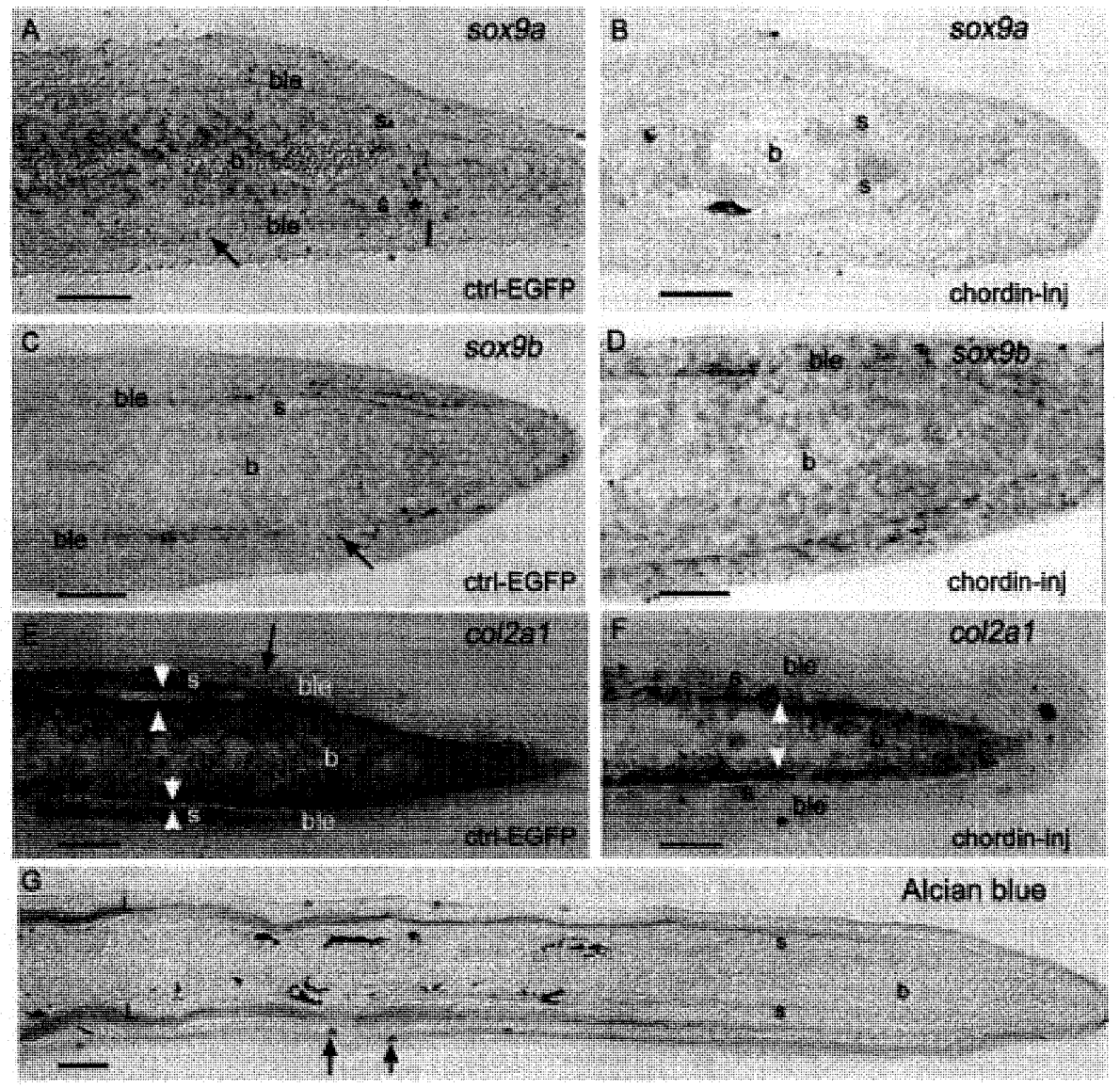
Figure 3.6: Expression analysis of known downstream targets of Bmp signaling following chordin injection.

Gene expression analysis using *in situ* hybridization on whole-mount and longitudinal sections of *EGFP* (A, C, F, H, J) and *chordin*-injected rays (B, D, G, I, K) at 4 dpa/1 dpi. (A-D) *runx2a* and *runx2b* are both expressed within newly differentiating scleroblasts (arrowheads in panels A and C) in control regenerates and are both downregulated following *chordin* injection (B, D). (E-F) *col10a1* expression on whole-mount 3 dpa regenerate (E) and 4 dpa regenerate 1 day following *EGFP* construct injection (F). At 3 dpa *col10a1* is already strongly expressed in the entire fin ray and presents a stronger expression in the distal part of the regenerate. At 4 dpa/1 dpi, in the EGFP-injected fin rays, a similar strong expression is observed with a broad domain in the distal part of the regenerate. (G) In contrast, in the *chordin*-injected rays, *col10a1* expression at 4 dpa/1 dpi is reduced and observed in a more restricted domain in the distal part of the regenerate. (H, I) *ihha* expression in the distal scleroblasts (arrowheads) of *EGFP*-injected rays (H) is unaffected in *chordin*-injected rays (I). (J, K) *shh* expression in the basal epidermal layer is unchanged in both *EGFP* (J) and *chordin*-injected rays (K). Asterisk in panel K indicates blood vessel. b, blastema; ble, basal layer of the epidermis; s, scleroblasts. Distal part of the regenerate is to the right (A-D, H-K) and to the top (E-G). Arrows indicate level of amputation. Arrowheads show expression of *col10a1* in each ray in panels E-G. Scale bars: (A-D, H-K) = 25 μm, (E-G) = 100 μm.

transcription factor mediating chondrocyte differentiation and cartilage formation (Bi *et al.* 1999; Yoon *et al.* 2004; Yan *et al.* 2005). In zebrafish, there are two duplicate orthologues of the single human *Sox9* gene, *sox9a* and *sox9b*. Their expression during embryogenesis partially overlaps in the craniofacial region and fins bud (Chiang *et al.* 2001). In the fin regenerate, *sox9a* expression is observed at 4 dpa in the proliferating blastema, in localized cells of the basal epidermal layer and in a few newly differentiating scleroblasts but is excluded from the more mature or more proximal scleroblasts (Fig. 3.7A). *sox9b* expression, on the other hand, is specifically detected in the basal epidermal cell layer of the distal part of the regenerate (Fig. 3.7C). Expression of *col2a1*, a known cartilage-specific extracellular matrix protein which is regulated by Sox9 (de Crombrugghe *et al.* 2000), has previously been reported in the regenerating fin (Johnson *et al.* 1995). Similarly, we detected *col2a1* expression in the scleroblasts, undifferentiated blastema cells and also a very strong expression in cells that appear to line the actinotrichia (Fig. 3.7E, white arrowheads). A weak expression of *col2a1* is sometimes seen in the basal epidermal layer depending on the level of the section (arrow in Fig. 3.7E). *col2a1* transcripts co-localize with *sox9a* in the scleroblasts, blastema cells and in some cells of the basal epidermis, whereas *sox9b* expression in the basal epidermal cell layer is much broader than that of *col2a1* and therefore may have a separate role than Sox9a in regulating *col2a1* expression. These results indicate that *col2a1* expression in the regenerate may be in part regulated by Sox9a and Sox9b, as during cartilage formation.

To gain insight into the regulation of the *sox9* and *col2a1* genes during fin regeneration, we examined the effect of ectopic Chordin expression on *sox9a*, *sox9b* and *col2a1* expression. While BMP signaling inhibition did not affect *sox9b* expression (Figs. 3.7C, D), *sox9a* expression was highly reduced following *chordin* injection (Figs. 3.7A, B) suggesting a differential mechanism of regulation of the duplicated *sox9* genes in the fin regenerate. We next looked at the effect of BMP signaling inhibition on the expression of *col2a1* since it is known to be regulated by Sox9 during cartilage formation. We found that *col2a1* expression was diminished within the blastema and also in the scleroblasts (Figs. 3.7E, F). The expression in the cells lining the actinotrichia was still present (white arrowheads, Fig. 3.7F). It is very likely that these cells are producing the extracellular matrix composing these actinotrichia which are fibrils strictly specific to fish and involved in structural support. It is therefore possible that *col2a1* expression in these cells may be controlled by a yet uncharacterized regulatory mechanism. Furthermore, the strong expression of *col2a1* in these cells prior to injection at 3 dpa makes it difficult to completely downregulate its expression 1 day after injection using our transfection approach.

Therefore, Sox9a is at least partly regulating *col2a1* expression within the fin regenerate as it does during cartilage formation. To ensure that no cartilage cells are present within the regenerate, sections of 4 dpa regenerates were stained with Alcian blue, a marker for glycosaminoglycans abundant in cartilage cells. We found no evidence of Alcian blue stained cells in the fin regenerate although the dermal bone matrix faintly stains blue (Fig. 3.7G). These results indicate that, in the regenerating fin, cartilage specific markers


A
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sox9a
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chordin-inj
C
sox9b
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s
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ble
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D
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sox9b
b
chordin-inj
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col2a1
s
ble
b
s
ble
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F
col2a1
ble
ble
chordin-inj
G
Alcian blue
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Figure 3.7

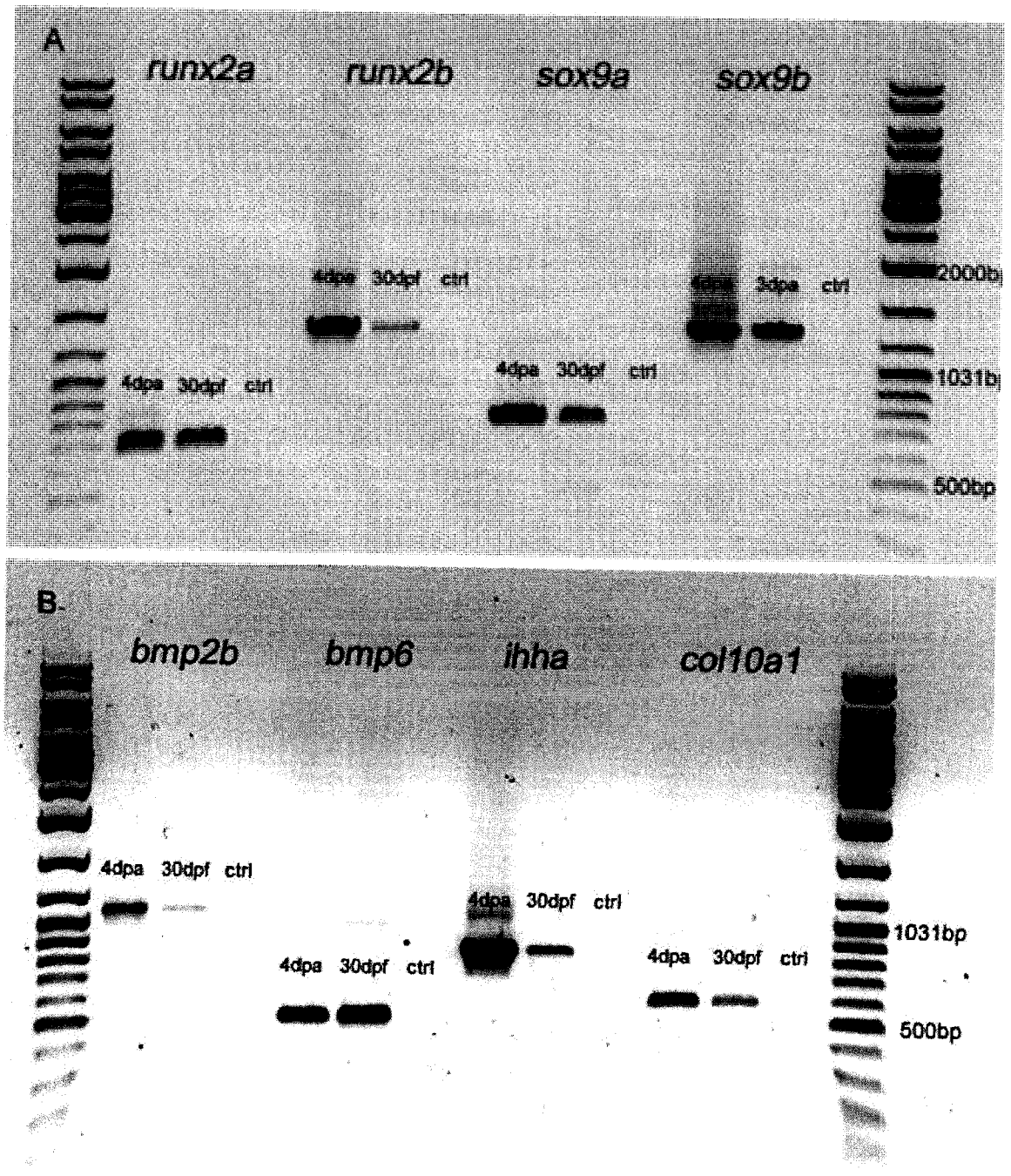
Figure 3.7: Expression analysis of cartilage markers within the fin regenerate.

(A-F) *In situ* hybridization on longitudinal sections of 4 dpa fin regenerates showing *sox9a* (A,B), *sox9b* (C,D) and *col2a1* (E,F) expression. (A) *sox9a* is expressed in the mesenchyme of the blastema, in a few cells of the basal layer of the epidermis (arrow) and in newly differentiating scleroblasts (asterisk). (B) 1 day following injection of *chordin*, *sox9a* expression is downregulated. (C) *sox9b* is expressed in cells of the basal epidermal layer in the distal part of the regenerate (arrow). (D) *sox9b* expression is unaffected in *chordin*-injected rays at 1 dpi . (E) *col2a1* is strongly expressed in the scleroblasts, in cells surrounding the actinotrichia (white arrowheads), the mesenchyme of the blastema and faintly in the basal epidermal layer (arrow). (F) *col2a1* expression is partially downregulated in the scleroblasts and mesenchyme following *chordin* injection but is still present in cells surrounding actinotrichia (white arrowheads). (G) Alcian blue staining shows that the regenerate is devoid of any cartilage cells although staining is observed in the lepidotrichial material and in a few mucous cells (arrows). ble, basal layer of the epidermis, b, blastema; s, scleroblasts. Distal part of the regenerate is to the right. Scale bars: (A-G) = 25 μm.

are expressed in cells that secrete a matrix that will form dermal bone and some are regulated by BMP signaling. Using RT-PCR on cDNA isolated from the caudal fins of 30-day-old larvae we found that all of the genes examined in this study, those specific to both bone and cartilage formation, are expressed during development of the larval fin rays and regeneration of the adult fin rays (Supplementary Fig. 3.2).

Discussion

In this report, we performed a functional analysis of the BMP signaling pathway in bone regeneration by partially characterizing the zebrafish *bmp6* cDNA and investigating the effects of BMP signaling inhibition by ectopic expression of Chordin in the blastema of regenerating rays. Inhibition of BMP signaling has two major and distinct consequences: it drastically slows down (1) the growth of the regenerate and (2) the function of the scleroblasts secreting the bone matrix. The downregulation of *runx2*a and *runx2b* gene expression in the scleroblasts following BMP signaling inhibition demonstrates that the observed skeletal defects are not simply due to the delayed outgrowth of the fin regenerate, which is likely caused by an inhibition of blastema cell proliferation. This study provides a more detailed exploration of the role of BMP signaling during regeneration highlighting its role in both growth of the regenerate and bone formation. We have also demonstrated that many cartilage specific markers are expressed within the bone secreting scleroblasts and the proliferating blastema of the fin regenerate where no cartilage is present.



Supplementary Figure 3.2

Supplementary Figure 3.2: RT-PCR analysis of various cartilage and bone genes on caudal fins of 30-day-old larvae and 4 dpa fin regenerates.

Total RNA was prepared from 50 caudal fins of 30-day-old larvae using Trizol (Invitrogen) (samples only contained the distal half of the fin to avoid the endochondral bones at the base of the fin rays) and from 20 fin regenerates. RNA was reverse-transcribed using Superscript II Reverse Transcriptase (Invitrogen). Gene specific primers (see below) to amplify cDNA fragments for the transcription factors *sox9a*, *sox9b*, *runx2a* and *runx2b*; the signaling molecules *bmp2b*, *bmp6* and *ihha*; and the extracellular matrix molecule *col10a1* were used for PCR analysis. cDNA from 4 day regenerates was used as a positive control for gene expression and a water control was used as a negative control. The PCR products were electrophoresed on an agarose gel beside a GeneRuler DNA Ladder Mix. (A,B) *runx2a, runx2b, sox9a* and *sox9b* (A) *bmp2b, bmp6, ihha* and *col10a1* (B) were all expressed in 4 day regenerates and 30 day larvae fins. For *runx2a, runx2b, sox9a, sox9b* and *bmp6* primers see materials and methods.

Other gene specific primers used:

ihha forward: 5'ATGCGTCTCCCCGTGGTGTT 3'

ihha reverse: 5'TCATCTATCATTGTCCATCATGC3'.

bmp2b forward: 5'TTGGACTCATTCCCGAGATCG3'

bmp2b reverse: 5'GTACTCGTCCAGGTACAGCA3'

col10a1 forward: 5'ATGTCCATTGCTCTTTCTCTG3'

col10a1 reverse: 5'CATAATGGTTTACAGAGCAAA3

Morphological effects of inhibiting the BMP signaling pathway on bone regeneration.

In the absence of BMP signaling, bone mineralization was delayed and the number of scleroblasts on the lateral edges of the regenerating bone was decreased. The thickness of the bone was especially reduced in the proximal-most region of the regenerate where very high densities of differentiated scleroblasts are observed in control regenerates. Our data suggest that the reduced amount of bone material is likely due to reduced differentiation or maturation of the scleroblasts, a defect in their function to secrete bone matrix and in their migration to the outer side of the lepidotrichia, which is part of their maturation process. This is consistent with previous studies in other organisms that showed that BMP signaling is required in both osteoblast differentiation and function (Yamaguchi *et al.* 1991; Thies *et al.* 1992; Rickard *et al.* 1994; Canalis *et al.* 2003; Wan *et al.* 2005). It has been demonstrated following tissue specific downregulation of BMP signals in transgenic mice overexpressing Noggin in osteoblasts that they have a reduced bone mineral density and reduced rate of bone formation (Devlin *et al.* 2003; Wu *et al.* 2003). In addition, transgenic mice expressing dominant-negative BMPR-IB under the control of the Col1a1 promoter have signs of osteopenia which include reduced bone mineral density, bone volume and bone formation rates (Zhao *et al.* 2002; Tsumaki *et al.* 2005). Differentiation of osteoblasts prepared from these mice were disrupted as indicated by reduced *Runx2* expression and mineralization (Tsumaki *et al.* 2005). The present analysis provides evidence that BMP signaling is required for scleroblast differentiation and function in the fin rays as seen in other organisms.

BMP signaling in blastemal proliferation and fin outgrowth

Inhibition of BMP signaling slows down the outgrowth of the regenerating fin, and this is accompanied by a downregulation of *msxB* expression in the distal blastema. This effect could be interpreted as a direct disruption of Bmp4 signaling in the distal blastema of regenerating fins (Murciano *et al.* 2002). Bmp4 can induce *Msx1* expression in several developing vertebrate systems (Watanabe *et al.* 1996), and the direct action of BMP on *Msx1* gene expression is further supported by the presence of Smad binding sites in the promoter of the mouse *Msx1* gene (Alvarez Martinez *et al.* 2002). Beck *et al.*, (2006) demonstrated that BMP signaling is important for regeneration of all tissues of the larval *Xenopus* tail and that transgenic tadpoles expressing Msx1 under the control of a HSP70 promoter were able to regenerate their tail when heat shocked at a stage where regeneration would not normally occur, suggesting that the *Msx1* gene is likely to be the main target of BMP signaling in this case (Koshiba *et al.* 1998; Beck *et al.* 2006). On the other hand, a study examining the role of BMP4 and Msx1/2 in fetal mouse digit tip regeneration demonstrated that Msx1 and BMP4 are necessary for regeneration but that Msx1 is likely acting upstream of BMP4 (Han *et al.* 2003). *msxB* and *msxC* are both expressed in the distal blastema of the fin regenerate (Akimenko *et al.* 1995) in a domain that overlaps with a population of cells that present a very reduced cell cycling (Nechiporuk *et al.* 2002). These cells have been proposed to constitute a population of progenitor cells for the more proximal part of the blastema which, in contrast to the distal blastema cells, is highly proliferative and assures the growth of the regenerate (Nechiporuk *et al.* 2002). Therefore, downregulation of *msxB* and *msxC* in the distal blastema could affect this cell population in a way that would prevent maintenance of the

blastema and would cause the inhibition of the regeneration process. To date, it has not been possible to clearly correlate any of the zebrafish *msx* genes to the mammalian *Msx1, Msx2* and *Msx3* genes (Akimenko *et al.* 1995). The expression patterns of *msxB* and *msxC* are reminiscent of the expression described for *Msx1* in the developing limb bud.

The BMP molecular pathway during ray regeneration

In higher vertebrate species, members of the BMP family have been shown to regulate osteoblast differentiation and bone formation through a direct downstream effector, Runx2/Cbfa1, which is essential for the transcription of bone specific genes (Banerjee *et al.* 1996; Ducy *et al.* 1997, 1999; Karsenty 2001; Kern *et al.* 2001; Canalis *et al.* 2003).

Runx2 null mice are completely devoid of any mineralized skeletal elements, from both dermal and endochondral origins (Ducy *et al.* 1997; Komori *et al.* 1997, 2005; Otto *et al.* 1997; Eames *et al.* 2004b). In addition, many studies have shown that Runx2 also regulates chondrocyte differentiation (Inada *et al.* 1999; Enomoto *et al.* 2000; Karsenty 2001; Ueta *et al.* 2001; Iwamoto *et al.* 2003). In zebrafish, the two duplicated *runx2* genes, *runx2a* and *runx2b,* show complementary expression patterns within the developing head skeleton of larvae (Flores *et al.* 2004). Our analysis revealed that, during the outgrowth phase of the fin regenerate, both *runx2a* and *runx2b* transcripts are expressed with *bmp2b* and *bmp6* in the scleroblasts, and Chordin ectopic expression results in the downregulation of *runx2a* and *runx2b* expression. Altogether, these results support the hypothesis that BMP signaling is regulating the expression of *runx2a/2b* that,

in turn, are likely mediating scleroblast differentiation and function by inducing downstream targets involved in bone development.

Collagen X is one of the known downstream targets of Runx2 in other organisms (Ninomiya *et al.* 1986; Zheng *et al.* 2003; Shen 2005). In mouse hypertrophic chondrocytes, *Col10a1* expression is regulated by BMP signaling through the Runx2 transcription factors. Zheng *et al.*, (2003) demonstrated, using in vitro and in vivo studies, that Runx2 directly transactivates *Col10a1* transcription through multiple binding sites found in the promoter of the mouse, human and chick *Col10a1* gene. *col10a1* is also expressed in the scleroblasts of the fin regenerate, and here we show that its expression decreases following BMP signaling inhibition. This reduced expression of *col10a1* in the absence of BMP signaling is probably due to the loss of *runx2a* and *runx2b* expression and correlates with the smaller number of fully differentiated scleroblasts.

The BMP and Ihh signaling pathways interact, both directly and indirectly, during various steps of endochondral bone formation (Seki *et al.* 2004; Yoon *et al.* 2004). In mouse, BMP and Ihh signaling act in parallel to maintain normal chondrocyte proliferation while BMP signaling seems to modulate the expression level of Ihh during chondrocyte differentiation (Minina *et al.* 2001). Runx2 directly induces the expression of Ihh, which is a positive regulator of chondrocyte proliferation (St-Jacques *et al.* 1999) Ihh also mediates osteoblast differentiation within the perichondrium. In both mouse and chick, overexpression of Ihh results in the upregulation of Bmp2 and Bmp4 within the perichondrium (Pathi *et al.* 1999; Minina *et al.* 2001). In addition, analyses of *Ihh*$^{-/-}$

mouse have shown that Ihh is essential for *Runx2* expression in the perichondrial/periosteal region (St-Jacques *et al.* 1999). Runx2 can directly induce Ihh in chondrocytes co-ordinating their proliferation and differentiation and therefore regulating the growth of the long bone. Our study shows that *ihha* expression in the newly differentiating scleroblasts is unaffected by BMP signal inhibition, therefore suggesting that BMP and Ihha signaling pathways may act in parallel. It is also possible that Ihha signaling is acting upstream of BMP in a similar way as in the chick and mouse perichondrium (Pathi *et al.* 1999). We have previously shown that ectopic expression of Shh between ray branches causes BMP-mediated ectopic bone deposition (Quint *et al.* 2002), and we have now demonstrated that Shh is unaffected by BMP inhibition. These results suggest that Hh signaling within the fin regenerate may be acting upstream of BMP signaling to mediate bone formation.

Differential regulation of *sox9a* and *sox9b* expression in the regenerating fin.

The expression patterns of the zebrafish duplicated *sox9* genes and the phenotypes of single and double mutants indicate that Sox9a and Sox9b have unique functions in the development of certain tissues (Chiang *et al.* 2001; Yan *et al.* 2002, 2005). The study of duplicated genes of zebrafish, such as the *sox9* genes, where subfunctionalization of the original genes function has occurred, provides many advantages for dissecting the mechanisms of regulation of these genes. Interestingly, *sox9a* and *sox9b* tissue distribution within the fin regenerate, in the mesenchymal cells of the blastema and in the basal epidermal layer surrounding the blastema, respectively, is reminiscent of the expression seen in the branchial arches of the zebrafish embryo where *sox9a* is expressed

in the arch mesenchyme which will contribute to the arch cartilage, while *sox9b* is restricted to the epithelium that surrounds each arch. These differential expression patterns suggest that *sox9a* and *sox9b* could be regulated by different pathways in the fin regenerate. To date little is known about the key regulators of *Sox9* gene expression. The few positive regulators that have been identified are members of the fibroblast growth factor (Murakami *et al.* 2000), bone morphogenetic (Zehentner *et al.* 1999; Semba *et al.* 2000) and hedgehog family of proteins (Tavella *et al.* 2004). For example, Healy *et al.*, (1999) demonstrated that exogenous application of Bmp2 to avian limbs resulted in an upregulation of *Sox9*, which was then followed by ectopic cartilage formation. They also showed that ectopic expression of the BMP antagonist Noggin in the limb resulted in loss of *Sox9* expression from developing digits. These results indicate that *Sox9* expression during chondrogenesis is dependent on BMP signaling. The expression of *sox9a* in the blastema of the fin regenerate and its downregulation following Chordin misexpression indicates that it is likely regulated by BMP signaling, possibly *bmp6* which has a similar expression pattern as *sox9a*. *sox9b* expression, on the other hand, is not downregulated following BMP inhibition and is therefore likely being regulated by a different pathway. Shh has previously been shown to regulate Sox9 in other organisms and tissues. For example, Sox9, which plays a role in the differentiation of the outer root sheath of the hair follicle and in formation of the hair stem cell compartment, is acting downstream of Shh signaling (Vidal *et al.* 2005). The expression of both *Shh* and *Sox9* in the outer root epithelial sheath, a continuation of the basal layer of the epidermis, is reminiscent of the co-expression of *shh* and *sox9b* we observe in the basal epidermal layer in the fin regenerate. Interestingly, a similar co-expression of

sox9b and *shh* is observed in the epithelial sheath surrounding the branchial arches in the developing zebrafish (Crump *et al.* 2004; Yan *et al.* 2005). It is therefore possible that *sox9b* expression in the fin regenerate may be regulated by Hh signaling.

Endochondral vs. intramembranous ossification

Skeletal elements are made via two different mechanisms: intramembranous or endochondral ossification (Hall *et al.* 1992, 1995, 2000, 2001; Eames *et al.* 2003; Mariani *et al.* 2003; Nakashima *et al.* 2003). In zebrafish, several bones of the craniofacial skeleton, parts of the pectoral appendage and the fin exoskeleton form by intramembranous ossification (Flores *et al.* 2004; Kang *et al.* 2004; Yan *et al.* 2005). In contrast, most of the axial and appendicular skeletons of all species including zebrafish are formed through endochondral ossification (Hall *et al.* 1992; Olsen *et al.* 2000; Nakashima *et al.* 2003). Both types of skeletogenesis begins when a subset of mesenchymal cells receives signals to form either bone or cartilage (Eames *et al.* 2003; Yan *et al.* 2005). It has been proposed that the fate of skeletal tissue can be predicted by the expression level of transcription factors at the time of mesenchymal condensation (Eames *et al.* 2004b). Sox9 has been shown to promote chondrogenesis, whereas Runx2 promotes both osteogenesis and cartilage formation (Ducy *et al.* 1997; Bi *et al.* 1999; Kim *et al.* 1999; Iwamoto *et al.* 2003). In our study we found the expression of both duplicated *runx2* and *sox9* genes in different cell populations in the fin regenerate, including the differentiating scleroblasts. Other molecular markers can also define the different modes of bone formation: cartilage cells express ECM components, Col2a1 and Col10a1 and the signaling molecules Bmp6 and Ihh, whereas osteoblasts specifically

express Col1a1, Bmp2, 4 and 6 (Eames *et al.* 2004b). Our results combined with those reported in Avaron *et al.,* (2006) demonstrate that many genes previously associated with endochondral bones are expressed in the regenerating fin rays which form directly by intramembranous ossification (Geraudie *et al.* 1982; Avaron *et al.* 2006). Examples of genes strictly associated with endochondral bone formation expressed within the regenerate include *sox9a, sox9b, ihha, col10a1* and *col2a1*, whereas other genes that define intramembranous ossification are also expressed and include *runx2a, runx2b, osteonectin* and *col1a1* (this study and Johnson *et al.* 1995; Padhi *et al.* 2004; Avaron *et al.* 2006). These studies suggest that the bone of the fin rays does not fit into the typical molecular classification, at the level of the transcription factors, secreted signalling molecules or ECM components which are expressed during their formation. These observations indicate that either the bone of the zebrafish fin rays may be an intermediate between endochondral and intramembranous type of bone, or intramembranous bone formation is not as simple as originally thought and may involve many of the genes previously only associated with endochondral bone formation. In support of the first hypothesis, histological studies have shown that teleosts have several types of cartilage that are absent from higher vertebrates species, many of these tissues having intermediate properties between cartilage and bone (Huysseune *et al.* 1988; Smith *et al.* 1990; Benjamin *et al.* 1991, 1992; Witten *et al.* 2002). Type II collagen, characteristic of hyaline cartilage, has been immunodetected in bone of 5 of 12 species of teleosts examined (Benjamin *et al.* 1991). The involvement of type II collagen in bones of teleosts (Benjamin *et al.* 1991) and of Sox9b in the formation of some intramembranous bones in young zebrafish larvae (Yan *et al.* 2005) further argues for some specificity in

the regulation of certain bone formation in zebrafish, at least, compared to higher vertebrate species. On the other hand, the recent finding that *Sox9* is expressed in the common precursors of the mouse chondrogenic and osteogenic progenitors (Akiyama Ddagger *et al.* 2005) suggests that many aspects of bone, and more specifically, intramembranous bone ontogeny remain to be uncovered.

BMP signaling has been shown to regulate many aspects of both bone and cartilage formation through distinct cascades of downstream signals. Here, we showed that Bmp2b and probably Bmp6 are involved in the regeneration of the fin exoskeleton and appear to regulate the expression of *sox9a*, *runx2a*, *runx2b* and *col10a1*. We unexpectedly found that components of the molecular pathways originally thought to either instruct chondrocyte or osteoblast differentiation and function are present, even though within the regenerate there are no cartilage cells detectable by Alcian blue staining. The fin regenerate being easily accessible to both surgical and molecular manipulations represents an ideal model to further investigate the nature of the lepidotrichia and the mechanisms of dermal bone formation and regeneration.

Acknowledgements

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.08.016.

Chapter 4: Manuscript 3

Summary

Often the first step in understanding gene function during zebrafish fin regeneration has been by analyzing the expression patterns of genes using whole-mount *in situ* hybridization (ISH) techniques. Then, to decipher in what exact cell type the mRNA is localized, the whole-mount tissue is often cryo-sectioned. In our laboratory, the method of whole-mount ISH was used frequently to characterize the expression of many genes, including *shh*, *ptc1* and *bmp2b*, within the fin regenerate. We began to realize that the majority of genes detected during fin regeneration, by our laboratory and others, were usually expressed in cells within the distal regenerate or in the epidermal layers that surround the lepidotrichia, and never in the mesenchymal cells of the proximal regenerate. Since the proximal blastema is where scleroblast differentiation, and bone thickening and patterning are taking place it was unclear why no genes were expressed in these cells. We hypothesized that this was likely due to the probe being unable to penetrate the regenerating bony rays. For that reason, we decided to re-examine the expression, of many already published genes, using ISH on cryo-sections technique and compare the expression patterns to those seen following whole-mount *in situ* hybridization followed by sectioning.

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Gene Expression Analysis on Sections of Zebrafish Regenerating Fins Reveals Limitations in the Whole-Mount *In Situ* Hybridization Method.

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Keywords: *in situ* hybridization; zebrafish; fin regeneration; cryo-section.

Contribution of Authors

Amanda Smith: I was responsible for all of the experiments (excluding experiments shown in Fig. 1 which were performed by Jing Zhang) and the writing of the manuscript, with technical assistance from Danielle Guay and writing assistance from Marie-Andree Akimenko.

Jing Zhang: Responsible for staining of fin regenerates with picrosirius red, followed by sectioning of stained tissues and picture taking. (Fig. 1)

Danielle Guay: *In situ* hybridization on sections with *col10a1*.

Elizabeth Quint: Designed protocol for *in situ* hybridization on sections.

Amanda Johnson: Assisted with project as a summer student in the lab of Dr. Akimenko.

Marie-Andree Akimenko: Supervised project and participated in large portion of writing of manuscript.

Abstract

The caudal fin of adult zebrafish is used to study the molecular mechanisms that govern regeneration processes. Most reports of gene expression in regenerating caudal fins rely on *in situ* hybridization (ISH) on whole-mount samples followed by sectioning of the samples. In such reports, expression is mostly confined to cells other than those located between the dense collagenous structures that are the actinotrichia and lepidotrichia. Here, we re-examined the expression of genes by performing ISH directly on cryo-sections of regenerates. We detected expression of some of these genes in cell types that appeared to be non-expressing when ISH was performed on whole-mount samples. These results demonstrate that ISH reagents have a limited capacity to penetrate between the regenerating skeletal matrices and suggest that ISH performed directly on fin sections is a preferable method to study gene expression in fin regenerates. Developmental Dynamics 237: 417-425, 2008. c 2007 Wiley-Liss, Inc.

Introduction

In the last 15 years, the caudal fin of the zebrafish has been shown to be a useful model to study the process of regeneration following amputation or injury (Becerra *et al.* 1996; Akimenko *et al.* 2003; Poss *et al.* 2003). The study of natural regeneration in many lower vertebrates has added to the growing fields of regenerative medicine in humans and stem cell research. Epimorphic regeneration is characterized by the formation of a blastema, a collection of proliferative, pluripotent progenitor cells that will eventually differentiate to give rise to all the cell types of the regenerated organ. Detailed descriptions of the regeneration process of the fins have been reported elsewhere (Becerra *et al.* 1996; Akimenko *et al.* 2003; Poss *et al.* 2003). Briefly, fin regeneration can be divided into three major phases: wound healing, blastema formation, and fin outgrowth. Fin regeneration begins with the formation of the wound epidermis characterized by the migration of epidermal cells to cover the wound (Becerra *et al.* 1996; Poleo *et al.* 2001; Santos-Ruiz *et al.* 2002). Next, mesenchymal cells near the cut site disorganize and migrate distally toward the amputation plane. These cells proliferate at a uniform rate and accumulate at the end of each bony stump to form individual regeneration blastemas. Soon after formation of the blastema, this cell population divides into two groups based on their proliferative rate: the distalmost blastema cells, a small subgroup of cells underlying the distal epidermis that are either slowly cycling or not at all, and, beneath the distalmost blastema, a large population of blastema cells that are highly proliferating (Nechiporuk *et al.* 2002; Poss *et al.* 2002; Santos-Ruiz *et al.* 2002; Nakano *et al.* 2004). At the same time, the proximal blastema cells start to differentiate to rebuild the lost structure. Among them are the bone-secreting cells or scleroblasts that differentiate

along the basement membrane underneath the epithelial cells. These scleroblasts secrete, in the epithelial/mesenchymal interface, a bone matrix that mineralizes and reconstitutes the main skeletal elements of the fin rays named lepidotrichia. Lepidotrichia are dermal bones composed of two concave hemirays facing each other and are located beneath the epidermis. The hemirays are made of a series of bone segments linked by ligaments (Santamaria *et al.* 1991; Akimenko *et al.* 2003; Poss *et al.* 2003). The space between the hemirays is filled with connective tissue and contains the nerves and blood vessels. The second skeletal element of the fin ray consists of an array of fine rigid collagenous fibrils called actinotrichia lining the inner side of the distal part of each hemiray (Santamaria *et al.* 1991; Becerra *et al.* 1996). Both the lepidotrichia and actinotrichia are replaced following fin amputation.

To date, several research groups have examined the expression patterns of members of many gene families to determine the molecular factors that are involved in the formation and growth of the ray blastemas as well as the cell differentiation and patterning of the regenerate (for example: Akimenko *et al.* 1995; Brulfert *et al.* 1998; Laforest *et al.* 1998; Poss *et al.* 2000a, 2000b; Borday *et al.* 2001; Nechiporuk *et al.* 2002; Padhi *et al.* 2004; Whitehead *et al.* 2005; Avaron *et al.* 2006; Smith *et al.* 2006). Most of these studies have been performed using the technique of *in situ* hybridization (ISH) of antisense RNA probes on whole-mount samples of fin regenerates that allow examination of the overall domain of expression of the gene. Sectioning of the hybridized samples following whole-mount analysis is often performed to examine the exact cell types expressing the genes of interest.

The whole mount ISH technique has been widely used in zebrafish for the analysis of both embryonic development and fin regeneration. However, Strähle *et al.*, (1994) recognized that whole mount ISH of zebrafish embryos and larvae, although useful, had its limitations including insufficient probe penetration. They developed a technique for ISH on sections of zebrafish embryos that we have adapted to sections of fin regenerates and we re-examined the expression patterns of previously published genes. We then performed parallel ISH on sections and on whole-mount regenerates that were sectioned after ISH. Our results on whole-mount preparations were comparable or superior to the published studies. However, ISH on cryosections revealed broader expression patterns for many genes compared to whole-mount ISH. Considering the morphological structure of the regenerating fin ray, it is most likely that the thickening bone matrix of the lepidotrichia and the presence of actinotrichia constitute barriers for the penetration of reagents including anti-sense RNA probes and antibodies in the central part of the fin regenerate when assayed on whole-mount samples.

This study demonstrates that performing ISH on sections is essential to accurately determine gene expression during fin regeneration and may lead to the reinterpretation of the function of some genes involved in the regeneration process.

Results and Discussion

Morphology of Collagenous and Mineralized Barriers of the Fin Regenerate

Based on whole-mount ISH, expression of many genes has been reported in the epidermis, the distal blastema (Akimenko *et al.* 1995; Laforest *et al.* 1998; Monnot *et al.* 1999; Poss *et al.* 2000a, 2000b; Whitehead *et al.* 2005; Thummel *et al.* 2006), and in the differentiated scleroblasts located on the external surface of the lepidotrichia (for example, see (Laforest *et al.* 1998; Borday *et al.* 2001; Padhi *et al.* 2004; Avaron *et al.* 2006; Smith *et al.* 2006), but rarely, if at all, in cells that occupy a more central and proximal position in the regenerate during the fin outgrowth phase. We hypothesized that the apparent lack of gene expression in the central and proximal blastema cells and in differentiated tissue could result from the poor penetration of ISH reagents to the central part of the regenerate due to the presence of the lepidotrichial bone matrix and possibly of the actinotrichia. When fin regeneration is examined at 28°C, bone matrix synthesis starts between 2 to 3 days post-amputation (dpa). Starting at 3 dpa, the fin regenerate acquires a characteristic architecture that is conserved along the rest of the regeneration process. At 4 dpa, the region immediately underlying the distal wound epidermis corresponds to the distal-most blastema that has been shown to be composed of non- or slowly cycling cells (Nechiporuk *et al.* 2002; Poss *et al.* 2002) (Fig. 4.1A). More proximally, the blastema cells located along the basement membrane are differentiating into scleroblasts and blastema cells more centrally located are highly proliferative (Akimenko *et al.* 2003; Poss *et al.* 2003). We used the picrosirius red staining, which labels collagenous structures, to visualize the basement membrane, actinotrichia and lepidotrichia (Santamaria *et al.* 1991, 1992) (Fig. 4.1). Beneath the distal epidermis and a few microns proximally to the distal-most part of the blastema, the distal tips of the actinotrichia become visible adjacent to the basement membrane (Fig. 4.1A). In more

proximal regions, the actinotrichia, whose diameter is increasing, are organized in a compact row of fibrils as can be easily observed on transverse sections through the distal and middle blastema (Fig. 4.1A-C respectively). In the middle part of the blastema, the actinotrichia become separated from the basement membrane by a layer of scleroblasts that are secreting the bone matrix in the space beneath the epidermis. The fibrils are, therefore, aligned along the scleroblasts, opposite to the secreted lepidotrichial matrix (Fig. 4.1C). These actinotrichia fibrils are absent in the very proximal regenerate close to the amputation plane (Fig. 4.1D). The bone matrix secreted by the scleroblasts accumulates along the basement membrane and becomes thicker in the more proximal part of the regenerate (Fig. 4.1D). As previously described, in the most proximal part of the regenerate, the scleroblasts are located both on the outer and inner surface of the lepidotrichia (Mari-Beffa *et al.* 1996; Smith *et al.* 2006)

Thus the central part of the ray regenerate is surrounded by an important amount of collagenous material, actinotrichia and/or lepidotrichia, on the majority of its length. The only region of the fin ray that is devoid of such material is the distal part of the regenerate and the epidermis, which are essentially the sites where most gene expression has been detected by whole-mount ISH. Therefore we set out to compare the ISH results obtained on whole-mount and on sections of fin regenerates of already published genes within the zebrafish fin regenerate.

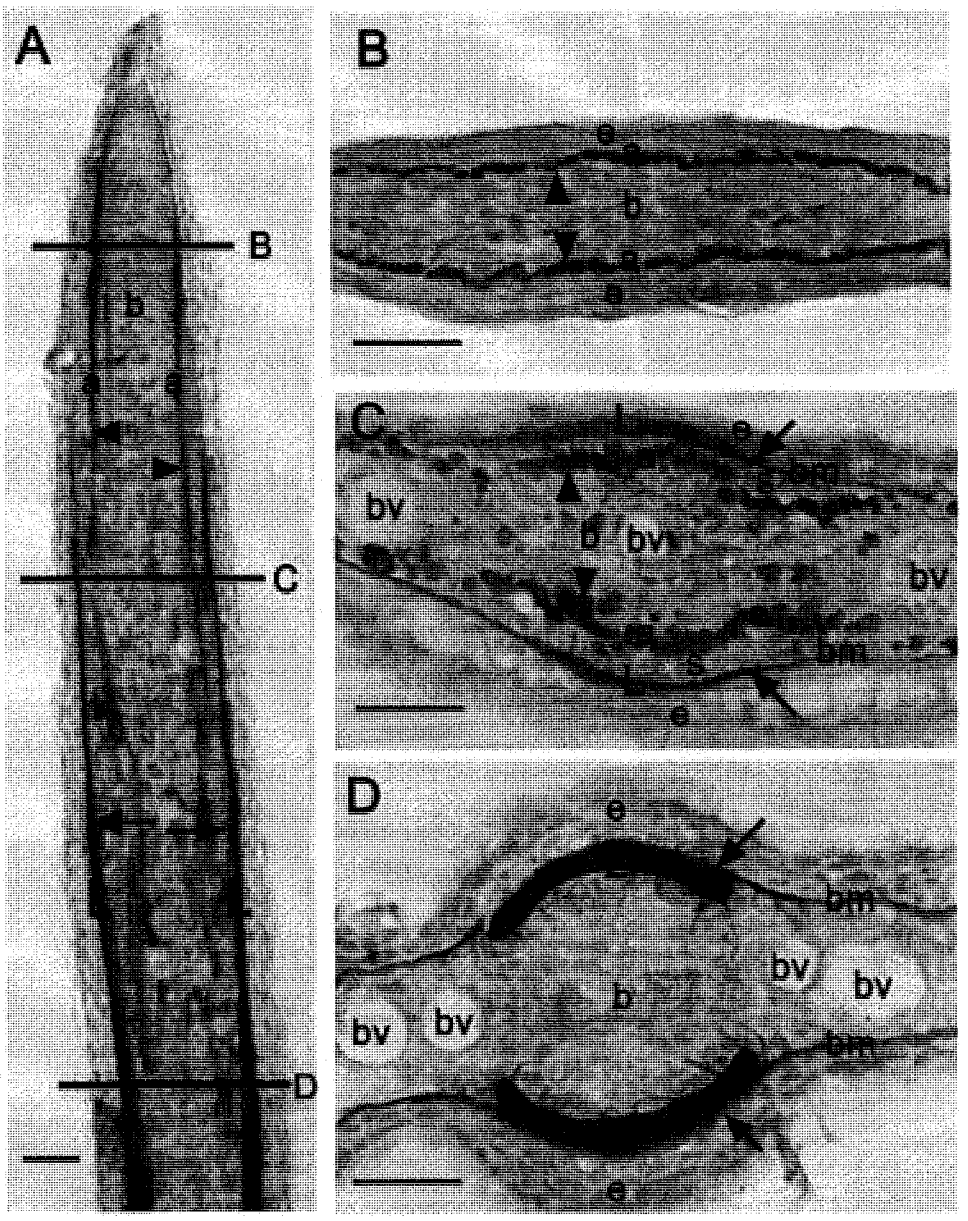


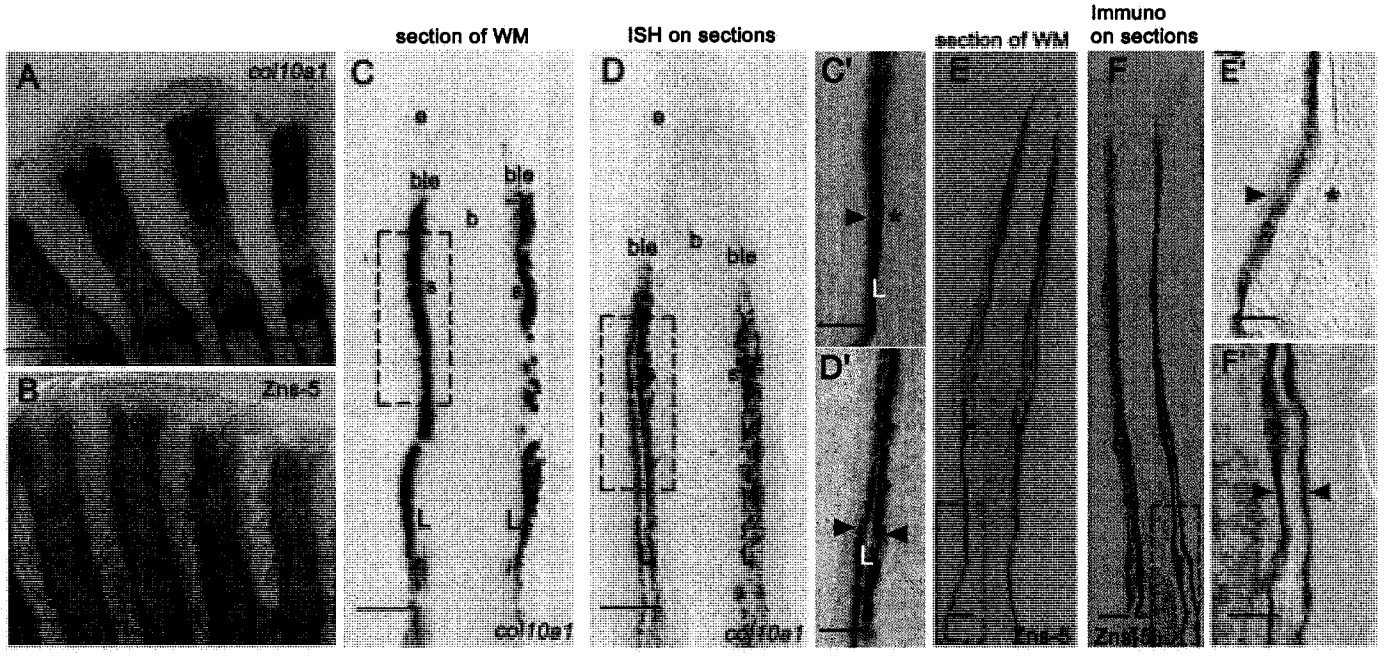
Figure 4.1

Figure 4.1: Picrosirius red staining of collagen on sections of 4-day regenerates.

Longitudinal (A) and transverse (B-D) cryo-sections of a 4 dpa regenerated fin ray that has been stained with picrosirius red, which labels collagenous structures, including the lepidotrichia (arrows), and the actinotrichia (arrowheads) and the basement membrane. The horizontal lines in A indicate the approximate positions of the transverse sections presented in B-D along the proximal-distal axis of the regenerate. The level of amputation of the fin is not shown in A. Note the presence of the tip of the actinotrichia in the distal part of the regenerate (B). More proximally, both actinotrichia of increased diameter and the lepidotrichia bone matrix are visible (C), while in the proximal part of the regenerate only thick lepidotrichia are present (D). L, lepidotrichia; a, actinotrichia; b, blastema; e: epidermis; bm, basement membrane; bv, blood vessels; s, scleroblasts. Scale bars = 25μm (A-D)

Expression of Genes Associated With Bone Formation

We recently examined the expression of many genes involved in bone formation during fin regeneration, including *collagen type X* (*col10a1*), *col2a1, indian hedgehog a (ihha), runx2a/b* and *sox9a/b* (Padhi *et al.* 2004; Avaron *et al.* 2006; Smith *et al.* 2006). This analysis was performed by ISH on cryo-sections. For example, we showed that *col10a1* is expressed in all scleroblasts of the regenerate at different stages of regeneration (Padhi *et al.* 2004; Avaron *et al.* 2006; Smith *et al.* 2006). Using *col10a1* as an example to compare the two ISH methods, we observed that its expression in the newly differentiating and more mature proximal scleroblasts, lining the internal side of the bone close to the amputation plane, of 4-dpa regenerates was only detected when ISH was performed on fin sections (Fig. 4.2D, D') and was not seen when a whole-mount hybridized sample was cryo-sectioned (Fig. 4.2A, C, C'). Using the Zns-5 antibody that recognizes an epitope in scleroblasts at all stages of differentiation, we also compared the results obtained after immunodetection on whole-mount samples and on cryo-sections. We observed that all scleroblasts located on both the inner and outer sides of the regenerating lepidotrichia were detected when antibody staining was done on cryo-sections (Fig. 4.2F, F'). In contrast, only the outer scleroblasts and some inner scleroblasts located in the distal but not in the proximal portion of the regenerate were labeled using whole-mount samples (Fig. 4.2B, E, E'). These results support our hypothesis that the connective tissue located between the skeletal elements is not easily accessible to the reagents when whole-mount samples are used for ISH or immunodetection.


A
col10a1
B
Zns-5
section of WM
C
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b
L
L
col10a1
ISH on sections
D
e
ble
b
ble
col10a1
C'
L
D'
L
section of WM
E
Zns-5
Immuno
on sections
F
Zns-5
E'
F'

Figure 4.2

Figure 4.2: Comparison of expression of two bone cell markers

Comparison of the expression pattern of two bone markers, *col10a1* (**A, C, D, C', D'**) and Zns-5 (**B, E, F, E', F'**), within the fin regenerate following two different *in situ* hybridization and immunostaining techniques. Whole-mount *in situ* hybridization (ISH) with *col10a1* RNA antisense probe (A) and immunostaining with Zns-5 antibody (B) on 4-dpa regenerates. Arrows in A and B indicate the level of amputation. C, C', E, E': Longitudinal cryo-sections of the whole-mount 4-dpa fin regenerates shown in A and B. D, D', F, F': ISH of *col10a1* probe (D, D') and immunostaining using Zns-5 antibody (F, F') performed on longitudinal cryo-sections of 4-dpa regenerates. C: *col10a1* expression on sections following ISH is found in the basal layer of the epidermis and also in the scleroblast cells lining the outer lepidotrichia. D: In addition, when ISH is performed on cryo-sections, *col10a1* expression is also detected in the scleroblasts lining the inner side of the regenerating lepidotrichia. C', D': Higher magnification of the area indicated by the dashed box in C and D, respectively. Black arrowheads in C' and D' indicate expression in scleroblasts. Note that the inner scleroblasts (right arrowhead in D') are only detected when an ISH is performed on cryo-sections. The asterisk in C' indicates the absence of detected expression in the inner scleroblasts when sections are done following a whole-mount ISH. E: On sections obtained after immunostaining on whole-mount samples, the Zns-5 antibody is staining all the outer scleroblasts along the entire length of the regenerate and the inner scleroblasts located in the distal part of the regenerate but not the more proximal one, which are only detected following immunostaining on cryo-sections (F). E', F': Higher magnification of the area indicated by the dashed box in E and F, respectively. Black arrowheads in E' and F' indicate expression in scleroblasts. Note that staining in the inner scleroblasts (right arrowhead in F') is only detected when the procedure is performed directly on cryo-sections. The asterisk in E' indicates the absence of detection in the inner scleroblasts when sections are done following immunodetection on a whole-mount sample. L, lepidotrichia; b, blastema; ble, basal layer of epidermis; s, scleroblasts. Scale bars = 100 μm (A,B), 50 μm (C,D), 25 μm (C',D',E,E',F,F').

Expression of Members of the *msx* Gene Family

Previous studies have demonstrated the expression of four homeobox genes, *msxa, msxb, msxc* and *msxd* within the regenerating fin (Akimenko *et al.* 1995). During regenerative outgrowth, *msxb* and *msxc* expression has first been reported in the distal blastema whereas *msxa* and *msxd* are expressed in the overlying epithelium. Subsequent studies provided additional insight on the expression and function of *msxb* during fin regeneration (Nechiporuk *et al.* 2002; Poss *et al.* 2002). Thus, during initial blastema formation, *msxb* is widely expressed among proliferating cells, and, during regenerative outgrowth, its expression becomes more restricted to a small number of non-proliferating blastema cells that specify the boundary of proliferation and provide direction for regenerative outgrowth (Nechiporuk *et al.* 2002; Poss *et al.* 2002). We also observed a similar expression pattern when we performed whole mount ISH with *msxb* anti-sense RNA probe and then cryo-sectioned the fin (Fig. 4.3A, C). However, when ISH was directly performed on cryo-sections of 4-dpa regenerates, expression of *msxb* was observed in a much broader domain than just in the distal slowly or non-proliferating blastemal cells (Fig. 4.3D). It now appears that *msxb* transcripts are, in fact, expressed in the majority of the proliferating blastemal cells at this stage. In addition, expression is also observed in newly differentiating scleroblasts along the epithelial/mesenchymal border (Fig. 4.3D). The same results were obtained when *msxc* expression was examined with the two methods (data not shown). The expression of *msxb* in a subset of differentiating scleroblasts is reminiscent of that described for the *msxC* orthologs in the regenerating fins of swordtails (Zauner *et al.* 2003) and suggests a possible role of *msxb* and *msxc* in scleroblast proliferation or differentiation. On the other hand, the *msxd*

domain of expression was very similar when using the two ISH methods at 4 dpa; the transcripts are detected in the distal epidermis, in a few cells of the distal blastema, and occasionally in the basal layer of the epidermis adjacent to the domain of newly differentiated scleroblasts (Fig. 4.3B, E, F). As all these domains of expression are outside of the critical central region of the regenerate, it is not surprising that the two methods provide the same results.

Interestingly, we observed previously unreported expression of *msxd* in the distal blastema and in the basal layer of the epidermis close to the site of ongoing differentiation of the scleroblasts. The expression in the blastema is reminiscent of the expression observed for *msxb* and *msxc* (Fig. 4.3B-D). However, *msxd* expression seems to be more concentrated to the distal blastema than *msxb* and *msxc* (compare Fig. 4.3D, F, and data not shown). Therefore our analysis would suggest that the previously proposed role of *msxb* in defining the limit between the distalmost slow-cycling blastema cells and the underneath proliferating blastema cell population, actually correlates better with the *msxd* expression pattern than with that of *msxb*. Another previously unnoticed expression of *msxd* was in the basal epidermal layer adjacent to the scleroblasts. It is only seen on very few sections per fin ray, which may explain why it has been missed previously. This provides a new and potentially exciting role for *msxd* in cells that are believed to play an important role in patterning of the regenerate possibly through epithelial/mesenchymal interactions.

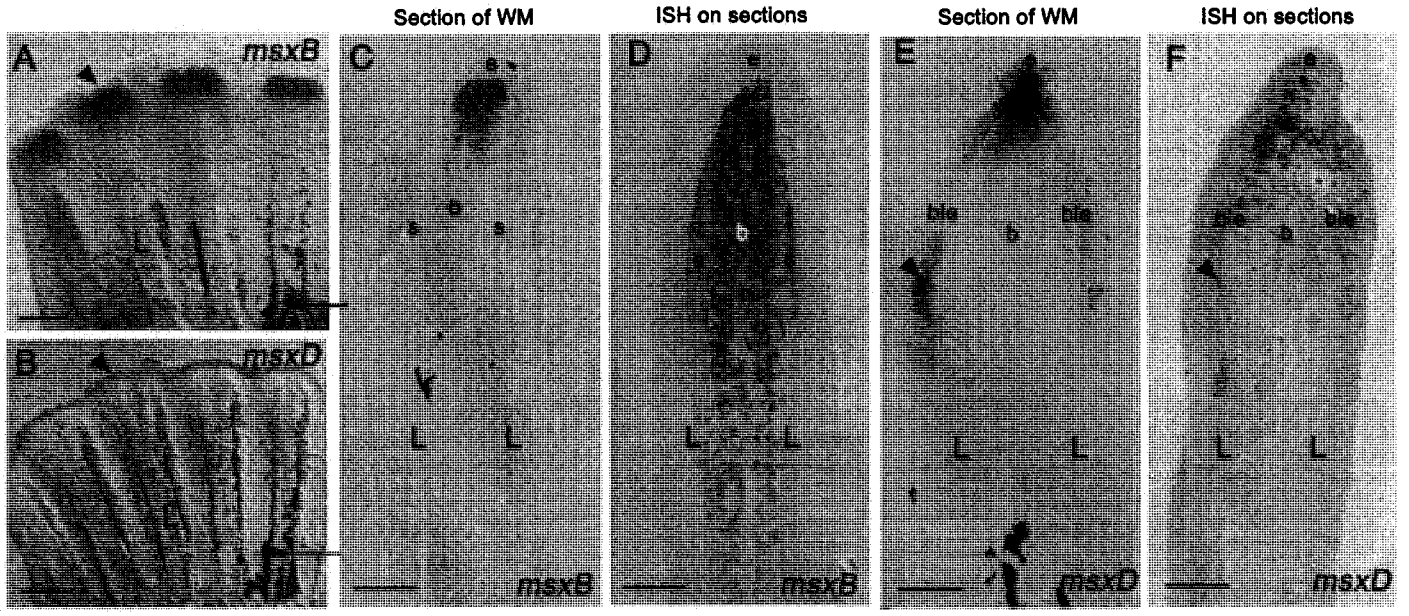
A
msxB
B
msxD
Section of WM
C
msxB
ISH on sections
D
msxB
Section of WM
E
msxD
ISH on sections
F
msxD

Figure 4.3

Figure 4.3: Comparison of the expression of msxb and msxd genes in 4-day fin regenerates using the two ISH methods.

A, B: Whole-mount ISH showing expression of *msxb* and *msxd* in the distal region of the fin regenerate at 4 dpa. *msxd* is also expressed in the distal part of the interray tissue. C: Upon sectioning of the whole-mount fins, *msxb* expression is seen in the distal blastema. D: ISH on sections reveals a broader expression domain for *msxb* encompassing the entire blastema as well as in newly differentiating scleroblasts. E: Sections of whole-mount fins hybridized with the *msxd* probe shows expression in the distal epidermis, in the distal blastema, and, on some sections, in the basal layer of the epidermis adjacent to the differentiating scleroblasts. F: *msxd* expression following ISH on sections is similar to that seen following whole-mount ISH. Arrowheads in E and F indicate the basal epidermal cells close to the differentiating scleroblasts expressing *msxd.* Arrows in A and B indicate the level of amputation. L, lepidotrichia; b, blastema; ble, basal layer of epidermis; e, epidermis; s, scleroblasts. Scale bars = 100 μm (A,B), 50 μm (C-F).

Broader Expression of *fgfR1* But Not *wfgf* When ISH on Cryo-Sections Is Performed Compared to Whole-Mount

Members of the fibroblast growth factor (Fgf) family have previously been shown to regulate blastema proliferation during fin regeneration (Poss *et al.* 2000b; Whitehead *et al.* 2005; Thummel *et al.* 2006). *Fibroblast growth factor receptor 1* (*fgfr1*) expression was found in mesenchymal cells underlying the wound epidermis during blastema formation and in distal blastema tissue during regenerative outgrowth using whole-mount ISH (Poss *et al.* 2000b). Upon sectioning of these regenerates, *fgfr1* transcripts were observed in a small population of cells comprising the distal blastema, as well as in the basal layer of the epidermis (Poss *et al.* 2000b). We found a similar expression for *fgfr1* when whole-mount ISH was performed (Fig. 4.4A) and then sectioned (Fig. 4.4C). However, when ISH with the *fgfr1* probe was performed on sections of 4-dpa regenerates, the transcripts were detected in the distal blastema as well as in more proximal blastema cells, therefore in a domain much larger than that detected with whole-mount ISH (Fig. 4.4D). The expression in the basal layer of the epidermis seen using the whole-mount method was also seen but in a larger cell population with a domain of expression that extends a lot more proximally. In addition, we observed the expression of *fgfr1* in scleroblasts on the inner side of the regenerating lepidotrichia (Fig. 4.4D). *fgfr1* expression in the scleroblasts was previously unseen, although it would not have been surprising since FGF signaling has been shown to play essential roles in both endochondral and intramembranous bone development (reviewed in: Ornitz *et al.* 2002). In contrast to *fgfr1* expression, *wound fgf (wfgf)* expression appeared to be the same using the two methods of ISH and was restricted to the basal epidermal cells of the distal part

of the regenerate as previously reported (Fig. 4.4B, E, F) (Poss *et al.* 2000b). Altogether our results show a broader expression domain of *fgfr1* but not of *wfgf* following ISH on cryo-sections of 4-dpa regenerates and support the idea that during whole-mount ISH, the *fgfr1* probe may not be able to penetrate deep into the blastema.

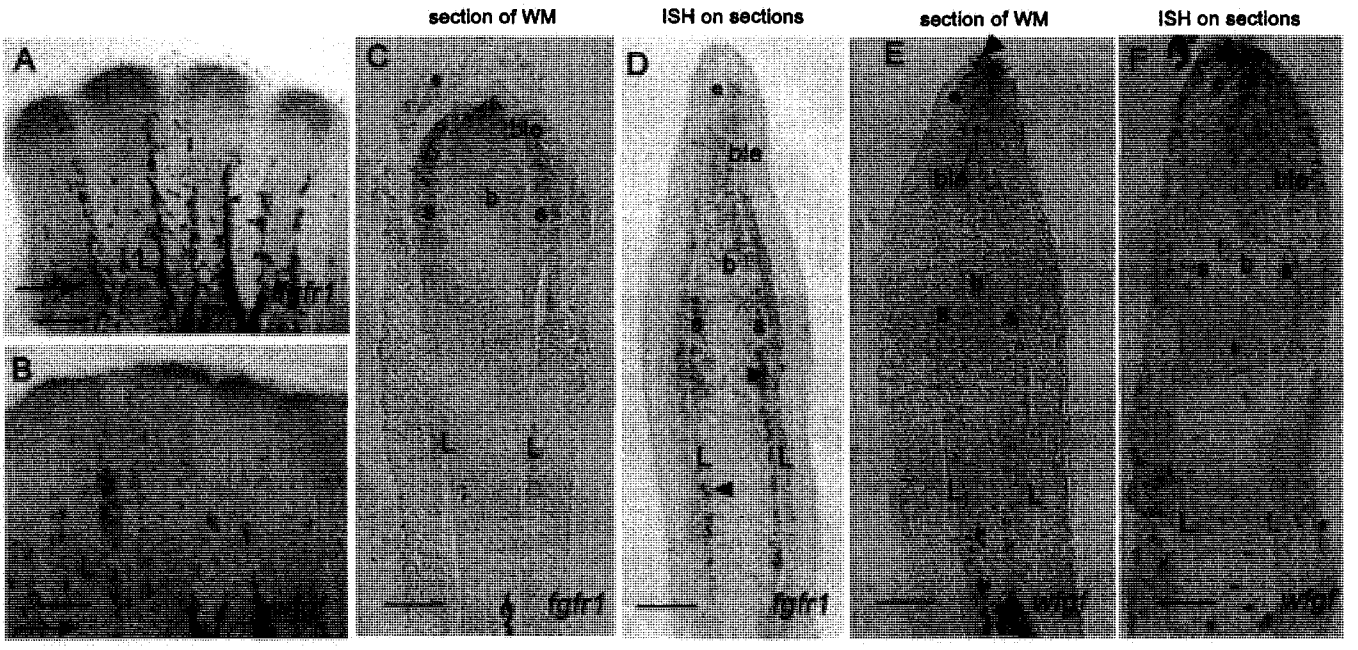
section of WM
ISH on sections
section of WM
ISH on sections
A
B
C
D
E
F
fgfr1
fgfr1
fgfr1

Figure 4.4

Figure 4.4: Comparison of the expression of fgfr1 and wfgf genes in 4-day fin regenerates using the two ISH methods.

A, B: Whole-mount ISH showing expression of *fgfr1* and *wfgf* in the distal region of the fin regenerate at 4 dpa. C: Upon sectioning of the whole-mount fins, *fgfr1* expression is seen in the distal blastema, in the basal layer of the epidermis, and in a few cells of the newly differentiating scleroblasts. D: ISH on sections reveals a broader expression domain for *fgfr1*: transcripts are observed in the blastema midway through the proximal-distal axis. It is observed; not only in newly differentiating scleroblasts, but also in differentiated scleroblasts in more proximal regions lining the inner side of the lepidotrichia (arrowheads in D indicate scleroblasts). E: Sections of whole-mount fins hybridized with the *wfgf* probe shows that *wfgf* expression is confined to the epidermis in the most distal tip of the regenerate (arrowhead). F: *wfgf* expression following ISH on sections is similar to that seen following whole-mount ISH (arrowhead). Arrows in A and B indicate the level of amputation. L, lepidotrichia; b, blastema; ble, basal layer of epidermis; e, epidermis; s, scleroblasts. Scale bars = 100 μm (A,B), 50 μm (C-F).

ISH on Whole-Mount and on Cryo-Sections Are Equivalent to Detect Gene Expression in the Basal Epidermal Layer

In regenerating fins of zebrafish, the transcription factor Lef1 involved in the Wnt signaling pathway has been reported to be expressed at the distal end of each ray, in four groups of cells, two groups on each face of the rays (Poss *et al.* 2000a). Sections of these hybridized fins demonstrated that *lef1* expression was restricted to a subset of cells of the basal layer of the epidermis adjacent to the newly differentiating scleroblasts and was weaker in the more proximal/mature regenerate (Poss *et al.* 2000a). *lef1* expression was also detected in cells of the distal blastema. We found no difference in the expression pattern of *lef1* on sections of 4-dpa regenerates when comparing the whole-mount and cryo-sections ISH techniques (Fig. 4.5). Similarly, using the whole-mount ISH technique, the signaling molecule sonic hedgehog (Shh) presents a pattern of expression in the basal layer of the epidermis almost identical to that of Lef1 (Laforest *et al.* 1998; Poss *et al.* 2000a). The same *shh* expression result was observed when ISH on cryo-sections was performed in a recently published comparison analysis of *ihha* and *shh* expression (Avaron *et al.* 2006) and data not shown). Therefore, it appears that the two methods of ISH will give the same results when genes are expressed in cells located on the external surface of the bone matrix (i.e., the epidermis and external scleroblasts) or in the distal blastema, most likely because the RNA probe and antibodies should efficiently reach these cells following an adequate proteinase K treatment when using a whole-mount ISH approach.

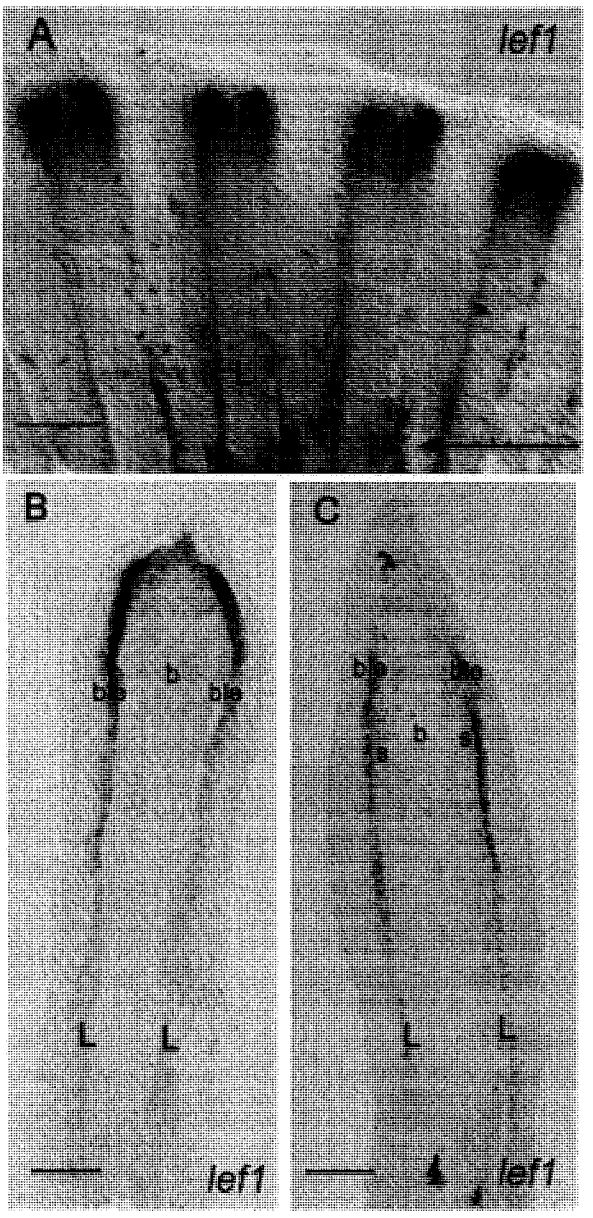

Figure 4.5

Figure 4.5: Comparison of the expression of *lef1* in a 4-day fin regenerate using the two ISH methods.

A: Whole-mount ISH with a *lef1* probe on 4-dpa regenerates shows expression splitting into two domains in the distal part of each fin ray indicating an imminent bifurcation event. B: Upon cryo-sectioning of the fin regenerate shown in panel A, *lef1* expression is observed in the basal layer of the epidermis and in the distal blastema. C: A similar expression pattern is observed when an ISH is performed on cryo-sections of 4-dpa regenerates. The arrow in A indicates the level of amputation. L, lepidotrichia; b, blastema; ble, basal layer of epidermis; s, scleroblasts. Scale bars = 100 μm (A), 50 μm (B,C).

Pros and Cons of the Two Methods of *In Situ* Hybridization

It is clear from the present analysis that if ISH of antisense RNA probes is performed on whole-mount samples, it will not efficiently detect gene expression in most of the mesenchymal cells of the fin regenerate with the exception of the most distal blastema cells during the outgrowth phase, most likely because of the presence of regenerating collagenous material that may constitute physical barriers to penetration of probe and *in situ* reagents in the fin tissue. Similar conclusions can be drawn for protein immunodetection although it seems that the antibody staining protocol may allow a detection of some cells centrally located that are missed by whole-mount ISH of antisense RNA probe (see Fig. 4.2). We show here that performing ISH and immunostaining on fin sections can circumvent the problem of accessibility to internal cells. In summary, analysis of a new gene or protein expression should be performed by ISH or immunodetection, respectively, on fin sections.

Another advantage of the ISH on sections is that tissues of the fin regenerate are better preserved than when the ISH is performed on whole-mount samples followed by sectioning. Very often in sections of ISH on whole-mount samples, the external layers of the epidermis are missing, only leaving the basal layer of the epidermis in the worse cases, likely due to constant manipulation of the sample and extended proteinase K treatment (for example compare Figs. 4.3E,F and 4.4C,D). In addition, it may occasionally occur that the basal layer detached from the connective tissue, creating gap artifacts on the sections (Fig. 4.3C). These artifacts do not frequently occur when ISH is performed on sections (Figs. 4.3D,F, 4.4D,F, 4.5C). ISH on sections not only preserves the integrity of the tissue but also the shape of the fin regenerate. On histological

preparations of fin regenerates, the distal part of the ray is narrower and thinner than the rest of the regenerate, a shape that is more consistently maintained after ISH on sections than on whole-mount samples (for example, compare Fig. 4.2C and D; 4.3C and D; 4.4C and D; 5B and C).

One advantage of the whole-mount ISH technique compared to the *in situ* on sections is that it easily reveals in certain cases, such as for *shh* and *lef1*, the dynamic changes of the pattern of expression during regeneration. *lef1* and *shh* have very similar expression patterns in the basal epidermal cells, and time course analysis of their expression using whole-mount ISH showed that the initial unique domain of expression in each ray observed at 3 dpa splits into 2 domains at 4 dpa before the formation of a bifurcation event of the rays. This observation led to the hypothesis that Shh and Lef1 may play a role in patterning of the fin rays through epithelial/ mesenchymal interactions. Using the technique of ISH on sections, such dynamic changes would have been more difficult to observe, as it would have needed the analysis of many slides.

In conclusion, ISH on whole-mount present clear advantages in many instances, for example to get an idea of the overall and dynamic expression patterns of a gene during the regeneration process. However, because of the limitations of the whole-mount procedures in detecting expression in internal structures, we strongly favor and recommend performing ISH on sections to examine gene expression patterns in regenerating fins.

Experimental Procedures

Animals

Adult zebrafish ranging from 6 to 12 months old obtained from our animal colony and maintained at 28°C were used for all experiments.

Fin Amputation and Fin Regenerate Preparation

Zebrafish were anesthetized using 0.6M tricaine and caudal fins were amputated below the origin of bifurcation. Regenerates were then collected at 4 days following amputation. They were fixed overnight at 4°C in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), then washed twice in PBS and twice in methanol for 5 min each and finally transferred to methanol. Fixed fin regenerates were stored at -20°C until processed for whole-mount *in situ* hybridization or for cryo-sectioning.

Antisense RNA Probes

Antisense RNA probes were synthesized *in vitro* as previously described using the following cDNA fragments; *fgfr1*, *wfgf* (2.5 and 2.4 kb, respectively) (Poss *et al.* 2000b); *msxB*, *msxC* and *msxD* (1.12, 2.02, and 1.15 kb, respectively) (Akimenko *et al.* 1995); *col10a1* (713 bp) (Smith *et al.* 2006); *lef1* (2.8 kb) (Poss *et al.* 2000a).

***In Situ* Hybridization on Cryo-Sections**

Following storage at –20°C, fixed fin regenerates were rehydrated in a methanol-PBS series, then embedded in 1.5% agar/ 5% sucrose dissolved in PBS. Embedded fins were cut into blocks for cryo-sectioning and placed in 30% sucrose overnight. Blocks were frozen in Shandon Cryomatrix (Thermo Scientific) mounting medium in isopentane kept at -80°C. Frozen blocks were sectioned with a 16-µm thickness. Sections were

transferred onto superfrost plus slides (Fisher) and stored at -20°C until needed for *in situ* hybridization. Frozen slides were thawed for 1 hr at room temperature (RT). Edges were sealed using a pap-pen to keep liquid on the slide. Probes were diluted in hybridization buffer (1X Salt solution [1X Salt: 0.2 M NaCl, 10 mM Tris HCl, 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 1 mM Tris-base, 5 mM EDTA], 50% deionized formamide, 10X dextran sulphate, 1 mg/ml yeast tRNA and 1X Denhardts) at a concentration of 1:200. The probe mix was denatured at 70°C for 5-10 min, then transferred on ice. Slides were placed in a sealed Tupperware box lined with paper towel and Whatman paper soaked with 1X Salt solution in 50% formamide to make a humidification chamber. Three hundred microliters of the probe mix was added to each slide that was then covered with a coverslip and placed in an oven at 65°C overnight. For washes, slides were transferred to a coplin jar and washed 1X 15 min at 65°C in solution A (1X SSC, 50% formamide, and 0.1% Tween 20) to allow coverslips to fall off, then 2X 30-min washes in solution A at 65°C, followed by 2X 30 min in 1X TBST at room temperature (RT). Slides were then blocked in 10% calf serum in 1X TBST (blocking solution) for >1 hr at RT. For incubation with the antibody, the slides were placed back in a sealed Tupperware with water-soaked paper towels at the bottom. Three hundred microliters of 1:2000 dilution of anti-DIG AP Fab fragment antibody (Roche) in blocking solution was added to each slide that were then covered with a coverslip and incubated overnight at 4°C. Slides were placed in coplin jars and washed 4-5X for 20 min in 1X TBST at RT. Slides were then equilibrated for 2X 10 min in NTMT staining buffer (100 mM NaCl, 100 mM Tris HCL pH 9.5, 50 mM $MgCl_2$ and 0.1% Tween 20). For staining, slides were placed in large Petri dishes lined with wet Whatman paper and covered with aluminium foil. Each slide

was covered with 400 µl of NTMT containing 1.4 µl of BCIP and 2.7 µl of NBT. Staining was performed for 10 min - 2 hr (depending on the probe). The reaction was stopped by 2-4X 10-min washes in PBST followed by fixation in 4% PFA overnight at 4°C. Slides were subsequently washed in distilled water and allowed to dry before mounting with coverslips using Aquamount (Fisher).

Whole-Mount *In Situ* Hybridization

Following storage at –20°C, fixed fin regenerates were rehydrated in a series of methanol and PBS washes and then washed three times in PBS-0.1% Tween 20 (PBST). Whole-mount *in situ* hybridization was performed as previously described by Poss *et al.* (2000a). Fins were then fixed in 4% PFA in PBS and processed for cryo-sectioning, as described above. Pictures of whole-mount and sections were taken using the Eclipse and Axiovision programs on Leica and Zeiss microscopes, respectively.

Immunostaining

Immunodetection on cryo-sections of 4-dpa regenerates using the Zns-5 antibody was performed as previously described in Smith *et al.* (2006). Immunostaining of whole-mount fin regenerates was done as follows. Fin samples were rehydrated in a methanol/PBS series and washed 3 times in PBS for 5 min. Fins were then digested in 8 µl of 10mg/ml of proteinase K in PBS for 30 min, then washed in PBST 2X for 5 min and fixed in 4% PFA in PBS. Whole-mount fins were blocked in 10% calf serum in PBST (blocking solution) for a minimum of 1 hr at RT and then placed in 1:200 dilution of the Zns-5 monoclonal antibody (Zebrafish Resource Centre, Eugene, OR) in blocking

solution overnight (O/N) at 4°C. The next day, fins were washed 3X 20 min in PBST and incubated again in blocking solution for at least 1 hr at RT and placed O/N in 1:150 dilution of biotinylated anti-mouse IgG secondary antibody (Vector Laboratories). Zns-5 antibody detection was visualized with a Peroxidase ABC-vectastain kit (Vector Laboratories, PK4001). Following antibody detection, fins were washed in PBS and processed for cryo-sectioning.

Picrosirius Red Staining

Cryostat sections were processed as for *in situ* hybridization on sections and then processed according to the protocol described by Junqueira *et al.* (1978). Briefly, the sections were stained in a saturated picric acid solution with 2% (w/v) Sirius Red (Sigma) for 30 min. Sections were washed quickly in water to remove excess staining solution. Sections were then dehydrated in 100% ethanol for 10 min twice and cleared in xylene and mounted with DPX mountant for histology (Fluka).

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Chapter 5: General Discussion

The study of the regeneration process in vertebrates, such as the zebrafish, can be useful to the medical field for a number of reasons. Firstly, since mammals only possess limited regeneration ability, we must acquire knowledge about the cellular and molecular processes that regeneration competent animals, such as fishes or urodele amphibians, possess that allow them to regenerate instead of forming scar tissue. One of the goals of this field of research is to eventually be able to regenerate human tissues and organs. The key to this ability to regenerate is that in response to an organ being damaged or lost, urodeles and fish form a 'blastema' of undifferentiated, proliferating cells that will perfectly replace the lost structure. Somehow through vertebrate evolution most animals have lost their ability to form a blastema, which is surprising since most genes and molecules, as well as their expression and function, have been conserved among vertebrate species (Masaki *et al.* 2007; Nakatani *et al.* 2007). Therefore, it is important to understand how those genes involved in blastema formation are regulated and to dissect the molecular signaling pathways involved in this process. The second interest in studying regeneration is the similarities it shares with the ontogeny process, the "normal" formation of adult structures through development. For instance, many studies of urodele limb regeneration and development have shown that these two processes are very similar both at the cellular and the molecular levels (Muneoka *et al.* 1992). One of the major differences is that during limb development the cells that contribute to the developing limb arise from undifferentiated progenitor cells, whereas in the regenerating limb fully differentiated cells de-differentiate before reforming the missing structure (Blomme *et al.* 1999; Nye *et al.* 2003; Hutchison *et al.* 2007). Also, the outgrowth phase, which involves

growth, patterning and the eventual differentiation of the blastema cells, is thought to be a recapitulation of developmental events. Supporting this idea, many developmental genes and signaling pathways are being reactivated during regeneration (Muneoka *et al.* 1992; Torok *et al.* 1998; Roy *et al.* 2000, 2002). The regenerating zebrafish caudal fin provides a convenient and useful model system to examine the cellular and molecular mechanisms underlying development processes. Many researchers choose to study the mechanisms controlling fin development by looking at fin regeneration, because the timing can be controlled, fin surgeries are relatively simple and the growth rate of the regenerating fin is three to five times faster than during ontogenetic growth (Iovine 2007).

Therefore, the zebrafish regenerating caudal fin is an excellent model system to examine the expression and function of genes and pathways involved in initiation of regeneration (specifically cell de-differentiation and blastema formation and proliferation), and also those involved in regenerative outgrowth and patterning of the fin, which are most likely similar to the factors involved in development of the fin. Thus, by examining the genes and pathways involved in differentiation and patterning of the scleroblast we will gain insight into the development and regeneration of the bony fin rays.

The regenerating fin is also a valuable system to study dermal bone formation for many reasons; the forming bone rays are visible and easily accessible for experimental manipulation, the function of signaling pathways can be easily tested using pharmalogical drugs and injection of inhibitors to directly and simply assess their role in the proliferation, differentiation and function of scleroblasts and overall bone morphology. Finally, the expression of molecular markers within various cell types can be assessed using *in situ* hybridization or immunostaining. Overall the zebrafish fin model allows

monitoring of various processes taking place during fin regeneration at morphological, cellular and molecular levels, while bypassing the potential problem of lethality with genes involved in early development.

Finally, a third and less studied aspect of the regeneration process in such animals as zebrafish is to learn more about the control mechanisms involved in triggering cell proliferation in an adult animal. Recent studies have shown that many embryonic pathways are believed to affect the survival and proliferation of tumor cells and to orchestrate a complex microenvironment that promotes tumor survival and progression (Marino 2005; Bailey *et al.* 2007). Many of these embryonic pathways are also activated during fin regeneration making it an excellent model system to study how they induce proliferation in adult organisms and investigate approaches to inhibiting these signaling molecules.

The Hedgehog, WNT, FGF and BMP extracellular signaling pathways are found across animal species and network together during embryogenesis, tissue regeneration, and carcinogenesis. Therefore our laboratory undertook the study of investigating the function of Bmp and Hh signaling pathways in zebrafish fin regeneration and focused on their roles in 1) blastemal proliferation and outgrowth, and 2) dermal bone regeneration, specifically scleroblast differentiation and function. Based on these experiments, and those of others, we were able to formulate a model (Fig. 4.1) of the signaling mechanisms active during fin regeneration that mediate both blastemal proliferation and bone regeneration.

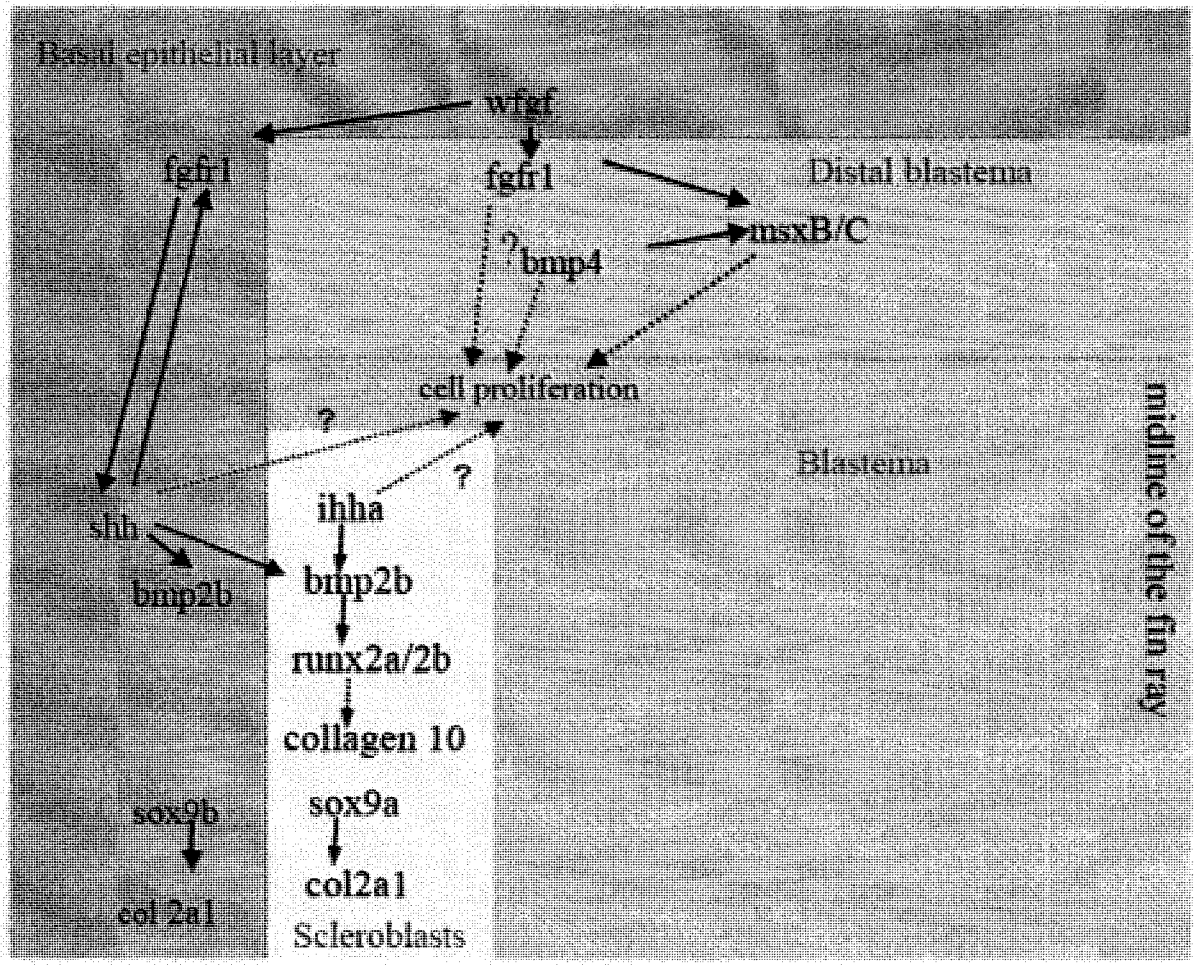
Basal epithelial layer
wfgf
fgfr1
fgfr1
Distal blastema
msxB/C
?
bmp4
cell proliferation
?
?
Blastema
midline of the fin ray
ihha
shh
bmp2b
bmp2b
runx2a/2b
collagen 10
sox9a
sox9b
col2a1
col 2a1
Scleroblasts

Figure 5.1

Figure 5.1: Model of molecular signaling pathways involved in both outgrowth of the regenerate, and bone formation and patterning.

Black arrows indicate pathway signaling and red and black dashed arrows indicate possible function. Pink area represents blastema region, blue area represents distal blastema, green area represents basal epithelial layer and yellow represents scleroblasts.

The role of Shh and Bmp signaling pathways in fin regeneration

Blastemal initiation, proliferation and outgrowth

The expression of both *shh* and *bmp4*, in the basal layer of the epidermis and the distal blastema cells, respectively, indicated their possible role in blastemal proliferation and growth (Laforest *et al.* 1998; Murciano *et al.* 2002). We demonstrated that inhibition of both of these molecules resulted in a significant decrease in blastemal cell proliferation and growth of the regenerate. Inhibition of Bmp signaling within the regenerate, through misexpression of chordin, resulted in a decrease in *msxb* and *msxc* expression, which is the suspected cause of this decrease in proliferation. Treatment of adult fish, whose fins have been amputated, with cyclopamine, a pharmacological inhibitor of Hh signaling, also resulted in an inhibition of fin outgrowth and a decrease in blastemal proliferation. Although it is unknown what direct pathway Shh signaling acts through to induce cell proliferation, one possibility is that it also acts indirectly to induce and/or maintain *msxb* expression in the distal blastema. It has been hypothesized that Fgf and Shh signaling pathways form a feedback loop within the regenerate to mediate blastemal proliferation, similar to what is seen during the development of many organs including the limbs, and it is in fact Fgf signaling that maintains *msxb* expression in the distal blastema to sustain proliferation during outgrowth (Vogel *et al.* 1995; Wang *et al.* 1995; Kettunen *et al.* 1998; Poss *et al.* 2000b). The overlapping expression of *fgfr1* and *shh* in the basal layer of the epidermis and *fgfr1* expression in the distal blastema could indicate such an interaction in the fin regenerate (Laforest *et al.* 1998; Poss *et al.* 2000b). Further evidence for this feedback loop and its importance in blastemal proliferation comes from the fact that inhibition of Fgfr1 using SU5402 results in not only a decrease in cell proliferation

and a block in regenerate outgrowth but also a downregulation of *shh* and *msxb* expression (Poss *et al.* 2000b). To date, *msxb* and *fgfr1* expression following Shh inhibition has not been described. Based on the studies done by our laboratory and others, the key to blastemal proliferation and growth seems to be the Msxb (or Msxc) transcription factor, since down-regulation of *msxb* expression results in decreased proliferation in the proximal blastema (Poss *et al.* 2000b, 2002; Nechiporuk *et al.* 2002, 2003; Beck *et al.* 2006). In conclusion, the secretion of the Bmp, Fgf and Shh signaling molecules from cells within the vicinity of the distal blastema induces the msx transcription factor within this region whose function is to regulate cell division within the blastema and promote outgrowth of the regenerate. Therefore the key to the regeneration process may be the induction of the msx transcription factors.

Bone regeneration and development

To test the function of Shh and Bmp signaling in bone ray regeneration, we ectopically expressed both *shh* and *bmp2b* between sister-ray branches, where they are not normally expressed. We found that ectopic expression of both *shh* and *bmp2b* led to ectopic bone deposition in regions between the basal layer of epidermis and mesenchyme where scleroblasts are normally located, suggesting that they may play a role in scleroblast differentiation and function. We also have shown that Shh is acting through a Bmp factor to mediate ectopic bone formation, since when we co-injected *shh* and *chordin*, a Bmp antagonist, we blocked ectopic bone formation. The role of Bmp signaling in bone regeneration was further explored by examining the effect of inhibiting Bmp signaling directly within the regenerate. We found that Bmp inhibition resulted in delayed bone

mineralization, a decreased number of scleroblasts on lateral edges of lepidotrichia, and reduced bone thickness. This indicated that Bmp signaling pathway likely plays a role in scleroblast differentiation and function. In other organisms Bmp2, 4, 6 and 7 are documented as the family members playing the most vital roles in skeletogenesis. *bmp2b* expression within the basal layer of the epidermis and scleroblasts, and the ectopic bone that forms following its misexpression strongly indicates its role in bone formation within the regenerate. We isolated and characterized the zebrafish *bmp6* gene and showed its expression in bone-secreting cells. Based on their expression patterns, it is likely the inhibition of Bmp2b and Bmp6 signaling that is the cause of the skeletal phenotypes observed in the regenerating fin following *chordin* injection, or a yet uncharacterized Bmp molecule within the regenerate. One possibility is *bmp7*, to date its expression and function have only been examined during early embryogenesis and further examination of its expression during zebrafish fin regeneration may be important to decipher what Bmp family members play roles in bone regeneration (Dick *et al.* 2000). Due to importance of members of the Bmp signaling family, including Bmp2, 4 and 7, in early embryonic patterning in most organisms, it has been very difficult to analyze their function in vivo during skeletogenesis since typical knockout experiments of any of these genes result in embryonic lethality (Winnier *et al.* 1995; Ying *et al.* 2001). Therefore, our inhibition studies within the regenerate highlights the value of the adult zebrafish caudal fin as a model to study loss of function of the Bmp signaling pathway in bone formation. Therefore, by inhibiting Bmp signaling within the regenerate we were able to identify the downstream signaling molecules involved in this pathway that are important for scleroblast differentiation and function.

Bmp signaling pathway members involved in bone regeneration and development

Based on what is known about the Bmp signaling pathway in bone development in other organisms we were able to characterize the Bmp pathway involved in scleroblast differentiation and function in the fin regenerate. We found that Bmp2b acts through the duplicated Runx2 osteogenic transcription factors expressed in scleroblast cells to mediate their differentiation and function. Furthermore, inhibition of Bmp resulted in a downregulation of *col10a1* expression, which is a known downstream target of the Runx2 transcription factor in chick and mouse (Yang *et al.* 1994; Drissi *et al.* 2003; Zheng *et al.* 2003). This pointed to the conclusion that the morphological effects seen in the fin rays following Bmp signaling inhibition are likely a result of blocking the activation of Runx2a/b and Col10a1 in scleroblast cells, therefore affecting their differentiation and function. Interestingly, we also detected the expression of many genes that were previously only associated with formation of the cartilage template during endochondral bone formation in other organisms. We found that markers expressed during chondrocyte differentiation within long bones of other organisms were at one point expressed in the fin regenerate that is devoid of cartilage. *sox9a*, *col2a1*, *ihha* and *col10a1* are markers of proliferating, pre-hypertrophic and hypertrophic chondrocytes, respectively, and have previously only been associated with cartilage formation. Interestingly, we found that they are all expressed in the scleroblasts of the fin regenerate, the equivalent to the osteoblast, which have not been shown to express chondrocyte markers. Expression analysis following Bmp inhibition concluded that many of these genes also appeared to be regulated by Bmp signaling. *chordin* injection resulted in a

downregulation of *sox9a* and *col2a1*, which is interesting since Sox9a has been shown to directly regulate Col2a1 in chondrocyte differentiation. These results indicate that Bmp signaling induces Sox9a, which in turn activates Col2a1 in the scleroblasts of the fin regenerate as during chondrocyte differentiation. Therefore it is possible that Col2a1 and Col10a1 have important functions in dermal bone formation in the fin regenerate and in order to have both collagens produced in the same cell type Bmp signaling must activate both *sox9a* and *runx2a/b* in order to activate the transcription and therefore production of Col2a1 and Col10a1.

The use of the zebrafish caudal fin to study dermal bone formation

The expression of genes thought to be cartilage-specific in the regenerating fin, which is devoid of cartilage could be explained by two possible scenarios: 1) the regenerating lepidotrichia represents a special type of intramembranous bone that possesses a cartilaginous phenotype and is an intermediate between endochondral and intramembranous bone and/or 2) the process of intramembranous ossification is not as well characterized as endochondral ossification which has been extensively studied and intramembranous bone could actually be closer to endochondral bones than previously thought. In a review by Fang and Hall, the authors suggested that chondrogenic capacity does exist in membrane (intramembranous) bone and that under certain circumstances cartilage or the cartilaginous phenotype is seen in membrane bone in vivo (Fang *et al.* 1997). The characteristics of one of these circumstances could fit with the bone of the zebrafish fins. Chondroid bone is found in some intramembranous bones and is described as a tissue which presents characteristics of both intramembranous bone and

cartilage and is thought to be an intermediate between the two (Haas 1962). This tissue has been found in some developing craniofacial bones and is made up of cells that are larger than osteogenic cells and express some chondrogenic markers such as type II collagen, but its extracellular matrix has bone-like (appearance or morphology) and contains (bone-specific) type I collagen (Goret-Nicaise *et al.* 1982, 1984, 1988; Lengele *et al.* 1990, 1996). Chondroid bone appears when membrane bones are undergoing fast growth and never becomes a genuine cartilage (Lengele *et al.* 1990). The dermal bone of the zebrafish fin regenerate fits many of these characteristics: the bone matrix-secreting scleroblasts express chondrogenic markers including *col2a1*, lepidotrichia grow very quickly especially during regeneration, and finally alcian blue staining showed that there are no cartilage cells present in the caudal fin regenerate of zebrafish. Interestingly, Fang and Hall also raised the possibility that chondroid bone may actually represent a particular differentiation stage of osteoblasts during intramembranous bone formation in which cells exhibit both bone and cartilage characteristics (Fang *et al.* 1997). Therefore it is possible that our study, which demonstrates the expression of both cartilage and bone molecular markers within the scleroblasts may represent a piece of evidence in favor of a previously uncharacterized stage of osteoblast differentiation important for intramembranous bone formation. In 2007, a study done by Abzhanov *et al.*, (2007) was conducted to better understand the development of dermal (intramembranous) bone within the craniofacial elements of the chick and mouse, and to achieve a molecular and cellular understanding of this process, comparable to what is available for endochondral bone development. They found that a number of markers that have previously been associated with endochondral skeleton development were in fact involved in the dermal

ossification process. In a way that resembles endochondral ossification, Abzhanov *et al.*, (2007) found a series of distinct cellular steps involved in dermal bone formation, including a transitional cell type, a chondrocyte-like osteoblast (CLOs). CLOs co-express both osteogenic and chondrogenic markers in mouse and chick and are precursors to the mature osteoblasts. They also express *osteopontin* (osteoblast marker), as well as *Coll II*, *Coll IX* and *IHH* (chondrocytes markers) (Abzhanov *et al.* 2007). Therefore the osteoblast, like the chondrocyte, has more than one stage of differentiation. The authors also examined the roles of Bmp signaling during intramembranous bone development and found that both Bmp2 and Bmp4 are necessary for intramembranous ossification, but also have the ability to induce endochondral ossification (Abzhanov *et al.* 2007). The results of our study (Smith *et al.* 2006) and those by Abzanhov *et al.*, in 2007, strongly suggests that intramembranous bone formation is similar between fish, chick and mouse. These findings once again stress the value of using the zebrafish caudal fin to study both the cellular and molecular mechanisms behind dermal bone formation.

The importance of using the proper *in situ* hybridization technique to analyze the expression of genes during zebrafish fin regeneration

In situ hybridization has been used as a major tool in deciphering the potential function of a gene during zebrafish development and regeneration. A large scale *in situ* hybridization screen of thousands of cDNA clones was performed through all stages of zebrafish development and genes with restricted expression patterns were selected and photographs were posted on a website available to all zebrafish researchers (www.zfin.org) (Crosier *et al.* 2001; Kudoh *et al.* 2001; Tamme *et al.* 2001). The majority of the work we have done attempting to characterize the Bmp and Shh signaling pathways active during fin

regeneration has been done using *in situ* hybridization to detect the expression of genes involved in these pathways and also examining their expressions following misexpression and inhibition of both Bmp and Shh signaling. Detection of mRNA transcripts, using *in situ* hybridization, is extremely widely used in the study of zebrafish development and regeneration due to the fact that there are very few antibodies available in zebrafish to detect protein localization. The majority of laboratories working on zebrafish development and regeneration perform *in situ* hybridization on whole-mount embryos or regenerates to observe the entire domain of expression and if they wish to examine the exact cell type expressing the gene they perform cryo-sections of the whole-mount *in situ*. In our studies, examining gene expression during fin regeneration, we found that this method did not give the full expression pattern of the gene of interest possibly because of the inability of the mRNA probe to cross the collagenous matrix that surrounds the mesenchymal core of the regenerate. By performing *in situ* hybridization on cryo-sections, we found that expression patterns of many already published genes were different than previously stated. One important example of this differential expression pattern obtained using the different *in situ* hybridization techniques, whole mount followed by sectioning vs. *in situ* on sections, is the expression pattern of the *msxb* gene. Nechiporuk *et al.*, (2002) reported that during regenerative outgrowth, *msxb* expression was limited to a small number of non-proliferating distal blastema cells. The majority of proliferating blastema cells did not express *msxb*, based on whole-mount *in situ* followed by sectioning (Akimenko *et al.* 1995; Nechiporuk *et al.* 2002). Based on these expression patterns they concluded that up-regulation of *msxb* expression may be necessary to inhibit proliferation in the DMB during regenerative outgrowth (Nechiporuk

et al. 2002). We found, following *in situ* on cryo-sections in the outgrowth phase, that *msxb* is actually expressed in a much wider domain with transcripts being detected in the majority of cells of the proliferating blastema and also in a subset of scleroblast cells in the more distal blastema. Therefore in the future, it will be necessary for researchers studying fin regeneration to perform *in situ* on cryo-sections when investigating the function of a gene in a certain cell type in order to obtain the complete expression pattern.

Regenerative medicine studies the ability to rebuild organs and tissues, and it requires a deep understanding of the fundamental principles underlying the regeneration, especially the several molecular pathways involved in this process. Therefore, understanding the coordinate action of signaling pathways that stimulate blastema maintenance and growth in species that are able to regenerate organs during adulthood establishes the basis required to develop methods, drugs or tools that could be effectively used to induce a regenerative response to injury, trauma or degenerative event in species normally unable to trigger such a response.

Also, understanding ways to inhibit proliferation-inducing pathways activated in adult organisms, such as cyclopamine and chordin treatments may be valuable in treating the growing number of cancers that are associated with the re-activation of developmental signaling pathways.

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