Unravelling the Induction and Development of

Intramembranous Bones in the Chicken Eye

By

Kellie Duench

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Approved:	Dr. Roger H. Sawyer External examiner
Approved:	Dr. Tamara Franz-Odendaal Senior Supervisor
Approved:	Dr. Genlou Sun Supervisory Committee Member
Approved:	Dr. Brian K. Hall Supervisory Committee Member
Approved:	Dr. Christian Suteanu Program Representative
Approved:	Dr. Kevin Vessey Dean of Graduate Studies

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Dedicated To:
Mom and Dad (The Bankers). I can't describe how much you've done for me, without you this thesis would not have been possible.
&
Janet Kennedy. You have passed on some of your strength to me. I take you with me. R.I.P.

Table of Contents

Title Page i
Dedication ii
Table of Contents iii
List of Figures v
List of Tables vii
Abstract viii
List of Abbreviations ix
Acknowledgements x

1.0 Introduction 1
1.1 The skeleton: an introduction 1
1.2 Intramembranous vs. endochondral ossification 2
1.3 A description of scleral ossicles and their development 4
1.4 Potential developmental pathways involved in scleral ossicle induction 10
1.4.1 Hedgehog family 10
1.4.2 The bone morphogenetic protein family 11
1.4.3 Other candidate genes 13
1.5 Objectives 15

2.0 Materials and Methods 16
2.1 Chicken embryos 16
2.2 Staging chicken embryos 16
2.3 Microsurgery 17
2.3.1 Tools 17
2.3.2 Shell-less culturing (*ex-ovo*) 18
2.3.3 Bead implantation 23
2.3.4 Alkaline phosphatase stain to visualize ossicle formation 25
2.4 Probe preparation and whole mount *in situ* hybridizations 25
2.4.1 Amplification of cDNA plasmids 25
2.4.2 Isolation of plasmid 27
2.4.3 Linearization of plasmid cDNA 28
2.4.4 cDNA purification and gel electrophoresis 29
2.4.5 Synthesizing the RNA probe 29
2.4.6 Whole mount *in situ* hybridization 31
2.4.7 Optomizing the whole mount *in situ* hybridization protocol 32
2.4.8 Sectioning whole mount *in situ* hybridization samples 33
2.5 Photography and image analysis 34

3.0 Results 35
3.1 Inhibition of *BMPs* using affi-gel beads soaked in noggin 35
3.1.1 Results of PBS vs. noggin soaked bead implantations 35
3.1.2 Embryo survival rates after microsurgery 36
3.1.3 Further interpretation of the noggin bead implantations 40
3.2 Determining if *indian hedgehog* is present during the induction of scleral ossicles 45
3.3 Determining where *shh* is received through the expression pattern of the Hedgehog family receptor *patched* 47
3.4 Determining if the other candidate genes *scleraxis* and *msx2* are present during the induction and development of scleral ossicles 51

4.0 Discussion 56
4.1 Application of exogenous noggin inhibits ossicle formation 56
4.2 *BMPs* involvement in the developmental pathway of scleral ossicles 59
4.3 Neighbouring ossicles demonstrate the ability to compensate for a missing ossicle 63
4.4 *Sonic hedgehog* is likely the only member of the *hedgehog gene* family involved in the induction and development of scleral ossicles 64
4.5 *Sonic hedgehog* acts as an autocrine and paracrine signal during the development of scleral ossicles 65
4.6 The role of *Scleraxis* during scleral papillae development 70
4.7 *Msx2* not involved in condensation growth 73

5.0 Conclusions 74

6.0 Bibliography 79

7.0 Appendices
A. LB/ampicillin plates 84

B. Plasmid isolation protocol and recipes 85

C. Standard RNA labelling reaction and preparation of probe dilutions 86

D. DIG high prime DNA labelling and Detection protocol and recipes 88

E. Whole mount.*in situ* hybridization protocol and recipes 91

List of Figures

Figure 1:	Diagrammatic representation of a cross-section through a single scleral papilla of a stage HH35 chicken eye.	8
Figure 2:	Schematic representation of the patterning of scleral papillae development.	8
Figure 3:	Timeline for the development of scleral ossicle modified from Franz-Odendaal (2008).	9
Figure 4:	The *ex ovo* culturing technique.	21
Figure 5:	Bead implantation surgery.	24
Figure 6:	Dot blot results for the *indian hedgehog* and *msx2* probes.	30
Figure 7:	Inhibition of *BMPs* by noggin soaked bead implantations results in the inhibition of ossicle formation	38
Figure 8:	Alkaline phosphatase stained embryos after bead implantation	43
Figure 9:	Location of *shh* and *ihh* mRNA through whole mount *in situ* hybridization expression.	46
Figure 10:	Location of *ptc* mRNA based on whole mount *in situ* hybridization expression in eyes of embryos from stages HH34-37	49
Figure 11:	Location of *scleraxis* mRNA based on whole mount *in situ* hybridization expression in eyes from stages HH34-37.	53
Figure 12:	Location of *msx2* expression in the chicken head.	55
Figure 13:	Possible locations for BMP signals during the induction and development of scleral ossicles.	62
Figure 14:	Schematic showing suggested autocrine and paracrine signalling pathway for *shh* during scleral ossicle development based on the *in situ* hybridization expression of the *ptc* receptor	69
Figure 15:	Schematic showing hypothesized *scleraxis* involvement in the development of scleral papillae based on *in situ* hybridization expression	72

Figure 16: Suggested molecular pathway involved in the induction and development of scleral ossicles 78

List of Tables

Table 1:	Spectrophotometer results for the *patched* DNA sample	27
Table 2:	Reaction conditions for linearization of amplified cDNA and synthesizing RNA probe.	28
Table 3:	Survival rates for *ex-ovo* embryos that underwent microsurgery	37
Table 4:	Comparison between the eyes of individual embryos that were affected by the implantation of a noggin bead	42
Table 5:	*Ihh in situ* hybridization expression pattern in embryos stages HH 33, HH 35/36, and HH 38	45
Table 6:	*Patched in situ* hybridization expression pattern in the eyes of stages HH 30 through HH 40	48
Table 7:	*Scleraxis* and *msx2 in situ* hybridization expression pattern in embryos at stages HH 30 through HH 40.	52

Abstract

"Unravelling the Induction and Development of Intramembranous Bones in the Chicken Eye"

By: Kellie Duench

Bone development is a complex process, involving multiple tissues and hierarchical inductive interactions. The study of skeletal development has focused on endochondral bones while intramembranous bones (e.g. scleral ossicles) have received less attention. Although, *sonic hedgehog* was recently demonstrated to be present during induction of scleral ossicles, other signalling molecules are still unknown. Using *in situ* hybridization and bead implantation I have demonstrated the presence or absence and the involvement of candidate genes and gene families during the development of scleral ossicles. *Scleraxis*, p*atched*, as well as the bone morphogenetic protein (BMP) family of genes, were found to be involved in the development of scleral ossicles. Conversely, it was determined that *indian hedgehog* and *msx2* are not present. This study has contributed to unravelling the signals involved during the induction and subsequent development of scleral ossicles and has contributed to the current understanding of intramembranous bone development.

September 29, 2010.

List of Abbreviations

BCIP - 5-Bromo 4-Chloro 3'-Indolyphosphate p-Toluidine salt

BMP – bone morphogenetic protein

DepC - Diethyl pyrocarbonate

dhh – desert hedgehog

dH2O – distilled water

EDTA- Ethylene Diamine Tetra-acetic Acid

EtOH – Ethanol

ihh – indian hedgehog

MeOH - Methanol

NBF – Neutral Buffered Formalin

NBT- Nitro- Blue Tetrazolium

PBST – Phosphate Buffered Saline Tween

PFA – Paraformaldehyde

PVA – Polyvinyl Alcohol

RPE – Retinal Pigmented Epithelium

shh – sonic hedgehog

TBST – Tris-Buffered Saline 0.1% Tween

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Mom, Dad, Graham (my comedic relief!), Jill, Grandma and Grandpa (S and D), and all my family – I say thank you. I know you continuously have me in your thoughts and prayers. I love and appreciate every one of you and couldn't do this without you. Chris, none of this would have been possible without you. For everything you have done and continue to do for me – Thank you! To the Ellis and Cloney families, thank you for accepting me like family (no matter how stressed I was) and always giving me your love and support. To all my best friends: Kathy, Stephanie, Nadalie, and Nicole, thank you for your continuous love and support. You are my angels.

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1.0 Introduction

1.1 The skeleton: an introduction

Skeletal development is a complex, step-wise, hierarchal process. The skeleton is made up of two main groups; appendicular and axial (Steele and Bramblet, 1988). The axial skeleton consists of bones in line with the centre axis, the spine. Therefore, the vertebral column, the ribs, and bones of the craniofacial skeleton including the calvariae (intramembranous bones of the skull vault) are all part of this group. The appendicular skeleton consists of all the bones in the appendages, lower limbs, upper limbs, and the sockets/girdles that hold the limbs in place (pectoral and pelvic girdles).

Within the axial skeleton, the bones of the craniofacial skeleton are derived from neural crest cells that migrate from the neural tube and invade the craniofacial region, to form the craniofacial mesenchyme. The mesenchyme is then involved in the signalling and induction for a variety of craniofacial bones, such as calvaria, bones of the jaw, and bones of the inner ear. Neural crest derived mesenchyme is not involved in all types of bone development. In the appendicular skeleton, the bones are typically derived from the somatic lateral plate mesoderm (Hall, 2005).

In summary, the cells that give rise to these two skeletal systems are different (mesoderm vs. neural crest derived). The processes by which these two skeletal systems ossify are also different. There are two main types of ossification. Most craniofacial bones of the axial skeleton undergo intramembranous ossification (Gilbert, 2000). These dermal bones form directly from the mesenchyme, without a

cartilage precursor. Conversely, the limb bones of the appendicular skeleton form through endochondral ossification. Endochondral ossification occurs through the formation of a cartilage template, which is eventually replaced by bone. During both intramembranous and endochondral ossification epithelial and mesenchymal tissues interact via inductive signalling events. Previous studies have largely focused on the development of endochondral bones (specifically in the limb) (see review, Johnson and Tabin, 1997). In comparison, far less is known regarding the development of intramembranous bones. The study of calvariae has produced the majority of the information regarding intramembranous bone development (Rice *et al.* 2003., Hornik *et al.,* 2004., Gross and Hanken, 2008). However, the developmental pathways for other intramembranous bones, such as the scleral ossicles of the eye, remain unknown.

1.2 Intramembranous vs. endochondral ossification

Intramembranous bones such as some calvariae and scleral ossicles, begin to form when neural crest derived mesenchymal cells migrate to the craniofacial region and interact with the epithelium, these interactions are known as an epithelial-mesenchymal interactions. Once the epithelial-mesenchymal interactions occur, the mesenchymal cells aggregate to form a skeletogenic condensation (Hall and Miyake, 2000). The condensation of mesenchymal cells requires a variety of conditions in order to continue developing. The condensation must reach a specific size, must upregulate tissue specific genes, and must obtain proper vascularisation (Hall and Miyake, 2000). Once these specific conditions have been met within the

condensation, the mesenchymal cells differentiate into osteoblasts (the matrix secreting cells), and ultimately osteocytes. Osteocytes are the main cell type in a fully developed bone. These cells are responsible for the maintenance of the bone, through waste and nutrient exchange with the blood. Osteocytes form when osteoblasts are surrounded by the osteoid matrix they have secreted (Reviewed in Franz-Odendaal *et al.*, 2006).

The development of endochondral bone begins in a similar fashion to intramembranous bone, with epithelial-mesenchymal interactions resulting in a mesenchymal condensation. The aggregation of mesenchymal cells will differentiate into chondrocytes, the cells responsible for laying down a cartilage matrix. This forms a template of the future bone that consists of only cartilage; at the center point of that early-bone the chondrocytes will begin to undergo apoptosis and hypertrophy. As these cells undergo apoptosis, space is created within the premature bone and the vasculature penetrates. Once blood flow reaches the developing bone, nutrients, oxygen and osteoprogenitor cells are brought to the location of osteogenesis. These osteoblasts will then transform the cartilage matrix into osteoid matrix, ultimately completing the development of the bone.

In both endochondral and intramembranous bone development cell-to-cell and tissue-to-tissue signals are critical. The first signalling event is an epithelial-mesenchymal interaction. Therefore, taking a closer look at the signals involved during epithelial mesenchymal interactions could provide insights into the key factors behind the inductive mechanisms that control the proper development of the skeleton.

The primary goal of my thesis was to search for the developmental signals involved in the induction and growth of specific intramembranous bones within the eye, the scleral ossicles.

1.3 A description of scleral ossicles and their development

In vertebrates, the eye is a complex and crucial organ. In many species, including mammals, most snakes, and many amphibians, the eye is void of any skeletal structures (Franz-Odendaal and Vickaryous, 2006). The sclera, in mammals, is a dense fibrous layer of connective tissue that is continuous with the cornea. Conversely, for reptiles and teleost fish, skeletal tissues are found within the sclera. The sclera is reinforced for support with scleral cartilage, scleral ossicles, or both. Scleral cartilage can form in a variety of morphologies (from a narrow ring of cartilage to a full cartilage cup). In many cases, the sclera also develops scleral ossicles. Scleral ossicles are typically intramembranous bones, however in teleosts they develop through perichondral ossification.

In reptiles, scleral ossicles hold a slightly different position in the eye from the scleral cartilage. While the cartilage is continuous with the cornea, and forms around the posterior part of the eye, the ossicles form at the anterior limit of the sclera (Franz-Odendaal and Vickaryous, 2006). The number and morphology of scleral ossicles can vary across different taxa. In general, each scleral ossicle will grow and overlap the adjacent ossicle. This overlapping results in a ring, the sclerotic ring. Ossicles are thought to be more important for accommodation in the eye, rather than structural support. This is suggested since birds with the necessity for extreme eye accommodation (such as diving birds) have very predominant ossicles. My research

does not pertain to cartilage therefore the following information will focus on scleral ossicles, more specifically scleral ossicles in the chicken.

In the chicken, scleral ossicles are intramembranous bones that develop through interactions with conjuctival papillae, which are small clusters of epithelial cells (Coulombre and Coulombre, 1962). Despite the fact that these papillae form from the conjunctival epithelium, they are commonly referred to as 'scleral' papillae (Hamburger and Hamilton, 1951) and will be referred to as such in this thesis. These papillae can be found in direct correlation (1:1 ratio) with the number and pattern of scleral ossicles that will form (Figure 1). There is a unique sequence by which the scleral ossicles develop (first investigated and described by Coulombre and Coulombre, 1962). More recently this pattern was described in *Gallus gallus* the strain of chicken used in my research (Franz-Odendaal, 2008). In *Gallus gallus* the number of papillae present can range from 13-16 per eye. First a small group of papillae (3-4) will form over the ciliary artery, followed by another group of papillae directly across from the first group. Temporal then nasal groups of papillae form until there is a complete ring (Figure 2). The number of papillae (and therefore scleral ossicles) is often asymmetric from right to left eye in the same embryo. 50% of embryos might have asymmetry in papillae and ossicle number; however there is rarely a difference of more than one papilla/ossicle between the right and left eye (Franz-Odendaal, 2008). The range and asymmetry of the numbers of scleral papillae make a classical numbering system challenging (Figure 2).

It has been demonstrated that Scleral ossicle induction occurs after papillae formation (Figure 3). This induction occurs in the same sequence as the development

of scleral papillae. After induction, the mesenchymal cells underneath the papillae aggregate forming a condensation and the papillae degenerate. These mesenchymal condensations will then differentiation into osteoblasts, which lay down osteoid matrix. As the ossicles grow, they eventually overlap giving rise to the sclerotic ring (Figure 3).

Early studies suggest that the induction of scleral ossicles occurs during HH stages 30 to 36 (Pinto and Hall, 1991). This study also demonstrated that the induction signal occurs by a diffusible factor from the epithelium. In a later study, it was demonstrated that *sonic hedgehog* is present in the papillae during HH stage 35 and 36 and may be a potential inductive signal from the epithelium (Franz-Odendaal, 2008). It is therefore suggested that the stages when induction is likely occurring are HH 35 and 36. Therefore, it is understood that an epithelial mesenchymal signalling event is important for the induction of scleral ossicles, similar to other intramembranous bones. It is also suggested that the signalling factor during induction is diffusible. However, very little is known about exactly what genes and signalling molecules are involved in the developmental pathway of scleral ossicles.

One of the most recent studies involving scleral ossicles in chick embryos investigated one developmental gene family, the Hedgehog family (Franz-Odendaal, 2008). Through implantation of affi-gel beads soaked in cyclopamine (a Hedgehog family inhibitor) it was demonstrated that the Hedgehog family of genes is crucial to the induction of scleral ossicles. When exogenous cyclopamine was placed next to a papilla during induction, the formation of the underlying ossicle was inhibited. This suggests a role for the Hedgehog family in the induction of scleral ossicles at HH stage

35 and 36. However other members of the Hedgehog family could not be ruled out, since cyclopamine inhibits the entire Hedgehog family including *indian hedgehog* and *desert hedgehog* (Pathi *et al.,* 1999). *Desert hedgehog* is involved with sexual development and has never been implicated in bone or cartilage development in any vertebrate. *Indian hedgehog* has been shown to be involved in bone development (for example during endochondral ossification in the limb) and therefore it could potentially be involved in the induction of scleral ossicles. Determining whether or not *indian hedgehog* is involved, as well as, what role *sonic hedgehog* is playing during the induction is the one of the objectives of my Master's research.

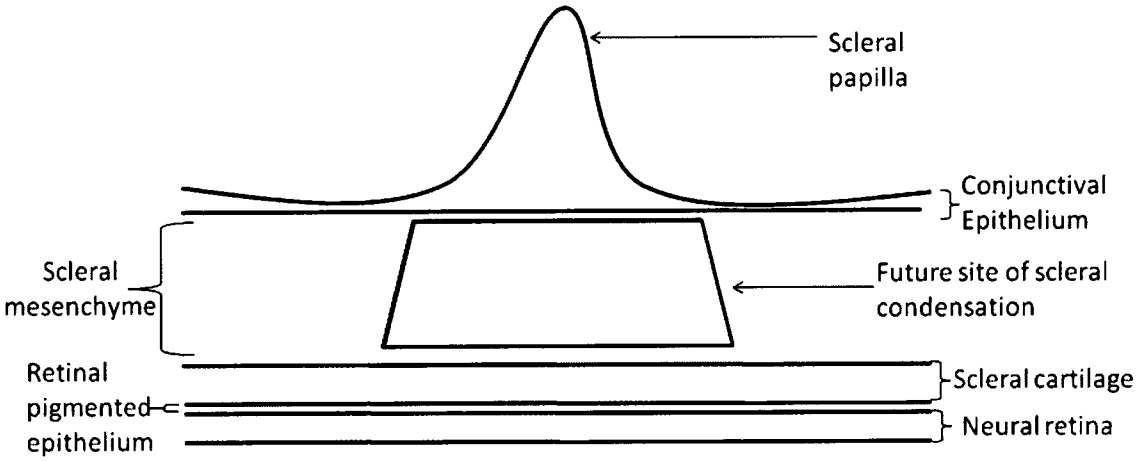


Figure 1: Diagrammatic representation of a cross-section through a single scleral papilla of a HH stage 35 chicken eye.

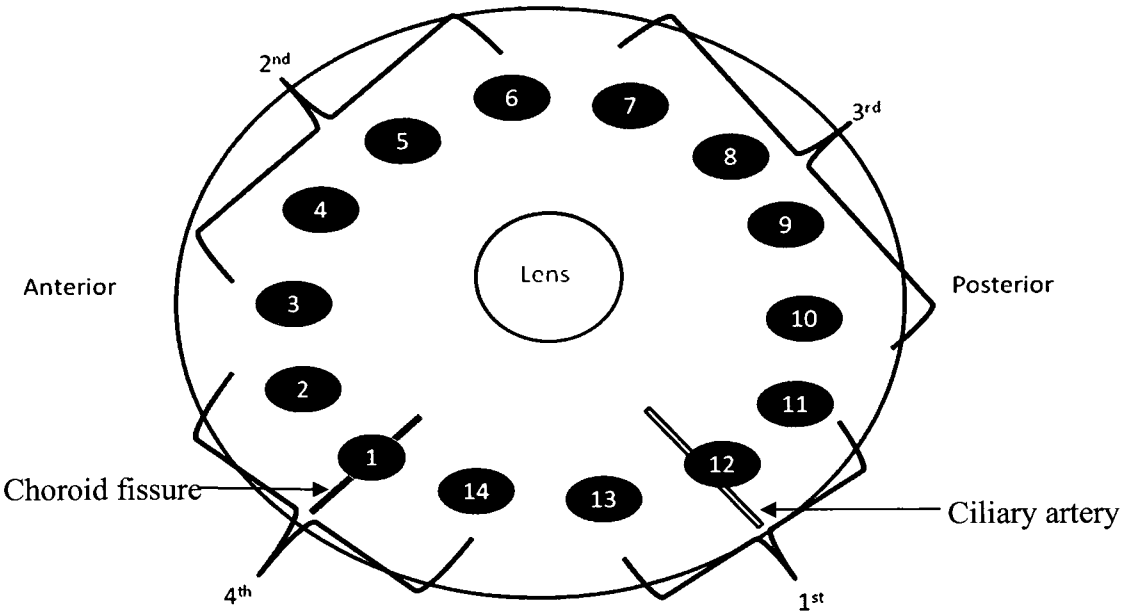


Figure 2: Schematic representation of the patterning of scleral papillae development in chicken. Numbers on the scleral papillae indicate conventional numbering of scleral papillae with 1 on the choroid fissure and 12 on the ciliary artery (Fyfe, 1980). Brackets indicate the sequence in which the papillae develop. The first papilla develops on the ciliary artery and then a couple more form adjacent to the first. Then, directly across from the first group, the second groups will form. Following that, a temporal group of papillae will form. Finally the last papillae are the group surrounding the choroid fissure.

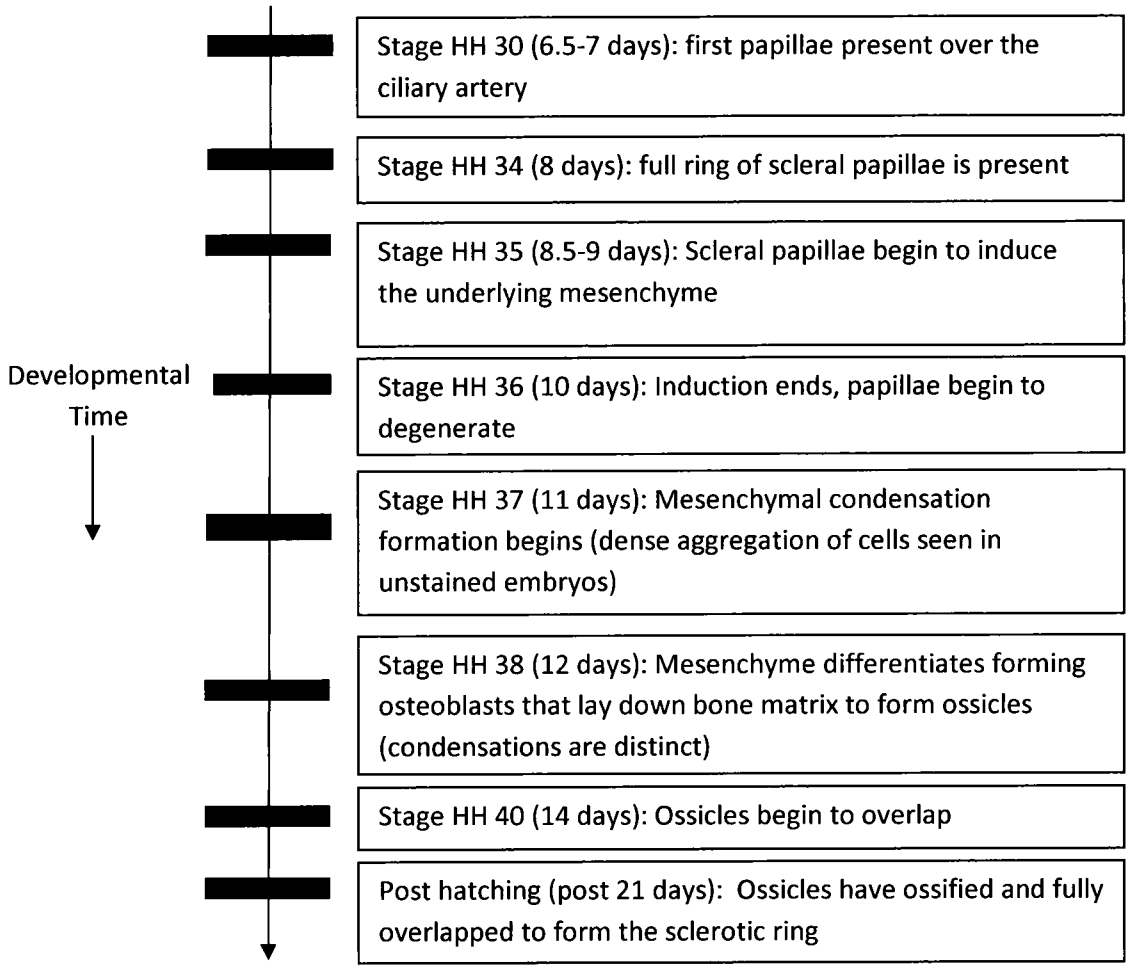


Figure 3: Timeline for the development of scleral ossicles modified from Franz-Odendaal (2008). Timeline shows Hamburger and Hamilton (1951) stages as well as embryonic days.

1.4 Potential developmental pathways involved in scleral ossicle induction

Apart from *shh,* there has been no demonstration of gene involvement during the induction of scleral ossicles (Franz-Odendaal, 2008). It was shown via real-time PCR that there is a slight increase in expression of both *tenascin C* and *bone morphogenetic protein 4* during the induction stages (HH 35 and 36) of scleral ossicle development. However, whether or not these genes play a critical role in the development of scleral ossicles is still unknown. Based on the involvement of several different genes during the induction of other bones (such as the calvariae, the dentary, and the limb bones) a number of candidate genes potentially exist.

1.4.1 Hedgehog family

Shh mRNA was found in the papillae during the induction of scleral ossicles, however it is unknown where the protein for that gene will be received since *shh* is able to signal at both long and short ranges (Drossopoulou *et al.,* 2000*)*. The location of the receptor for *shh* would give further insight into the role *shh* plays during the induction of scleral ossicles. *Patched* (*ptc*) is a transmembrane receptor that is associated with a G-protein coupled transmembrane receptor molecule *smoothened (smo)* (Carpenter, *et. al.,* 1998). *Ptc* is the ligand receptor for the entire Hedgehog family of genes. *Ptc* normally inhibits the function of *smo* in the absence of any hedgehog signal. However, when a hedgehog protein binds to the ligand receptor *ptc, smo* is no longer inhibited and a transmembrane transduction reaction occurs activating the downstream *hedgehog* target. In order to determine which cells and tissues are receiving the *shh* signal, the location and distribution of its receptor (*ptc*) is often used (Traiffort *et al.,* 1998., Harfe *et al.,* 2004).

As previously stated a prior study used cyclopamine to inhibit the entire Hedgehog family of genes and thereby preventing the formation of a scleral ossicle (Franz-Odendaal, 2008). Since cyclopamine inhibits the entire Hedgehog family, not just *shh* alone, it is unknown whether or not *shh* works in cooperation with another vertebrate Hedgehog gene such as *indian hedgehog (ihh). Indian hedgehog* is important during endochondral ossification in the appendicular skeleton. *Ihh* activates *runx2* which induces the differentiation of chondroblasts to osteoblasts (Yang, 2009). There is no evidence that *ihh* is involved during intramembranous bone development. It has been suggested that *ihh* regulates *runx2* during endochondral bone development, however during intramembranous bone development *shh* plays the role of activating *runx2* and subsequently initiating osteoblast differentiation (Yang, 2009). Therefore previous studies suggest that *shh* is likely the only Hedgehog gene playing a role in this intramembranous ossification pathway, however *ihh* can not been ruled out as a possible factor in the induction of scleral ossicles.

1.4.2 The bone morphogenetic protein family

The bone morphogenetic protein (BMP) family of genes is part of a much larger family of proteins known as the transforming growth factor beta super family (TGFβs) (Dudley and Robertson, 1997). All of these proteins are secreted proteins. Originally it was thought that there were only seven members of the BMP family, but today there are twenty recognized *BMPs* (Feely *et al.,* 2005). Studies indicate that many B*MPs* are involved in specific aspects of bone growth and development. *BMP* involvement includes; recruitment of mesenchymal cells into early bone condensations (Hall and Miyake, 2000), ensuring that neural crest derived mesenchymal cells are committed to

become osteogenic (or chondrogenic) (Abzhanov *et al.*, 2007), and the regulation (in correlation with *msx* and *fgf* genes) of differentiated and undifferentiated cells during the growth of osteogenic fronts in calvariae development (Kim *et al.*, 1998). *BMPs* are also produced as secreted signalling proteins during epithelial-mesenchymal interactions, for example during tooth development. *BMP*2, 4, and 7 signals are produced by the epithelium and induce the underlying mesenchyme as well as the epithelium itself to gain competence for further inductive events (Thesleff, 2003). The BMP family of genes is involved in a wide variety of inductive events during bone development and are therefore excellent candidates for involvement in the development of scleral ossicles. Since there are so many different possibilities for which precise member of the BMP family is involved in this developmental pathway, the most efficient way to investigate *BMP* involvement is through inhibition of the entire family of genes.

The bone morphogenetic protein family of genes has two common inhibitors, *noggin* and *gremlin. Noggin* is a secreted protein found in developmental pathways which inhibit the *BMPs* by binding to the protein and rendering it inactive (Zimmerman *et al.,* 1996). Noggin has increased affinity for *BMP2, BMP4,* and *BMP7. Gremlin* is also a secreted protein that works as an antagonist to the bone morphogenetic protein family (Hsu *et al.,* 1999). *Gremlin* acts in a similar fashion to *noggin* by binding to the secreted *BMP* proteins rendering them inactive. *Gremlin* is suggested to have the same increased affinity for *BMPs* 2, 4, and 7. Despite having very little gene sequence in common, it is thought that *gremlin* and *noggin* likely share structural similarities that allow them both to bind specifically to *BMPs.* Although,

noggin and *gremlin* are effective inhibitors of *BMP2, 4,* and *7*, *noggin* is most commonly used for implanting the protein on beads *in vivo* (Chang *et al.,* 1999, Botchkarev *et al., 2001,* Chung *et al.,* 2007, Hosoya *et al.,* 2008*).*

1.4.3 Other candidate genes

The *msx* family of genes are homeobox genes that are homologous to *Drosophila muscle segment homeobox* gene (*msh).* In vertebrates, this gene family consists of three members (*msx1, msx2, msx3*), however only *msx1* and *msx2* are found widely expressed throughout development, particularly where epithelial mesenchymal interactions occur (Alappat *et al.,* 2003). In addition, *Msx1* and *msx2* have previously been reported to be involved during the development of the facial skeleton, in the developing dentary as well as in the sutures and developing condensations of the calvariae. Specifically, *Msx2* is found in the suture mesenchyme and dura mater of the developing calvariae. In wild-type mice, *msx2* is required to maintain the proliferation of osteoblasts at the osteogenic fronts of the developing calvariae (Liu *et al.,* 1999). If *msx2* expression is increased, the population of osteoblasts present at the osteogenic front undergoes considerable growth. However, if *msx2* is inhibited then differentiation occurs prematurely resulting in craniosynostosis (the premature closure of the sutures in the skull) (Dodig, *et al.*, 1999). It has been suggested that *msx2* has a regulatory function controlling the balance between proliferation and differentiation of osteoblasts during calvariae development due to its downstream activation of *runx2* (Rice *et al.,* 2003). For this reason, it could be hypothesized that *msx2* might also play a role in the expansion and growth of scleral ossicles in the chicken eye as they start to overlap to form the sclerotic ring.

Msx1 expression is found to have a very similar pattern to *msx2* in a variety of prenatal tissue interaction sites in the craniofacial region such as, the developing calvariae, distal regions of the facial primordial, and the teeth (Alappat *et al.,* 2003). The large difference in expression between *msx1* and *msx2* is demonstrated after birth. *Msx1* continues to be expressed after birth in the late stages of skull morphogenesis while there is a sharp decline in the expression of *msx2.* Therefore when investigating the *msx* family of genes, *msx2* is more specific to pre-natal development and is therefore of more interest than *msx1 w*hen working with embryos.

Scleraxis is a member of the basic helix-loop-helix (bHLH) family of transcription factors. This family of transcription factors has been shown to play an important role in developmental processes such as cell differentiation and proliferation. *Scleraxis* is typically found in the area of chondrogenesis that precludes the development of endochondral bones such as the limb and hyoid bones (Cserjesi *et al.,* 1995). *Scleraxis* is also common in other connective tissue areas such as; tendons and ligaments, chest wall, diaphragm, tongue, and heart valves. Although *scleraxis* is not found in the developing calvarial bones or any other intramembranous bone, it has been found in the mesenchymal cells of the frontonasal region (Brown *et al.,* 1999). Despite no published account of the presence of *scleraxis* during intramembranous bone development, we were informed of its potential presence in scleral papillae (personal communication, J. Richman, UBC).

1.5 Objectives

The broad objectives of this study are to gain further knowledge and understanding about the development of scleral ossicles and intramembranous bones, in general.

The specific objectives are:

1. Determine if the *bone morphogenetic protein* family of genes is involved in the development of scleral ossicles.
2. Determine if *indian hedgehog* is present during scleral ossicle induction.
3. Determine the location and distribution of the Hedgehog ligand receptor, *patched*.
4. Determine if *msx2* and *scleraxis* are present during scleral ossicle development.

These objectives were investigated through *in situ* hybridization and affi-gel bead implantation in the chicken embryo.

2.0 Materials and Methods

2.1 Chicken embryos

Fertilized chicken eggs of the strain *Gallus gallus* were obtained from Cox Bros. Farm in Truro, Nova Scotia. Eggs were kept at 4 °C degrees for no more than one week before being incubated at 37°C with approximately 40% humidity. Eggs were turned once daily.

2.2 Staging chicken embryos

Chicken embryos were staged using the Hamburger and Hamilton (1951) staging chart. Embryos were staged at two different time points. First, embryos were staged before *ex-ovo* culturing. The ideal stage for *ex-ovo* culturing was HH stage 19 (3-3.5 days of incubation). HH stage 19 was characterized by the following morphological traits. Somites had extended into the majority of tail (37 -40), however the end of the tail remains unsegmented. This extension of the somites distinguishes a HH stage 19 embryo from a HH stage 18 embryo since at HH 18 the somites are not as far into the tail (30-36). Also, the tail bud remains curled. The allantois is still quite small, and has no vasculature. The main difference between HH 19 and HH 20 is that at HH stage 19 the eyes have no pigment while at HH stage 20 a greyish hue is visible.

Embryos are staged again before bead implantation surgery to ensure that the embryos are at HH stage 35 (8.5-9 days incubation), and have not yet reached HH stage 36 (10 days incubation). This stage is crucial because bead implantation needs to occur at the beginning of induction (HH 35). The main characteristic that was used to

distinguish between these two stages was the nictitating membrane. If the nictitating membrane was completely covering any of the papillae, the embryo was considered too old for bead implantation because at HH stage 36 the nictitating membrane begins to cover the most anterior papillae. In order to ensure the embryos were not too young, HH stage 34 embryos were recognized by the nictitating membrane being half way between the outer rim of the eye and the scleral papillae. This membrane was much closer to the scleral papillae by HH stage 35. Also, at HH 34 the distance between the tip of the beak and the eye was smaller than at HH 35 due to the lengthening of the visceral arches at HH 35. These characteristics ensured that the embryo was at HH stage 35 when bead implantation occurred.

2.3 Microsurgery

2.3.1 Tools

Fine needles were made for the bead implantation surgery. Glass pipettes were pulled over a flame and using a diamond glass cutter the pipette was etched until a clean break was made, leaving one end with a very fine hole. Fine (0.1 mm) and coarse (0.25 mm) tungsten wire (Alfa Aesar, H08S018 and C12N03) was cut approximately two centimetres in length and was carefully threaded into the tip of the pulled pipette. The glass tip and wire were then briefly placed back over the flame, causing the glass to melt, sealing the wire in place. The needles were sharpened using electric current as follows. First, a 1N NaOH solution was placed into a plastic beaker. Then a straightened paperclip was placed over the edge of the beaker, partially submerged in the NaOH solution. One wire was plugged into an AC power source and

connected to the paperclip. Another wire was also plugged into the power source, leaving the other end free. The power source was turned on and both the needle and the free end of the wire were partially submerged in the NaOH. The electrolytic properties of the solution and the paperclip will cause a sharpening at the tip of the needle.

Affi-gel beads (BioRad 153-7302) were used for all experiments. Noggin-soaked beads were prepared by first choosing approximately 30 beads of similar size from a stock of beads, which are stored in PBS at 4°C. These beads were placed into a 0.1 ml sterile eppendorf tube and spun at 13000 RPM for five minutes in a centrifuge in order to remove all of the PBS. The beads were then placed at room temperature for an hour to ensure full dehydration (noted by shrivelled beads). Following this, 1 µl of recombinant mouse noggin (reconstitute as 25 µg of noggin in 25 µl of DepC treated 1X PBS, to give a final 1µg/µl concentration) (R&D Systems, 1967-NG) was placed in the tube with the beads. The beads were then left to incubate at room temperature for one hour to allow the beads to absorb the noggin. Following the one hour, the tube was placed on ice until bead implantation was complete. This allowed for all 30 beads of approximately the same size to absorb the same quantity of the 1.0µg/µl noggin solution. Control beads were rinsed 20 times in DepC treated 1X PBS.

2.3.2 Shell-less culturing (*ex-ovo*)

Ex ovo culturing was performed instead of windowing the eggs due to the restricted access to the embryo through windowing. Easy access to a specific papilla at HH stage 35 was required. At HH stage 35, in a windowed embryo, the head of the

embryo could be partially or completely inaccessible making the surgery impossible. Also, the small hole in the egg present when using a windowed embryo makes it challenging to use forceps and fine needles. Due to the limitations of the windowing method, embryos were prepared for *ex ovo* culturing at HH stage 19 as follows.

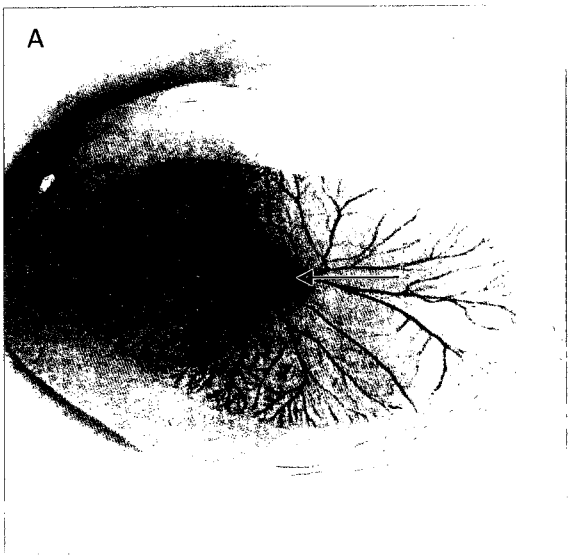
Before removing the embryo, the shells were sprayed with 70% ethanol and allowed to dry. Using a mortar and pestle, an empty shell was crushed into a fine powder and the shell was placed at the bottom of a weigh boat (Fischer Scientific, 08732113, 88 x 88 x 23 mm). The egg was carefully cracked and the embryo was placed directly into a sterile weigh boat. The embryo was inspected to ensure that the yolk was still perfectly intact (Figure 4A). If the yolk had broken, the embryo was discarded as it will not survive *ex ovo*. The embryo was also observed to ensure that it was viable; the heart was beating, vasculature appeared normal, and no obvious abnormalities were present. Using a pipette 40 µl of penicillin/streptomycin (5,000 U Penicillin: 5 mg Streptomycin, Sigma P4458) was placed over the surrounding albumin of the embryo to help fight infection. A humidity chamber was also prepared (Figure 4B and C) for each embryo as follows: a small stack of Kim-wipes and an absorbent pad made of cotton were placed in the bottom of a Tupperware container (12 cm x 12 cm x 6 cm). Distilled water was added to moisten the Kim-wipes and cotton (approximately 200 ml of water). The embryo was then placed in its weigh boat on top of the moist padding and half of a square sterile petri-dish (9.5 cm x 9.5 cm) was placed overtop to form a loose lid for the weigh boat. The Tupperware container was then covered with its lid. Two corners of the lid were sealed ensuring a partial seal that still allowed for good air flow inside of the chamber. The embryo was then

carefully placed back into a 37°C incubator until desired stage for bead implantation. Containers of water were placed in the incubator to help control humidity. All water in the humidity chambers and in the incubator was replenished as needed during the incubation period.

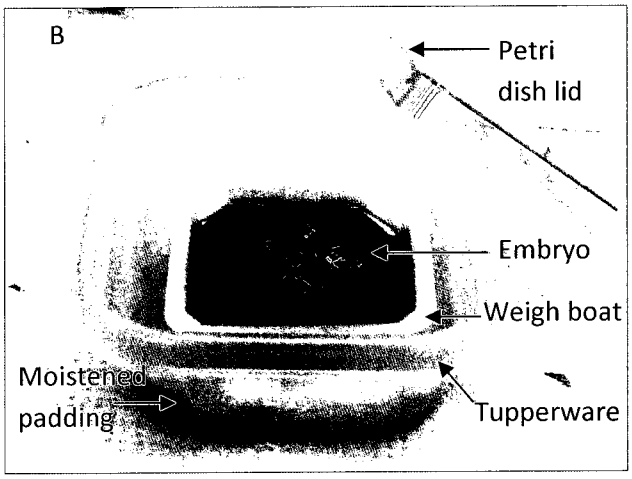
Many different techniques were attempted in order to optimize the *ex-ovo* culturing. Different absorbent padding, different antibiotic doses, different entry points through the membranes, as well as increased sterile techniques were all tried in order to increase the survival rates of the embryos *ex ovo.* Despite these efforts, embryos survived well up until HH stage 37 at which point a large die off would consistently occur.

Figure 4: The *ex ovo* culturing technique. (A) A HH stage 25 embryo after removal from the shell, arrow points to embryo with vasculature and yolk that are not broken. (B) A HH stage 40 embryo in an open humidity chamber (C) The complete humidity chamber prior to placing in the incubator.

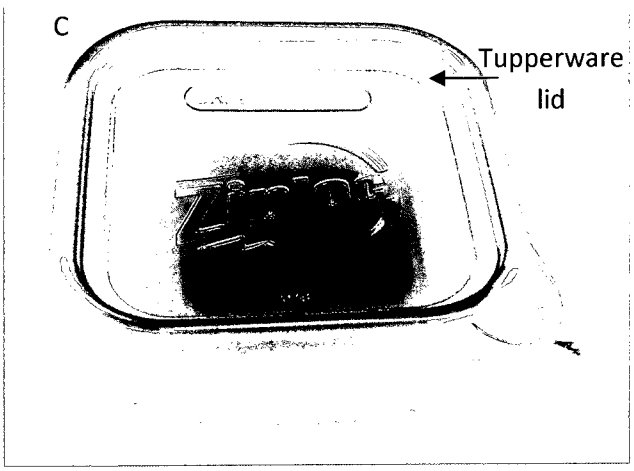
A



B


Petri
dish lid
Embryo
Weigh boat
Moistened
padding
Tupperware

C


Tupperware
lid

2.3.3 Bead implantation

Tungsten needles and fine forceps were disinfected with 100% EtOH, and then flamed before surgery. An *ex ovo* cultured embryo at HH stage 35 (10 days of incubation) was removed from the incubator and placed under a Nikon (SMZ1000) dissecting microscope. Using forceps, a small hole was torn in the membranes surrounding the embryo. The placement of this hole was typically in an area free of major blood vessels and directly over the eye (Figure 5). Once access to the eye had been gained, a fine tungsten needle was used to make a small hole in the conjunctival epithelium of the eye directly next to a scleral papilla. If access was ideal, the bead was placed next to scleral papilla 8 or 9 (Figure 2). Using fine forceps a prepared affi-gel bead was placed on the torn membranes, near the opening to the embryo. Using a tungsten needle the bead was moved over to the epithelium of the eye and gently pushed directly underneath the conjunctiva. A 45 µl dose of penicillin/streptomycin (5,000 U Penicillin: 5 mg Streptomycin, Sigma P4458) was again placed on the albumin of the embryo to prevent infections. The petri-dish and Tupperware lids were placed back over the embryo and the embryo was placed back in the 37°C incubator. Embryos continued to develop until HH stages 39-41 (13-15 days of incubation). The embryo was then decapitated and the head was fixed in 10% Neutral Buffered Formalin (Fischer Scientific – 245685) overnight at room temperature followed by processing through a graded EtOH series. In total, 153 noggin-soaked beads and 172 1X PBS-soaked beads were implanted into individual eyes (1 per embryo) adjacent to papilla 8 or 9 at HH stage 35, the onset of induction.

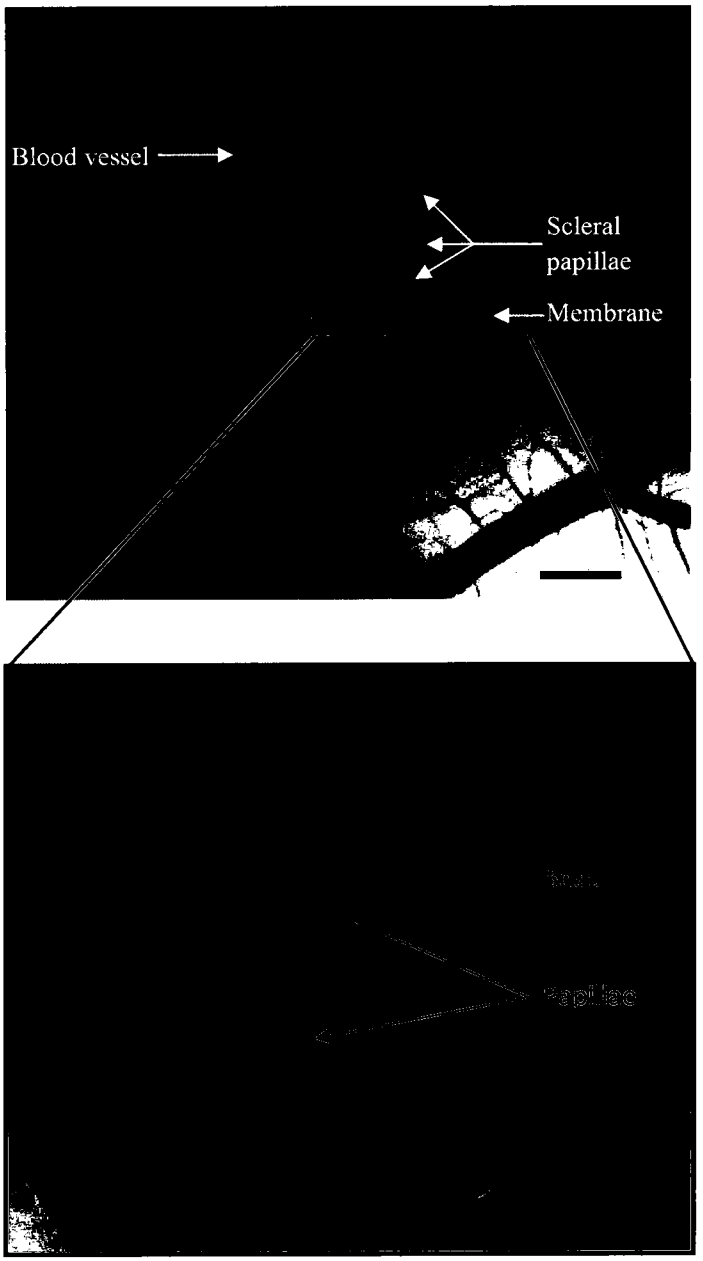


Figure 5: Bead implantation surgery. (A) Embryo HH stage 35 exposed through a small hole torn in the overlying membranes in order to gain access to the underlying eye for bead implantation. (B) High magnification of the affi-gel bead adjacent to a scleral papilla. Scale bar in A is 100 μm.

2.3.4 Alkaline phosphatase stain to visualize ossicle formation

The alkaline phosphatase stain protocol was followed according to Edsall and Franz-Odendaal (2010). Previously fixed specimens were rehydrated through a graded EtOH series to 100% distilled water, followed by three 15 minute washes in distilled water. The specimens are then incubated in tris-maleate buffer pH 8.3 (0.605 g trizma base, 0.55 g maleic acid, 25 ml distilled water) for one hour at room temperature. All of the following steps occurred in the dark. Embryos were incubated in alkaline phosphatase substrate solution (0.1 ml of 10mg/ml napthol-AS-TR-phosphate (Sigma – N6125) in N, N-dimethylformamide (Sigma – D4551), 8 mg diazonium salt (Sigma –D9805), 10 ml tris-maleate buffer pH 8.3) for one hour at room temperature. To stop the enzyme reaction, the samples were washed three times for 15 minutes in saturated sodium borate water. Embryos are then bleached (to remove eye pigment) in 10% hydrogen peroxide made with 1% KOH overnight. Finally embryos were processed through a graded glycerol series and stored in 80% glycerol. The alkaline phosphatase stain was performed with embryos at HH stage 38 and older. HH stage 37 embryos were too young; the condensation formation has started but the differentiation of the osteoblasts is not complete (Figure 3).

2.4 Probe preparation and whole-mount *in situ* hybridizations

2.4.1 Amplification of plasmids containing cDNA

Plasmids containing *indian hedgehog* and *patched* cDNA were obtained from Dr. John Fallon (Department of Anatomy, University of Wisconsin Medical School) and plasmids containing *MSX2* and *scleraxis* cDNA were obtained from Dr. Joy

Richman (Department of Oral Health Sciences, University of British Columbia). Plasmids were removed from the filter paper by adding 100 µl of TE buffer (10mM tris-HCl, 0.1M NaCl, pH 8.0). After five minutes, the sample was mixed using a vortex and then spun at room temperature for one minute at 3000 RPM. The tube was then incubated at room temperature for 30 minutes and the filter paper was carefully removed from the solution. The solution with the plasmid was stored at -20 °C until required.

Plasmids were transformed into *Escherichia coli* bacteria competent cells (HB101 Competent cells, Promega L1011). Polypropylene culture tubes and sterile tips were chilled in a -20°C fridge before use. Competent cells were removed from the -80°C freezer and placed on ice to thaw. Using a chilled pipette tip, competent cells were gently mixed and 100 µl was placed in a chilled 5 ml tube. 10 µl of the plasmid solution was added to the competent cells. The solution was chilled on ice for 20 minutes, heat shocked for 50 seconds in a 42°C water bath, and then placed back on ice for two minutes. Then, 900 µl of cold 2.5% Luria-Bertani (LB) broth (Difco, 244320) was added to the competent cells/plasmid mixture. This mixture was incubated for one hour at 37°C with vigorous shaking. During this time 0.01% ampicillin in 2.5% LB agar plates (Appendix A) were warmed upside-down in a 37°C incubator. Finally, 100 µl, 10 µl, and 1 µl volumes of the LB broth with the competent cells mixture were placed on each plate and a flamed loop was used to distribute the cells evenly. The plates were incubated overnight at 37 °C.

The following day transformed *E. coli* were amplified. Using a sterile pipette tip a single colony was placed into a 10 ml RNAse free falcon tube containing 4 ml of LB broth. The tube was then sealed and incubated at 37°C with vigorous shaking, overnight.

2.4.2 Isolation of plasmid

In order to isolate the plasmid containing cDNAs, a mini preparation protocol was performed. The *E. coli* cells were lysed and the plasmid was removed (Appendix B). To verify the concentration of the plasmid obtained from the mini preparation protocol, the UV absorbance at 260 nm and 280 nm was recorded using a spectrophotometer (Thermo scientific, Genesys 10*uv*). The spectrophotometer reading was taken using 4 µl of plasmid sample in 116 µl of distilled water, a dilution of 29 times. A 260 nm reading was used to determine the concentration of the sample and the 260 nm/280 nm ratio was used to determine the purity of the sample (Table 1). A ratio of approximately 1.8 - 2.2 was considered acceptable. The concentration of the samples was determined using the formula: [DNA] = (50ng/µl/absorbance unit x Dilution Factor x OD260)/1000.

Table 1: Spectrophotometer results for the plasmid containing *patched* cDNA

Gene	**OD260**	**OD280**	**Purity**	**Concentration**
Patched	1.574	0.784	2.01	$= \frac{(50ng/\mu l/abs \times 29 \times 1.574)}{1000}$ = 2.28µg/µl DNA

2.4.3 Linearization of plasmids

The plasmids were linearized using predetermined restriction enzyme cut sites within the designed plasmid. The linearization procedure was typically done with a total volume of 100 µl, except in specific cases when it was possible to do the digestion with a total volume of 20 µl. To stop the linearization reaction, heat inactivation was performed by placing the tubes in a 65°C water bath for 15 minutes. Samples were stored at -20°C.

Table 2: Reaction conditions for linearization of amplified plasmids containing cDNA and synthesizing RNA probe.

cDNA	Restriction Enzyme and Buffer	Conditions	Polymerase	Sense or Antisense Probe
sonic hedgehog (*shh*)	NotI + Buffer D (Promega)	37°C water bath, overnight	T7	Antisense
patched (*ptc*)	SalI + Buffer D (Promega)	37°C water bath, 2 hours	T3	Antisense
patched (*ptc*)	SalI + Buffer D (Promega)	37°C water bath, 2 hours	T7	Sense
indian hedgehog (*ihh*)	EcoRI + Buffer H (Promega)	37°C water bath overnight	T3	Antisense
msx2	BamHI + Buffer E (Promega)	37°C water bath overnight	T7	Antisense
Scleraxis	SmaI + Buffer J (Promega)	25°C water bath overnight	T7	Antisense
Scleraxis	EcoRI + Buffer H (Promega)	37°C water bath, 4 hours	T3	Sense

2.4.4 cDNA purification and gel electrophoresis

A high pure PCR product purification kit was used to clean up the linearized plasmids (Roche, 14056400). After plasmids had been purified, two elutions were collected for each sample. 2 µl (at least 1 µg of DNA) of each elution were loaded onto a 1.5% agarose gel in 1X TBE (5X stock – 27 g Trisma buffer, 13.75 g of boric acid, 0.372 g of EDTA, in 500 ml dH2O), with 0.05% ethidium bromide (Sigma, E1510). A one kilobase ladder (Promega) was also loaded onto the gel. Gel electrophoresis was performed at 110 volts for approximately 45 minutes. DNA bands were observed to ensure samples had been appropriately linearized and purified.

2.4.5 Synthesizing the RNA probe

The RNA probe was made using a Digoxigenin RNA labelling kit (Roche 11401420) (Appendix C). 1µg of each DNA sample was used to create a labelled RNA probe. Specific polymerases (T3 or T7) were required depending on the site of restriction enzyme digestion that occurred during the linearization step (Table 2). To stop this reaction, 2 µl of 0.2M EDTA was added to each tube. Probe was stored at -20°C until ready to use.

In order to verify that the probe had been labelled correctly, five dilutions were made (1 ng/µl, 10 pg/µl, 3 pg/µl, 1 pg/µl, and 0.3 pg/µl) as suggested by the digoxigenin high prime RNA labelling protocol (Appendix D). Briefly, the detection protocol involves placing 1 µl of each dilution and 1 µl of a pre-labelled RNA probe from the kit (positive control) onto a nylon membrane (Roche, 57112314). The membrane is baked at 120°C to ensure that the probe is not washed off the membrane

during the protocol. The membrane is then washed in Maleic acid buffer (0.1 M Maleic acid and 0.15 M NaCl) and placed in a blocking solution (2% Sheep serum, 3% skim milk powder, 1X TBST). After blocking, a quick 1X TBST rinse is done and the membrane is placed in an antibody solution (1:5000 anti-digoxigenin-AP in 1X TBST). The membrane is then placed in washing buffer (0.1M Maleic acid, 0.15M NaCl, 0.3% Tween) and equilibrated before placing in detection buffer (0.1M Tris-HCl and 0.1M NaCl). The membrane is incubated at room temperature in the detection solution, and viewed every five minutes. The colour reaction takes anywhere from half an hour to 12 hours. The colour of each dilution can be analyzed to determine the approximate concentration of the probe from the kit. If the intensity and colour of each sample is similar to the intensity and colour of the probe from the kit then this demonstrates that the probes have been efficiently synthesized and labelled (Figure 6).

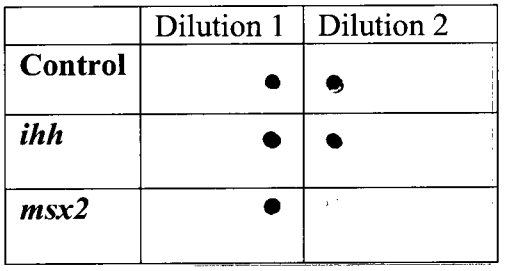


Figure 6: Dot blot results for the *indian hedgehog* and *msx2* probes. Dots for *ihh* and *msx2* are similar in colour and intensity to the control dots, which indicate efficiently labelled synthesized probes. Only the first two dilutions at 1 ng/µl and 10 pg/µl are shown.

2.4.6 Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization protocol was adapted from Nieto *et al.* (1996) (Full detailed protocol is given in Appendix E). Briefly, embryos were staged using the Hamburger and Hamilton (HH) staging chart. HH stages 30 through 40 were collected and fixed in 4% Paraformaldehyde (Sigma, P6148) in PBST, overnight at 4°C. The following day, heads were processed through a graded methanol series to 100% methanol and left to de-hydrate for at least one hour. After dehydration heads were bisected. Half-heads were then rehydrated and bleached to remove eye pigment. The half heads were stored at -20°C in 1X hybridization solution (0.005% yeast tRNA and heparin in prehybridization solution). Embryos were then permeabilized using a 50 µg/ml Proteinase K solution, and post fixed in a 1:1 solution of 4% PFA in 1XPBST: 0.25% Gluteraldehyde (in 1XPBST). The half heads were hybridized in probe overnight at 60°C with shaking. The following day, the embryos were washed and placed in a blocking solution (20% heat inactivated sheep serum in 1X TBST). After the antibody is pre-absorbed (in chick powder), the embryos are placed in the antibody solution (1:10,000 anti-digoxigenin-AP antibody (Roche – 12930020) in blocking solution) overnight at 4°C with shaking.

To reduce background signal, the embryos were washed in a 2 mM levamisole solution. Embryos were then placed in colour detection for 24 to 36 hours in the dark. The colour reaction was stopped with 5 mM EDTA in 1X PBST. The embryos were fixed in 4% PFA, and dehydrated through a MeOH series. Once in 90% MeOH, they were left overnight in order to leach the background signal from the embryos. Finally,

the embryos are placed in a 1:1 solution of glycerol: distilled water and stored in the dark at 4°C.

This protocol was performed with two controls. One control was an embryo that would receive sense probe instead of the normal antisense probe. This was to ensure that the addition of a non-specific probe, did not give the same expression pattern as the antisense probe that binds specifically to the gene of interest. The other control was an embryo that would receive no probe. This was to ensure that the other steps in the protocol were not creating the expression pattern shown by the antisense probe experiments. This entire protocol was performed at least three times for each gene that was investigated.

2.4.7 Optimizing the whole-mount *in situ* hybridization protocol

In order to determine which candidate genes are involved in the development of scleral ossicles, *in situ* hybridizations were performed. To ensure that the whole-mount *in situ* hybridization protocol was working correctly, *shh* probe was used. *Shh* expression was found in the developing feather bud starting at HH stage 35 (Harris *et al.*, 2002, Franz-Odendaal, 2008). Once the protocol was optimized, the other candidate genes were investigated.

For each new gene (*ihh, msx2, scleraxis,* and *ptc*), the initial embryo stages that were investigated using *in situ* hybridization were HH stages 33, 35/36, and 38. These stages were chosen in order to test a stage before induction (HH 33), during induction (HH 35 or 36), and after induction when condensations are present (HH 38) (Figure 2).

If the gene was detected in any of these stages, then subsequent *in situ* hybridizations were performed including all the stages between HH 30 and HH 40.

2.4.8 Sectioning whole-mount *in situ* hybridization samples

Tissue was dissected from the eyes that had undergone *in situ* hybridization. These tissue samples were sectioned using a microtome or a cryostat, in order to determine the cellular location of the *in situ* hybridization signal.

Microtome sectioning: Tissue samples were dehydrated through a graded EtOH series to 100% alcohol. The tissue was placed in Citrosolve (Fischer Scientific, 22-143975) overnight. The tissue samples were then placed in molten paraffin wax (Fischer Scientific, 8889-502004) and the wax was changed twice. After 12 hours, the samples were embedded in wax and placed at -20°C overnight. The tissue was serially sectioned at 5 μm and the sections were left to set on the slides overnight at 37 °C. The following day, the slides were de-waxed through a series of Citrosolve rinses and coversliped. Finally the slides were examined under the compound microscope (Nikon Eclipse 50*i*).

Cryostat sectioning: Tissue samples were hydrated to 100% distilled water. A 1% agar solution was heated until the agar dissolved. The liquid agar solution was poured into a small petri dish and after one minute of cooling, the tissue sample was place into the agar liquid. The agar then hardened and was cut into a small block. The block of agar containing the tissue sample was placed in a 30% sucrose solution overnight, at room temperature. The sucrose solution was then drained and the block was placed in the tissue freezing medium (Jung – 020108926) onto the chuck. The agar block was

frozen onto the chuck in the cryostat for 10 minutes. Sections were cut at 5-10 μm and placed on slides. Slides were stored in the -20°C freezer. The slides were conversliped and viewed under the compound microscope (Nikon Eclipse 50*i*).

2.5 Photography and image analysis

Both the compound (Nikon Eclipse 50*i*) and the dissecting (Nikon SMZ1000) microscopes are camera capable (Nikon digital camera DXM1200C). In order to capture the digital photos the camera was connected to a computer and the program Nikon NIS-element (BR 3.0) software was used. This program was used to add scale bars to all photographs. Also, the surface area measurements were performed using this software. Once the pictures had been captured, cropping and contrast optimization were performed in Corel Draw X3 (version 13.0).

3.0 Results

3.1 Inhibition of *BMPs* using affi-gel beads soaked in noggin

3.1.1 Results of PBS vs. noggin-soaked bead implantation

In order to determine if *bone morphogenetic proteins (BMPs)* are involved in the development of scleral ossicles, affi-gel beads soaked in noggin, a *BMP* inhibitor, were implanted into the eye adjacent to a scleral papilla. At HH stage 35 (Day 8.5-9, the onset of induction), the bead implantation was performed. At HH stage 36, when induction is thought to be occurring the noggin-soaked bead had no effect on the maintenance of the adjacent papilla. The papilla next to the noggin bead maintains its normal developmental pattern despite the inhibition of *BMP* in the surrounding tissues (Figure 7A&B). At HH stage 40 (when condensations are present) the condensation that would form directly beneath the papilla adjacent to the noggin-soaked bead was missing (Figure 7C&D). The other condensations in the ring that were present during this time were very difficult to view under the microscope and with the camera. Since these condensations are translucent and often in the early stages of development, interpreting whether or not ossicles had been affected was very challenging (Figure 7C &D). This problem was overcome by using an alkaline phosphatase stain to mark the preosteoblasts and osteoblasts present in the ossicle condensations (section 3.1.3). Bead implantation at HH stage 35 was also performed with control affi-gel beads that had only been soaked in PBS. 24 hours later, at HH stage 36, the implantation of PBS- soaked beads had no effect on the adjacent developing papilla. This result also occurred in the noggin-soaked bead

experiments. Conversely, these control beads had no effect on the development of the underlying scleral ossicle in comparison to the noggin bead implantation (Figure 7E&F).

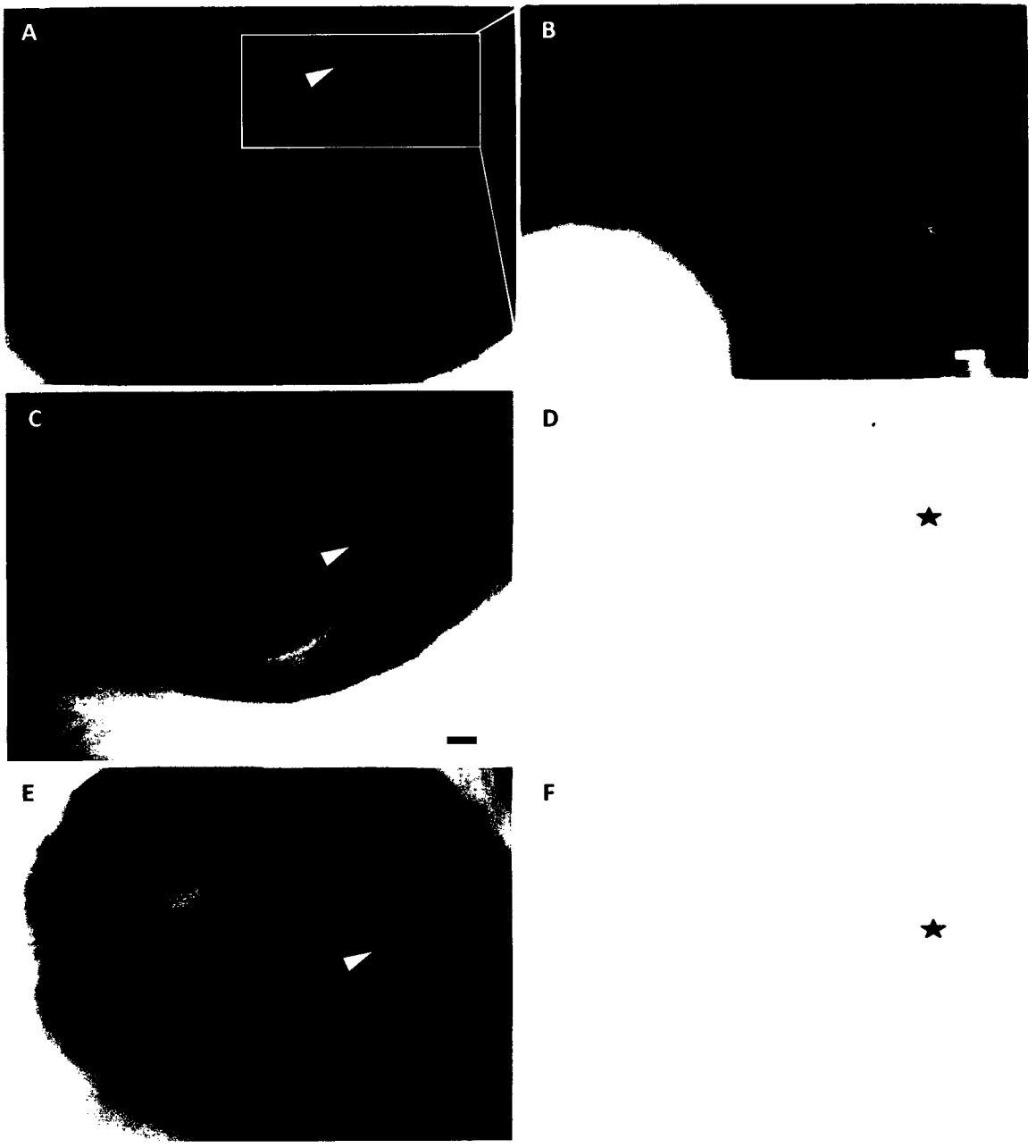
3.1.2 Embryo survival rates after microsurgery

The survival rates for the *ex-ovo* culturing, and therefore the bead implantation, were low. Most of the embryos would survive 24 hours post surgery (HH stage 36). 120/153 individuals that had a noggin bead implanted in the eye survived 24 hours past the surgery. Approximately the same percentage (~80%) of the individuals that had a PBS-soaked bead implanted in the eye survived 24 hours past surgery (Table 3). The critical stage in the shell-less cultured embryos was HH stage 37 (11 days). At this stage the majority of the embryos would die. Approximately 95% of all the embryos that underwent surgery (PBS- or noggin-soaked bead implantation) did not survive past HH stage 37 (Table 3). Embryos that underwent noggin bead implantation and survived past HH 37 (6/120) demonstrated that the ring of condensations was affected by this bead implantation. All six of the embryos that survived the noggin bead implantation demonstrated this result. Finally, embryos that had a control bead implanted and survived past HH stage 37 (11/137) did not have any altered condensations in the eye.

Table 3: Survival rates for *ex-ovo* embryos that underwent microsurgery. # indicates number.

	# of surgeries at stage HH 35 (onset of induction)	**# of embryos that survive surgery (stage HH 36 – induction)**	**# of embryos that survive to stage HH 38 – 41 (condensation formation stages)**	**# of embryos with altered condensations**
noggin-soaked beads	153	120 (78%)	6 (5%)	6 (100%)
PBS-soaked beads	172	137 (80%)	11 (8%)	0 (0%)

Figure 7: Inhibition of *BMPs* by noggin results in the inhibition of ossicle formation. (A) HH 36 embryo with noggin bead implantation (white arrowhead), all papillae are present despite noggin exposure for the past 24 hours (bead implantation was performed at HH 35). (B) Magnified view of the insert from (A); noggin-soaked affi-gel bead and adjacent papillae. (C) HH stage 40 after noggin bead implantation (white arrowhead) demonstrating a missing condensation. (D) Diagrammatic representation of 13 condensations from (C), an asterisk depicts the original placement of the noggin bead. (E) HH stage 40 embryo with a control PBS-soaked bead (white arrowhead) (F) Diagrammatic representation of 14 condensations from (E), an asterisk depicts the placement of the bead. Scale bar in A is 500μm, B is 250μm, and C&E are 500μm.


A
B
C
D
E
F

3.1.3 Further interpretation of the noggin bead implantations

Embryos that survived until HH stage 38 through 40 when condensation formation was occurring were difficult to interpret. In order to view the condensations after bead implantation more clearly, an alkaline phosphatase stain adapted from Edsall and Franz-Odendaal (in press) was used. This stain works through an enzymatic reaction that occurs with alkaline phosphatase, one of the main enzymes secreted by preosteoblasts and osteoblasts when depositing bone. After staining with alkaline phosphatase, six embryos demonstrated signs of missing ossicles due to the implantation of a noggin-soaked bead (Figure 8). Figure 8A depicts one of these embryos that had faint alkaline phosphatase staining suggesting a condensation was present, however complete ossification had not yet occurred (white arrow). In another embryo that was fixed at HH stages 38 there was a large gap in the ring of scleral ossicles (Figure 8E, F). In the embryo that survived the longest (HH stage 41) the gap created by the missing ossicle was reduced because the adjacent ossicle grew very large to complete the ring of scleral ossicles (Figure 8C&D). As shown in Figure 8, noggin bead implantation caused a range of effects on scleral ossicles, such as complete inhibition of condensation formation (Figure 8C&E) as well as effects on condensation size causing delay in osteoblast differentiation (Figure 8A).

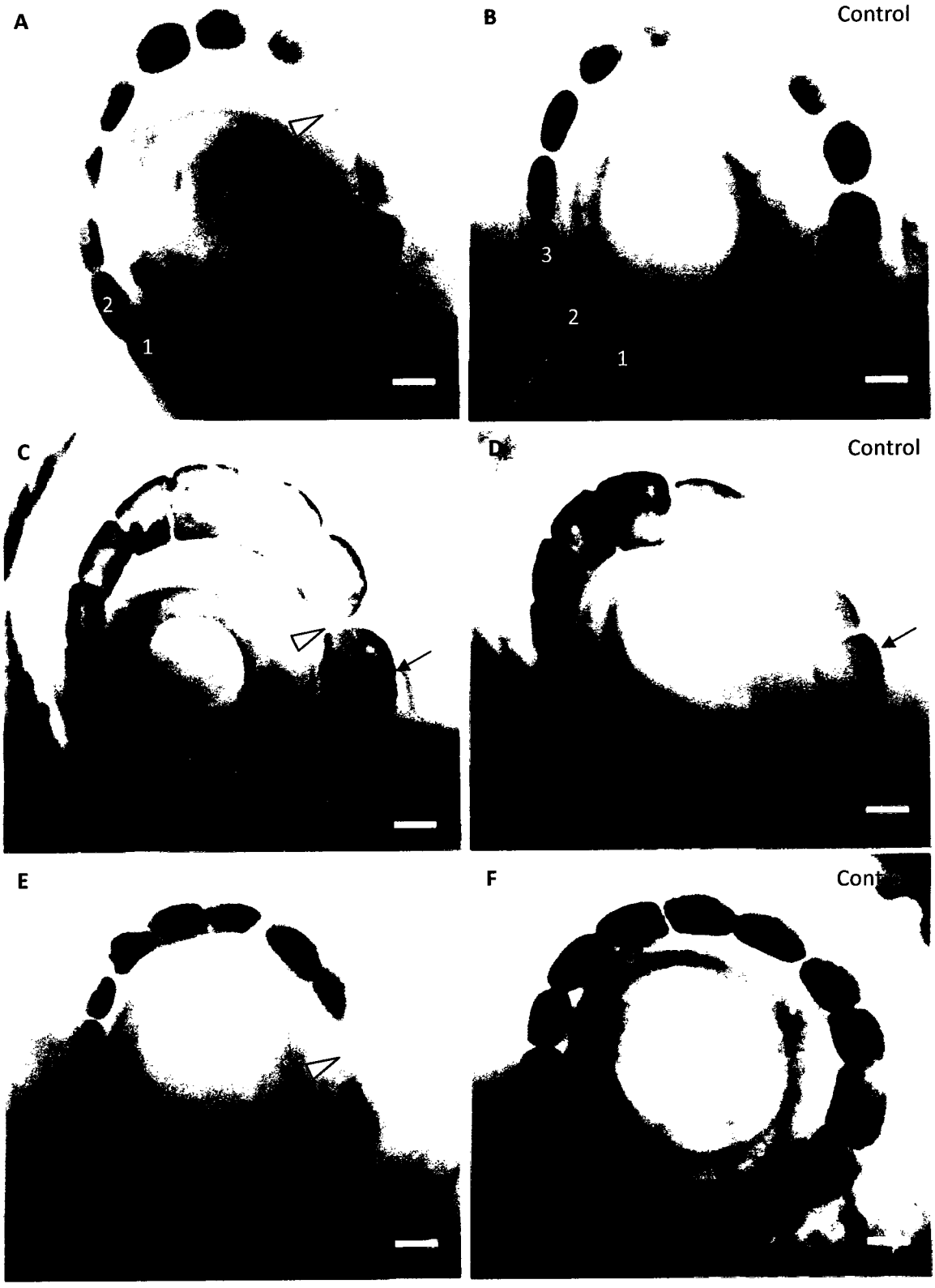
Upon discovering that certain ossicles grew larger in the eyes with a missing ossicle, a comparison in surface area was performed. If it appeared that the ossicle directly next to the missing ossicle was larger than the control, then the surface of that ossicle was measured and compared to the control side (Figure 8C&D) (Table 4). The surface area of the ossicle in the control eye was smaller (0.886 mm^2) than the ossicle that was adjacent to a missing ossicle (1.465 mm^2). If it appeared that the ossicles directly

adjacent to the missing ossicles were not larger than the controls, then the next group of induced ossicles were compared (Figure 8A&B, ossicles 1, 2, and 3) (Table 4). The surface area of the group of ossicles that were induced directly after the missing ossicle is larger (m= 0.416 mm^2, n=3) than the control ossicles in the same location (m=0.258 mm^2, n=3). In some cases, the ossicles in the control eye were approximately equal in surface area to the ossicles in the experimental eye (Figure 8E&F) (Table 4). In this case, compensation for the missing ossicle did not occur. These results indicate that, in some cases, the condensation(s) that are induced immediately after the group of ossicles where bead implantation was conducted are able to grow larger (Table 4).

Table 4: Comparison between the eyes of individual embryos that were affected by the implantation of a noggin bead (m = mean).

Affected Individual Embryos	**# of ossicles (control eye)**	**#of ossicles (experimental eye)**	**Surface area of ossicles on the control eye**	**Surface area of the ossicles on the bead implantation eye**
Embryo 1 (Figure 8 A&B)	15	14	1) 0.237 mm^2 2) 0.278 mm^2 3) 0.258 mm^2 m = 0.258 mm^2	1) 0.496 mm^2 2) 0.405 mm^2 3) 0.347 mm^2 m = 0.416 mm^2
Embryo 2 (Figure 8 C&D)	14	13	0.886 mm^2	1.465 mm^2
Embryo 3 (Figure 8 E&F)	15	14	0.623 mm^2	0.594 mm^2
Embryo 4 (Figure 7C)	15	13	Not measured	Not measured
Embryo 5 (not shown)	15	15	1) 0.541 mm^2 2) 0.597 mm^2 3) 0.626 mm^2 m = 0.588 mm^2	1) 0.920 mm^2 2) 0.770 mm^2 3) 0.791 mm^2 m = 0.827 mm^2
Embryo 6 (not shown)	15	14	1) 0.369 mm^2 2) 0.288 mm^2 3) 0.229 mm^2 m = 0.295 mm^2	1) 0.793 mm^2 2) 0.836 mm^2 3) 0.756 mm^2 m = 0.795 mm^2

Figure 8: Alkaline phosphatase stained embryos after bead implantation. (A) HH stage 39 embryo after bead implantation (white arrowhead) with a missing condensation or delayed ossification. The following group of ossicles to be induced have grown larger (numbers 1, 2, 3). (B) Control eye of the HH stage 39 embryo with a complete ring of condensations. (C) HH stage 41 embryo after noggin bead implantation with a small gap where bead implantation occurred (white arrowhead) and larger ossicle (black arrow) compensated to fill the gap. (D) HH stage 41 embryo control eye with a complete ring of condensations. (E) HH stage 38 embryo after noggin bead implantation (white arrowhead) with a missing condensation. (F) Control eye with a complete ring of condensations. Scale bars represent 200 μm in A-F.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3.2 Determining if *indian hedgehog* is present during the induction of scleral ossicles

In order to ensure the *in situ* hybridization protocol was optimized, *shh* probe was used and found in the feather buds in a similar pattern observed by previous studies (Harris *et al.,* 2002) (Figure 9A). Therefore the protocol was determined to be producing reliable results. In order to determine if *shh* is the only Hedgehog family member present during the induction of scleral ossicles, *indian hedgehog* probe was also used. *Indian hedgehog* was not found at HH stages 33, 35/36, and 38 (Figure 9B and Table 5). Although the scleral papillae and surrounding eye tissue had no *ihh* expression, signal was demonstrated in other locations of the embryo such as the developing mandible (Figure 9C). *Ihh* has a unique expression pattern, where expression stopped at the most distal end of the beak. This suggests that *ihh* is likely not involved in the development of scleral ossicles.

Table 5: *Ihh in situ* hybridization expression pattern in embryos HH stages 33, 35/36, and 38

Stages	***indian hedgehog***
Stage HH 33	- No expression in the eye
Stage HH 36	- No Expression in the eye; expression located in the mandible
Stage HH 38	- No Expression in the eye

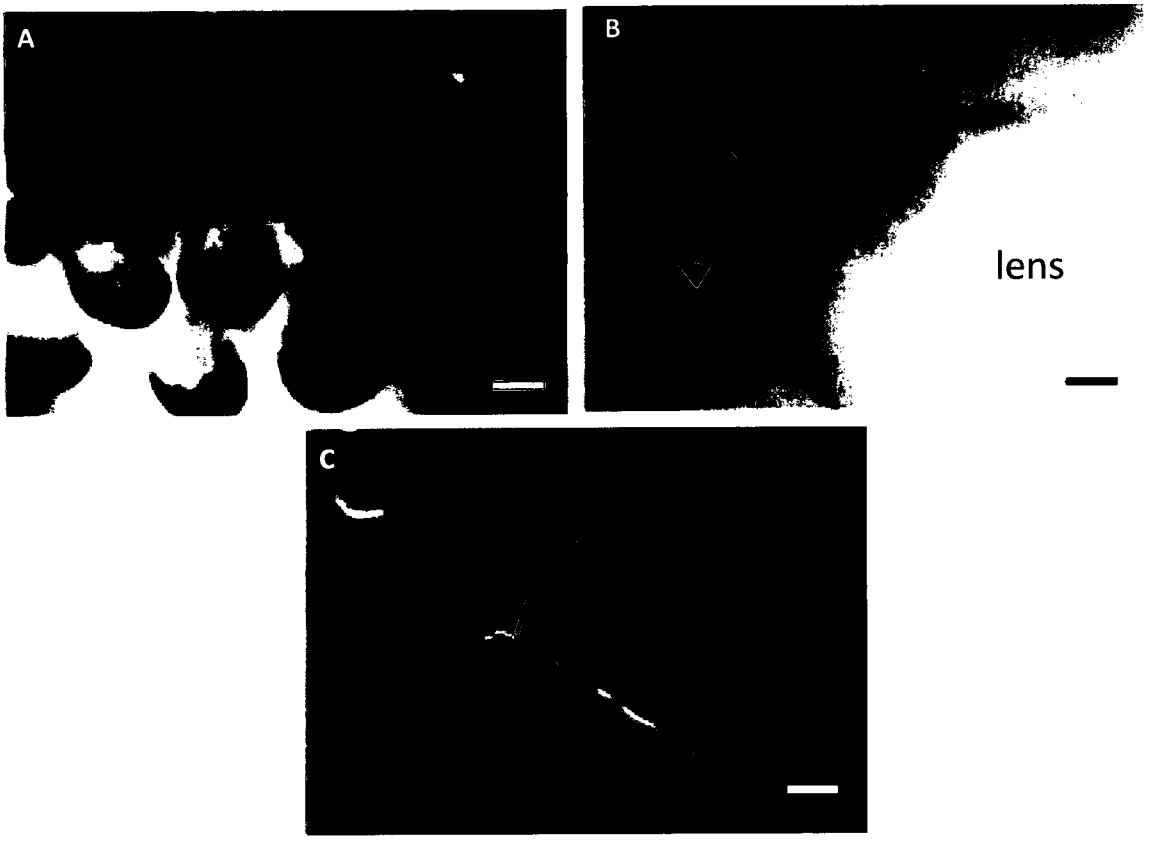


Figure 9: Location of *shh* and *ihh* mRNA through whole-mount *in situ* hybridization expression. (A) Predicted *shh* expression pattern found in the developing feather buds of a HH stage 36 embryo; (B) no *indian hedgehog* expression present in the papillae (black arrowheads) or surrounding eye tissues of a HH stage 36 eye; (C) *ihh* in the developing mandible with unique loss of expression at the distal tip of the mandible (dashed line). Scale bar in A represents 100 μm, and 400 μm in B and C.

3.3 Determining where *shh* is received through the expression pattern of the Hedgehog family receptor *patched.*

After an initial *in situ* hybridization on HH stages 33, 35, 38 in which *ptc* expression was identified at HH 35, a more extensive investigation was carried out on a wider range of stages (HH 30-HH 40) (Table 6). No *ptc* expression was shown in HH stages 30 through 34 (Figure 10A). *Ptc* expression is first detected in the papillae at HH stage 35 (Figure 10B). As the embryo develops through HH stage 35 and into HH 36, the expression declines in the papillae and appears to begin in the surrounding mesenchyme. At HH stage 36 the expression of *ptc* is both in the papillae and in the surrounding mesenchyme of all the papillae that have not started degenerating (Figure 10C). At HH stage 37 the papillae have completely degenerated and condensation formation begins. No *ptc* expression was detected at this stage (Figure 10D). Similarly no *ptc* expression was shown in HH stages 38 through 40. Two controls were also performed; a sense-probe control and a no-probe control. In both of these control experiments no *ptc* expression was detected (Figure 10E&F). This result suggests that *shh* and *ptc* are both present during the inductive stages (HH 35 and 36) of scleral ossicle development and that *shh* likely signals to both the epithelium and the mesenchyme.

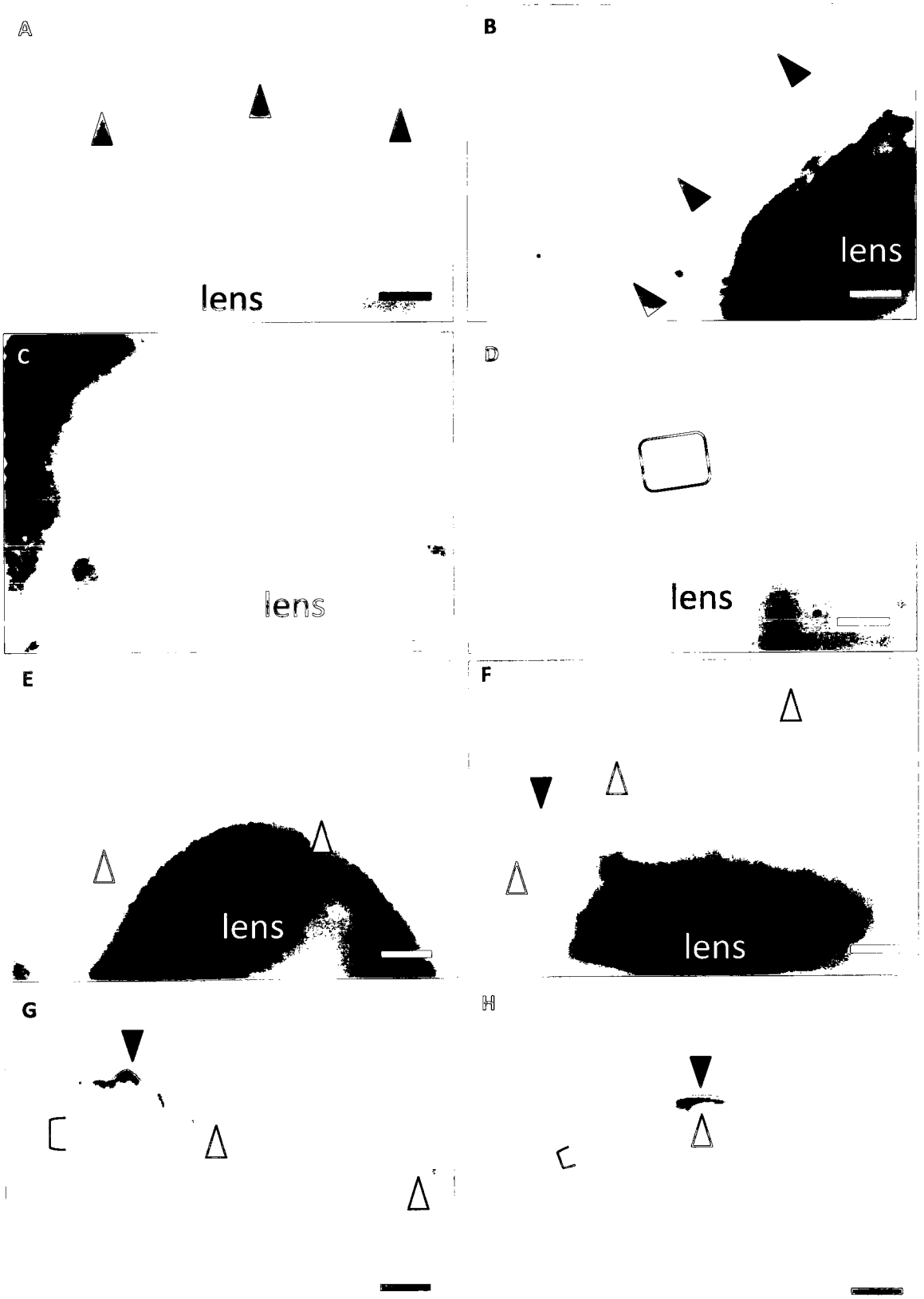
To further analyze this *ptc* expression pattern, whole-mount *in situ* hybridization tissue was sectioned using a cryostat. In serial sections, *ptc* expression is clearly visible in the papillae and in a region of epithelium adjacent to the papillae (Figure 10G&H). In addition, *ptc* was detected in the mesenchyme below the papillae as well as in the adjacent mesenchyme (Figure 10G&H). These results suggest that *shh* signal is received

by a wider range of epithelium than just the papillae and is also received by the mesenchyme directly below and adjacent to the papillae.

Table 6: *Patched in situ* hybridization expression pattern in the eyes of HH stages 30 through 40

Embryonic Stage	***Ptc* Expression**
HH 30 (6.5-7 days)	No expression
HH 31 (7-7.5 days)	No expression
HH 32 (7.5 days)	No expression
HH 33 (7.5-8 days)	No expression
HH 34 (8 days)	No expression
HH 35 (8.5-9 days)	Expression in the papillae and starting to appear in the surrounding mesenchyme.
HH 36 (10 days)	Expression reduced in the papillae and expression in the surrounding mesenchyme.
HH 37 (11 days)	No expression
HH 38 (12 days)	No expression
HH 39 (13 days)	No expression
HH40 (14 days)	No expression

Figure 10: Location of *ptc* mRNA based on whole-mount *in situ* hybridization expression in eyes of embryos from HH stages 34-37 (A) HH stage 34 embryo with no expression in the papillae (black arrowheads); (B) HH stage 35 embryo with expression in two papillae as well as a third papillae beginning to show expression in the underlying mesenchyme (black arrowheads); (C) HH stage 36 with expression located both in the papillae and surrounding the papillae in the underlying mesenchyme; (D) HH stage 37 embryo with early condensations (an outline around one condensation) and no *ptc* expression; (E) HH stage 36 sense probe hybridization with no expression shown in the papillae (white arrowheads); (F) HH stage 36 hybridization performed without probe and therefore no expression is shown in the papillae (white arrowheads), however a small amount of precipitate (not signal) from the colour reaction is shown (black arrowhead); (G) cryostat section of the HH stage 36 hybridized tissue showing epithelial signal in the papillae (black arrowhead) as well as underlying mesenchymal signal (white arrowheads), bracket indicates the scleral cartilage; (H) cryostat section of HH 36 hybridized tissue located directly next to a distinct papillae showing more intense expression in the epithelium adjacent to the papillae (black arrow) and localized mesenchymal expression directly beneath the epithelium (white arrow), bracket indicates scleral cartilage. Scale bars in A-C represent 250 μm, D is 500 μm, E and F are 200 μm, G and H are 100 μm.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3.4 Determining if other candidate genes *scleraxis* and *msx2* are present during the induction and growth of scleral ossicles

In order to determine if *scleraxis* is present during the induction of scleral ossicles whole-mount *in situ* hybridization was performed. After an initial *in situ* hybridization on HH stages 33, 36, and 38 in which *scleraxis* expression was identified at HH 36, a more extensive investigation was carried out on a wider range of HH stages 30- 40 (Table 7). No *scleraxis* expression was detected in HH stages 30-34 (Figure 11A). HH stage 35 was the first stage when expression was detected and this expression continued to HH 36 (Figure 11B&C). At HH stage 37 the papillae have completely degenerated and condensation formation begins. No *scleraxis* expression was detected at this stage (Figure 11D). Similarly no *scleraxis* expression was shown in HH stages 38 through 40. A sense probe and a no probe control were both performed and no expression was detected (not shown). To further analyze the *scleraxis* expression pattern, tissue after whole-mount *in situ* hybridization was sectioned using a microtome. In 5 μm serial sections, *scleraxis* expression is clearly visible in the papillae and in the adjacent epithelium alone and not in the underlying mesenchyme (Figure 11E). These results suggest that *scleraxis* is important during HH stages 35 and 36 when the papillae are the longest and that *scleraxis* is not involved in the induction of scleral condensations or the growth of scleral ossicles.

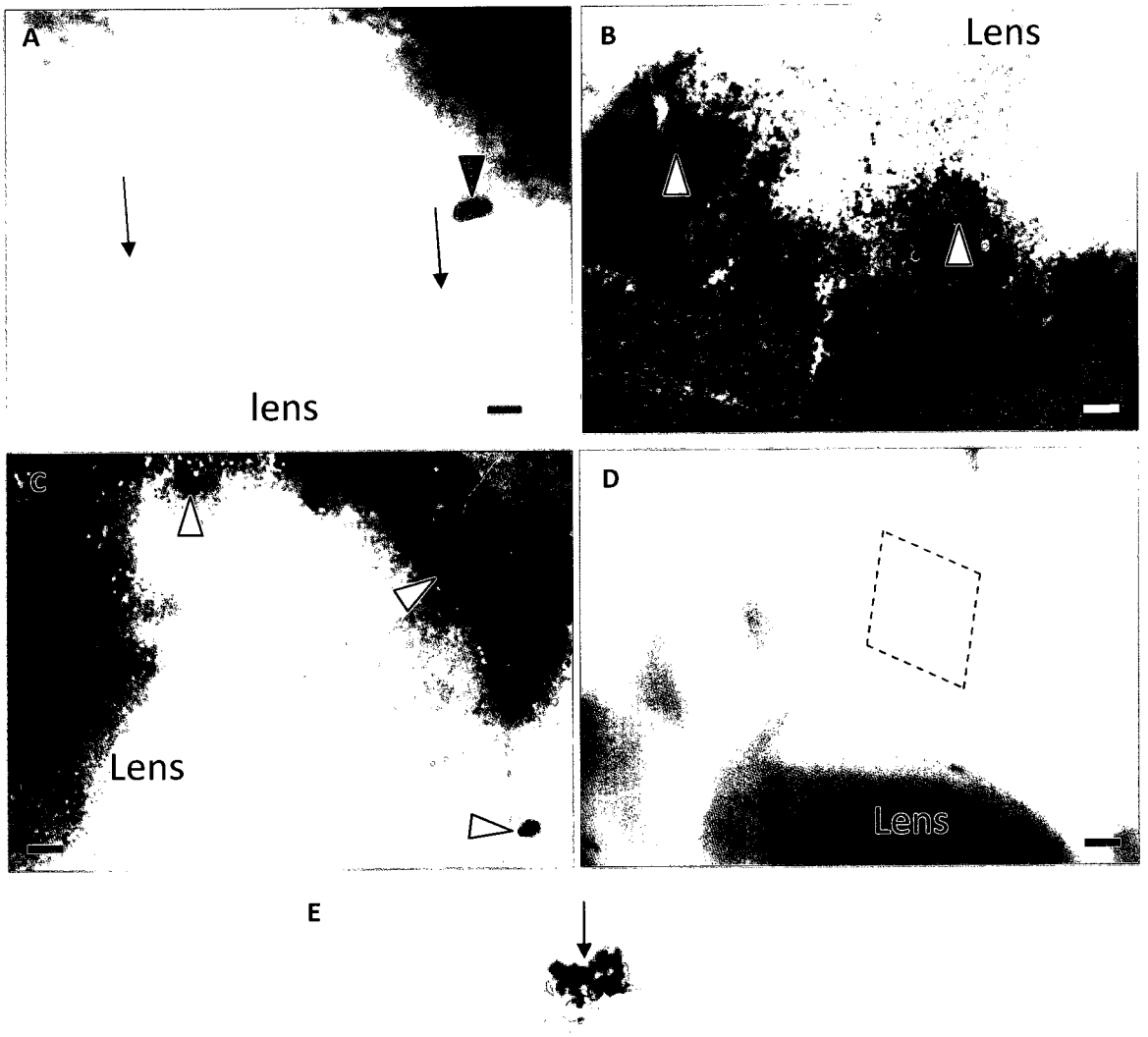
Due to its role in maintaining the proliferation of osteoblasts in the osteogenic fronts of developing mice calvaria, *msx2* was investigated for its potential role in the expansion and growth of scleral condensations. In order to determine if *msx2* is present during the condensation growth period, an initial *in situ* hybridization on HH stages 35/36

(induction stage), HH 38 (early condensation stage), and HH 40 (late condensation stage) was performed. *Msx2* expression was not found in the eye at any of the stages investigated (Figure 12A) but was detected in the developing mandible in a unique expression pattern with the most distal tip of the beak lacking expression (Figure 12B). This result suggests that *msx2* is likely not involved in the induction or growth of scleral condensations.

Table 7: *Scleraxis* and *msx2 in situ* hybridization expression pattern in embryos at HH stages 30 through 40.

Stage	***scleraxis* expression**	***msx2* expression**
HH 30 (6.5-7 days)	No expression	-
HH 31 (7-7.5 days)	No expression	-
HH 32 (7.5 days)	No expression	-
HH 33 (7.5-8 days)	No expression	-
HH 34 (8 days)	No expression	-
HH 35 (8.5-9 days)	Expression in the papillae	No expression in the eyes
HH 36 (10 days)	Expression in the papillae	-
HH 37 (11 days)	No expression	-
HH 38 (12 days)	No expression	No expression in the eyes; expression in the mandible and feather follicles
HH 39 (13 days)	No expression	-
HH 40 (14 days)	No expression	No expression in the eyes; expression in the mandible and feather follicles

Figure 11: Location of *scleraxis* mRNA based on whole-mount *in situ* hybridization expression in eyes from HH stages 34-37. (A) HH stage 34 embryo with no signal present in the papillae (arrow), black arrowhead represents precipitate from the colour reaction and is not signal; (B) HH stage 35 embryo showing expression of *scleraxis* specifically in the two adjacent papillae (white arrowheads); (C) HH stage 36 embryo with expression in the papillae (white arrowheads); (D) HH stage 38 embryo showing ossicles (outlined by the hashed line) without any *scleraxis* expression; (E) 5 μm section of a piece of hybridized tissue showing the *scleraxis* signal in the papilla (arrow) and not in the underlying mesenchyme. Scale bar in A and B represent 100 μm, C is 200μm, D is 100 μm, and E is 40 μm.


A
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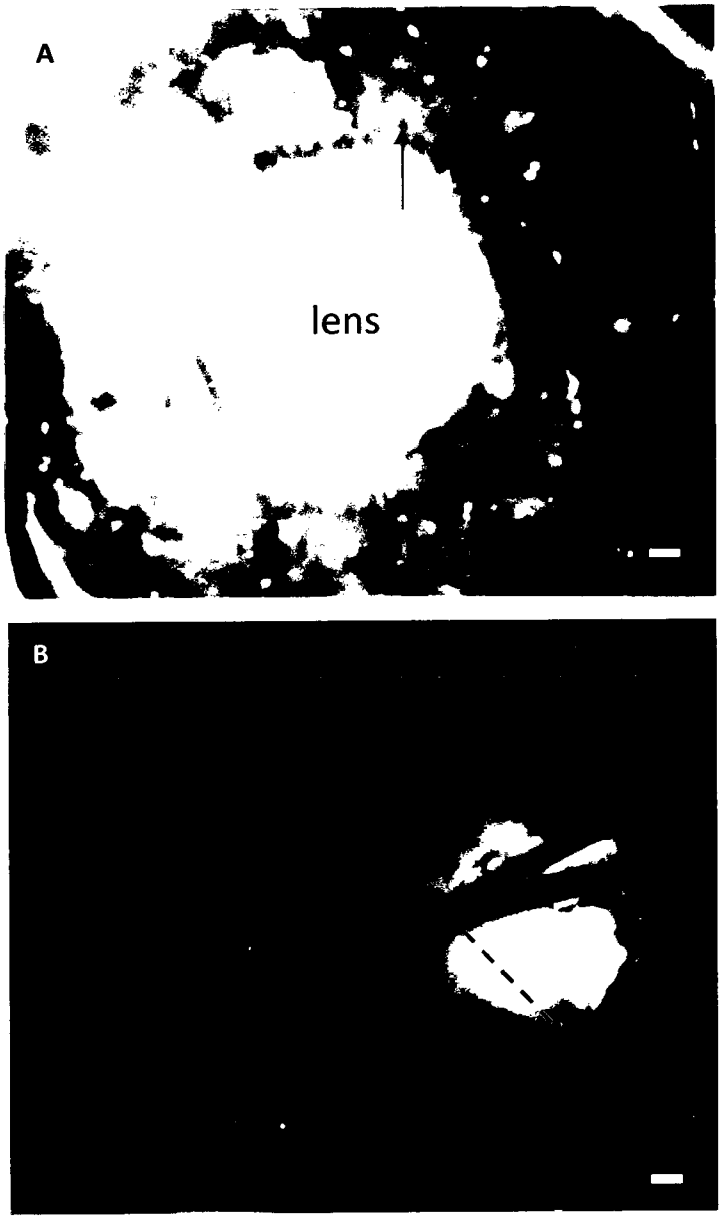

Figure 12: Location of *msx2* expression in the chicken head. (A) Anterior region of a HH stage 39 eye was removed to confirm the lack of expression in the condensations (arrow). (B) At HH stage 40 beak with a unique expression pattern, the most distal part of the mandible is clear of expression (line indicates border of signal); feather follicles also show expression. Scale bars in A and B represent 200 μm.

4.0 Discussion

4.1 Application of exogenous noggin inhibits ossicle formation

Noggin is a secreted protein used in developmental pathways such as the limb, ear, and skeleton, for the inhibition of the bone morphogenetic protein family. Noggin inhibits *BMP2, BMP4,* and *BMP7* by binding to these proteins and therefore rendering them inactive (Zimmerman *et al.,* 1996). This specific property of noggin, makes it a very useful feedback molecule in pathways that include the *BMP* signalling molecules.

In limb development, *noggin* plays an important role. *Noggin,* with the BMP family, the BMP receptors, and other TGFβs form a feedback loop that is crucial in anterior-posterior patterning of the limb. Digit chondrogenesis is regulated by this feedback. *Noggin* is crucial in this feedback loop for the maintenance of the proper size and shape of the developing cartilaginous digits (Merino *et al.,* 1998).

Noggin was also used in an experimental setting to demonstrate the effects of *BMP* knockout during the development of the inner ear. *BMP4* is expressed during a variety of time points in the developing inner ear in chicken embryos (Chang, *et al.*, 1999). In order to understand the specific role *BMP4* has in the development of the inner ear, noggin can be used to inhibit its effects. A noggin-soaked aff-gel bead was implanted into the developing inner ear. This application inhibited the *BMP4* normally expressed in the inner ear, and produced a variety of mutant phenotypes. These mutant phenotypes demonstrated that *BMP4* plays a crucial role in the sensory and non sensory structures (semicircular canals) of the inner ear. Also, it was determined that *BMP4* plays a crucial role in the patterning of the cristae of the inner ear. With the ability to knock out *BMPs*

important information can be gained about their role in development including systems such as scleral ossicles.

During condensation formation, *BMP2, 4,* and *7* are important molecules for the recruitment of mesenchymal cells into the skeletogenic condensation (Hall and Miyake, 2000). Recruitment of cells into a condensation is a required step for the proper development of skeletal tissues. However, there is a limit to the number of cells required in a condensation in order for cellular differentiation to occur. Therefore noggin is likely present during condensation formation to inhibit the BMP*s* at the appropriate time and subsequently favour differentiation indirectly resulting in condensation formation.

In my research, the exogenous application of noggin during the development of scleral ossicles had a number of effects. Firstly, the ossicle number was typically lower in the bead implantation eye than in the control eye (Table 4). This result alone is however not conclusive of noggin's effect, since Franz-Odendaal (2008) showed that there is a naturally occurring variance in the ossicle number between the right and left eyes of chicken embryos . However, there was also a noticeable difference in the morphology of the condensations in the experimental eye compared to the control eye. A gap in the ring of ossicles was shown in the eyes of embryos that had a noggin-bead implantation (Figure 8). Moreover, this gap (corresponding to a missing ossicle) could always be found in the mesenchyme underneath the epithelial location of the prior bead implantation. In one of the embryos, it appeared as though a condensation was present (Figure 8A) however ossification had not yet occurred. The faint alkaline phosphatase staining could represent a bead implantation that has delayed the condensation from reaching a critical size needed for differentiation of osteoblasts to occur. This delay in

ossification was likely due to slight differences in the timing of the bead implantation. HH stage 35 is defined as day 8.5 to 9, therefore it has a 12 hour range. A bead implantation toward the end of this temporal range could have led to delayed osteoblast differentiation within the condensation rather than the inhibition of condensation formation. Inhibition of a completely ossified ossicle was shown in 100% (6/6) of the embryos that survived to HH stage 38 (or later).

These results are strengthened by the consistent controls performed with each bead implantation. The amount of noggin on each bead was controlled by maintaining a concentration of 1.0 μg/μl per 30 beads of approximately the same size. There were two separate controls for these bead implantations. The first control was achieved by comparing the right and left eyes of the same embryo. One of the eyes had a noggin bead implantation, while the other eye is not affected by the local application of noggin. This way we can compare the number and size of the ossicles in the right and left eye for each embryo (Figure 8 A&B, C&D, and E&F). A second control experiment was performed to further demonstrate that the bead implantation alone was not the cause of the inhibitory effect shown in this experiment. Affi-gel beads of the same size were soaked in PBS alone and then implanted into the eyes in the exact same manner as the noggin beads. When PBS-soaked beads were implanted into the eye, no inhibition of ossicles was observed (Figure 7). In this case, 100% (11/11) of the embryos that survived post HH stage 38 with PBS-soaked beads showed no ossicle inhibition. Therefore the inhibition of ossicle formation must be attributed to the exogenous application of noggin, which inhibits BMPs in the local area of ossicle development.

4.2 *BMP* involvement in the developmental pathway of scleral ossicles

The *BMP* family is known to be involved in a variety of developmental systems. During condensation formation, *BMP2, 4,* and *7* are important molecules for the recruitment of mesenchymal cells into the condensation (Hall and Miyake, 2000). Secondly, *BMPs* are produced as signalling factors in a variety of epithelial signalling centres such as during tooth development (Thesleff, 2003). The *BMP* signal induces the underlying mesenchyme to condense or become competent for further inductive events. The mesenchyme then produces a reciprocal BMP signal, *BMP4*. *BMP4* signals back to the epithelium to regulate the formation of the dental placode. Later, this *BMP4* signal produced in the mesenchyme will aid in the formation of the enamel knot. Finally, *BMP2, 4,* and 7 are all produced in the enamel knot and released as signalling factors. Some of those BMPs act in an autocrine manner, signalling to the epithelium itself and helping to maintain the enamel knot. Other BMPs signal to the mesenchyme, which will eventually result in further morphogenesis of the dental epithelium.

A second signalling centre that uses the *BMP* family of genes is in the developing limb, in the zone of polarizing activity. This area is responsible for the patterning of the hand bones (thumb to little finger). In this region it is suggested that *sonic hedgehog* and *BMP2* work together to control the patterning and development of the bones in the hand (Mariana and Martin, 2003). Finally, *BMPs* (specifically *BMP* 2 and 4) are important molecules in patterning and development of feathers (Noramly and Morgan, 1998). *BMP2* works as an inhibitory molecule to ensure the "track-like" pattern of the developing feathers. *BMP2* and *4* then continue to regulate feather growth by determining the size and shape of the feather bud. The size and shape of the feather bud is

likely not determined by *BMPs* alone; but may also involve the fibroblast growth factor family.

The role of *BMP* in these epithelial-mesenchymal interactions makes it an excellent candidate gene to be involved in the development of scleral ossicles. The precise role that the *BMP* family of genes is playing during the development of scleral ossicles is still relatively unclear and requires further investigation. However, a few important insights can be gained from the results shown in this study. Firstly, the papilla that was exposed to noggin was not affected. When papillae are exposed to cyclopamine, a Hedgehog inhibitor, they degenerated (Franz-Odendaal, 2008). However, this was not the case when *BMP* inhibition occurred. This would suggest that the *BMP* family of genes is not required for the maintenance of the developing papillae. Despite having no effect on the development and maintenance of the papillae, inhibition of *BMPs* resulted in the loss of the underlying ossicle and the expansion of other ossicles in the eye. The loss of a scleral ossicle suggests that the BMP family is likely involved in the induction or subsequent development of scleral ossicles.

Since the noggin-soaked bead was placed directly under the epithelium, the noggin protein can diffuse to the adjacent epithelium and to the underlying mesenchyme. Therefore, BMP signalling could be present in the papillae, underlying mesenchyme, or deeper retinal pigmented epithelium (RPE) (Figure 13). The latter seems unlikely considering that the absorbed noggin on the bead only diffuses a short distance. This distance is demonstrated by the fact that only the ossicle directly underneath the bead is inhibited. The noggin on the bead is unable to diffuse further and cause an effect on surrounding ossicles. In addition the entire scleral mesenchyme and the scleral cartilage

are relatively thick in comparison to the distance between papillae, therefore it is unlikely that the noggin would diffuse across this distance to have an affect at the RPE. Therefore I hypothesize that the BMP signals that were inhibited by noggin are likely present in the epithelium, or underlying mesenchyme, or both. Further investigation is required, in order to determine exactly where BMP is expressed. Expression of *BMP 2* and 4 was shown to be slightly increased (3.5 fold and 2.7 fold respectively) at HH stage 36 through real-time PCR (Franz-Odendaal, 2008). However, this increase was not considered important at the time when compared to the drastic increase (30 fold) of *shh* expression at that stage. Also, previous *in situ* hybridization results, by an honours student in the lab (M. Bauer), showed that there was no expression of *BMP2* in the papillae or underlying mesenchyme during the inductive stages of scleral ossicle development. My results have shown that the BMP family of genes (most likely BMP2, 4, or 7) are involved in the induction and/or development of scleral ossicles. Therefore I suggest that *BMP7* is the most likely BMP member involved in scleral ossicle development.

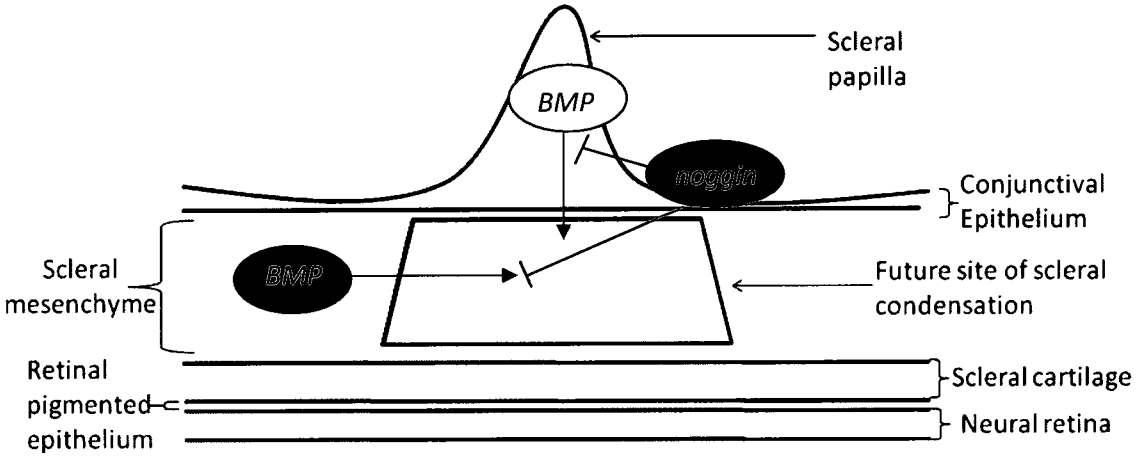

Figure 13: Possible locations for BMP signals during the development of scleral ossicles. Blue arrow represents epithelial-mesenchymal interaction from the papilla. Green arrow represents *BMP* involvement in the recruition of mesenchymal cells into the ossicle condensation. Red circle demonstrates placement of a noggin-soaked bead and the inhibitory effects that are possible on each of the BMP signalling pathways.

4.3 Neighbouring ossicles demonstrate the ability to compensate for a missing ossicle

Alkaline phosphatase stain was used in this study to visualize ossicles after bead implantation. This allowed for a comparison between the experimental vs. control eye of the embryo. In general, it appears that timing of the bead implantation is very important. If, the bead is implanted early during the induction stage then the papillae around papilla 8 or 9 are likely still in the process of inducing the underlying mesenchyme. Therefore, the papillae directly adjacent to papilla 8 or 9 after bead implantation will be able to alter its signalling and induce the formation of a larger ossicle. This would suggest that the ossicle directly adjacent to the missing ossicle is much larger to compensate for the gap in the ring (Figure 8 C). However, if bead implantation is performed slightly later (HH 35.5), induction of the underlying mesenchyme could already be underway at papillae 8 or 9 (Figure 2). In order to compensate for the inhibition occurring at papillae 8 or 9, the next group of papillae to induce the underlying mesenchyme would have the means to induce larger condensations (Figure 8A, group of ossicles marked by 1, 2, and 3). In general, the ossicles in the surgery eye were larger than the identical ossicle in the control eye (Table 4). There was one exception to this observation. In one individual, the control side actually had larger ossicles (Figure 8 E&F). In this eye the gap in the ring of ossicles on the bead implantation side remained present later in development, which demonstrates that the surgery eye was not capable of compensation for the missing ossicle.

The concept of compensation suggests that the formation of a complete sclerotic ring is important. This is further demonstrated in the *scaleless* mutant, in which despite forming an average of three papillae (and often only one or two ossicles), the developing

ossicles are grossly enlarged in an attempt to form a complete ring (Palmoski and Goetinck, 1970). The importance of this ring is implied, since the eyes are a crucial organ for most vertebrates. Moreover scleral ossicles are important for the accommodation that occurs in the eye during depth perception. Therefore the unique patterning of papillae/ossicle development could be explained evolutionarily. The delayed induction of individual groups of papillae enables the ring of ossicles to gain many opportunities to compensate for the potential loss or injury of an ossicle.

4.4 *Sonic hedgehog* is likely the only member of the Hedgehog gene family involved in the induction of scleral ossicles

Our lab has shown that *shh* is present during the inductive HH stage 35 and 36 of scleral ossicle development and that the inhibition of the Hedgehog family through exogenous application of cyclopamine (a Hedgehog inhibitor), prevents ossicle formation (Franz-Odendaal, 2008). These results suggest that *shh* likely plays a role in the development of scleral ossicles. However, these findings do not eliminate the possibility of other members of the Hedgehog family playing a role during this induction. *Indian hedgehog* is another common vertebrate Hedgehog gene, which plays a role in a skeletal development, albeit typically endochondral in nature.

Following the optimization of the *in situ* hybridization protocol, *ihh* was investigated. However, *ihh* expression was not shown at HH stage 33, 36, or 38 (Figure 9B). The lack of *ihh* expression in the induction of scleral ossicles, suggests that the only confirmed member of the Hedgehog family that is involved in this pathway is *shh*. *Desert hedgehog* is the other vertebrate Hedgehog family member. It is typically expressed

during sexual development and is therefore not likely playing a role in skeletogenesis. Also, a search of the *Gallus gallus* genome indicates that *dhh* is not present in chickens and possibly all birds and is therefore not a likely candidate.

Both expression of *ihh* and *shh* were found in the developing mandible at HH stage 36. While *shh* expression was shown throughout the mandible, *ihh* demonstrated a unique expression pattern in which the most distal end of the mandible was devoid of signal (Figure 9C). This pattern may suggest a signal gradient that occurs during the development of the mandible, but this interesting pattern is beyond the scope of this study.

4.5 *Sonic hedgehog* acts as an autocrine and paracrine signal during the development of scleral ossicles

Sonic hedgehog (*shh*) is common in many developmental systems, such as the developing craniofacial skeleton and the limb. *Shh* is involved in the development of the entire frontonasal and maxillary processes (Hu and Helms, 1999). Fluctuations in the normal *shh* signalling during the development of these craniofacial bones is thought to play a role in a variety of human genetic disorders such as cleft lip/palate and hypertelorism. Inhibition of *shh* causes the truncation of the frontonasal and maxillary processes primordia affecting the mediolateral axis of the face resulting in cleft lip. Conversely, overexpression of *shh* can cause the structures in the midline (such as the nasal bone) to duplicate and can also lead to a widening of the frontonasal and maxillary processes known as hypertelorism. This suggests that accurate expression of *shh* is required during the development of craniofacial bone.

A second example where correct *shh* signalling is required, is in the limb. *Shh* is produced by the zone of polarizing activity (ZPA) and acts as a morphogen to produce the anterior-posterior patterning seen in the digits (Freeman, 2000). Recent studies suggest however, that a more complex signalling pathway occurs during the development of the digits (McGlinn and Tabin, 2006). *Shh* is now thought to have both paracrine and autocrine roles. The most anterior digit (digit 1) is not affected by *shh* and never produces *shh* signal. Moving in a posterior direction, digit 2 and half of digit 3 never produce *shh* signal. However these digits rely on *shh* signal for their patterning, suggesting that *shh* signal is made at a more posterior location and received by these anterior digits, in a paracrine signalling manner. Finally the most posterior digits 4 and 5 (and half of digit 3) produce *shh* signal and also receive that signal for patterning, in an autocrine signalling manner. In the 4 digits where *shh* is received, it acts on both the mesenchyme and the epithelium to form the correct patterning of skin, bones, and muscle along the anterior-posterior axis. These findings demonstrate that *shh* can act as both an autocrine and paracrine signal in developmental systems.

In order to better understand the role that *shh* plays during the induction of scleral ossicles, the location and distribution of the Hedgehog receptor *patched (ptc)* was determined. Although *ptc* is the ligand receptor for the entire Hedgehog family of proteins, it was previously determined that only *shh* is present during ossicle induction. The location of the *shh* receptor indicates the tissue on which *shh* is acting. During the induction of scleral ossicles, the expression of the Hedgehog receptor *ptc* in both the epithelium and the mesenchyme suggests that *shh* is likely acting in both an autocrine and paracrine manner (Figure 14). There are several pieces of evidence for this signalling

pattern based on the results obtained in this study. Firstly, *in situ* hybridization was performed on embryos HH stages 30 through 40. *Ptc* expression was only shown in HH stage 35 and 36 (Table 6), which corresponds directly to the expression of *shh* shown by Franz-Odendaal (2008). Secondly, the expression of *ptc* in the papillae and the mesenchyme seems to have a temporal component. Meaning that, first *ptc* is expressed strictly in the papillae (HH stage 35) (Figure 10B) suggesting that *shh* appears to act on the papillae itself first. Then the expression is shown both in the papillae as well as the underlying mesenchyme (HH stage 36).

During HH stage 35 when the expression is located in the papillae, expression was also shown in the epithelium adjacent to the papillae (Figure 10H). This epithelial expression demonstrates that *shh* has two possible roles. Firstly, *shh* could be signalling to the papillae as a positive feedback signal to produce more *shh.* Secondly *shh* could be acting on the papillae and surrounding epithelium in order to maintain the structure of the papilla itself. Previous proliferation studies show that early in papillae development, the entire papillae is proliferating; as the papillae matures only the outer most cells of the papillae are proliferating (Franz-Odendaal, 2008). Therefore *shh* could be signalling to maintain the proliferation occurring throughout the papillae at HH stage 35 and as the papillae matures into HH stage 36 the proliferation is decreased correlating to a decrease in *shh* activity as well as *ptc* expression in the papillae. At the point (HH stage 36) *ptc* expression is also located in the underlying mesenchyme (Figure 10C). This finding suggests that not only is *shh* maintaining the papillae structure, but it is also signalling to the mesenchyme to likely induce condensation formation. All of these findings suggest

that *shh* signalling during the development of scleral ossicles has a dual method of action with an autocrine and paracrine component (Figure 14).

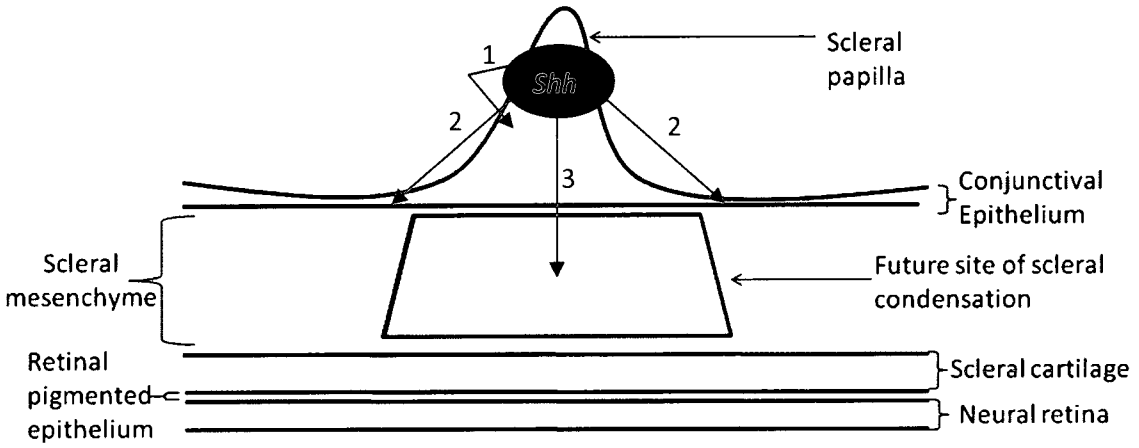

Figure 14: Schematic showing suggested autocrine and paracrine signalling pathway for *shh* during scleral ossicle development based on the *in situ* hybridization expression of the *ptc* receptor. *Shh* is produced in the papillae and then signals in an autocrine manner to the epithelium itself (arrow 1). *Shh* signals to the epithelium of the papillae and surrounding epithelium (arrows 2). S*hh* undergoes paracrine signalling to the underlying mesenchyme (arrow 3).

4.6 The role of *scleraxis* during scleral papillae development

Scleraxis is a member of the basic helix-loop-helix (bHLH) family of transcription factors and has been shown to play an important role in cell differentiation, proliferation, and development of connective tissue such as tendons and ligaments (Espira *et al.*, 2009). Studies have shown that the expression pattern of *scleraxis* in early embryogenesis is located mainly in the mesoderm, for example, in developing somites (Burgess *et al.,* 1995). Specifically *scleraxis* expression was shown in the somites that give rise to the ribs and vertebrae. However, as soon as ossification beings in these areas, *scleraxis* expression is consistently downregulated (Cserjesi *et al., 1995).* Also *scleraxis* is known to be a marker of mesodermally derived mesenchyme for the axial and appendicular skeleton, particularly in areas that form ligaments and tendons. These findings suggest that *scleraxis* is important in support structures such as; connective tissue of the spine and ribs, tendons, and ligaments.

In situ hybridization results in this study demonstrated that the expression of *scleraxis* was limited to HH stages 35 and 36 (Table 7). These stages are when the papillae are the largest and when induction is occurring. Despite papillae being present at HH stage 30 through 34, *scleraxis* was not expressed in any of these stages (Figure 11A). The absence of *scleraxis* expression in these early papillae stages is likely due to the fact before HH stage 35 the papillae are still relatively small and remain close to the conjunctiva. By HH stage 35 the papillae are large and form a distinct elongation of conjunctival epithelium (Figure 15). This elongation of the epithelium likely needs more structural support. Later in HH stage 36 the group of papillae over the ciliary

artery may start to degenerate. *Scleraxis* expression is not found in papillae that begin degenerating. Based on the sectioning results *scleraxis* expression is located in the papillae alone, not in the underlying mesenchyme (Figure 11E). All of these findings, as well as evidence from the literature, suggest that in mature papillae *scleraxis* is necessary for support.

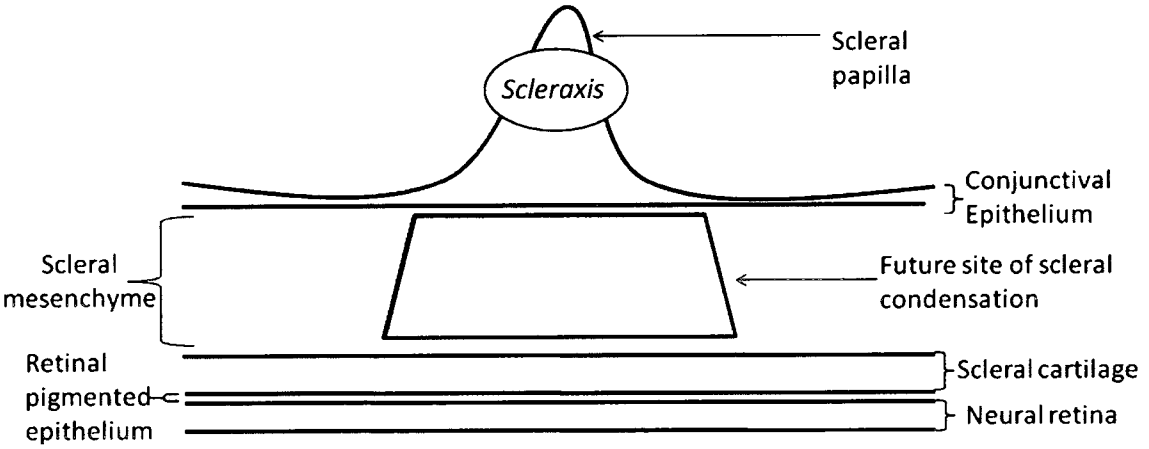

Figure 15: Schematic showing hypothesized *scleraxis* involvement in the development of scleral papillae based on *in situ* hybridization expression. *Scleraxis* is localized to the epithelium when the papillae structure is large (purple).

4.7 *Msx2* not involved in condensation growth

In vertebrates, the *msx* gene family consists of three members (*msx1, msx2, msx3*), however only *msx1* and *msx2* are widely expressed throughout the development of skeletal tissues such as the calvariae (Alappat *et al.*, 2003). Specifically, *msx2* is found in the suture mesenchyme and dura of the developing calvariae. In a wild type mouse skull, *msx2* is required to maintain the proliferation of the osteoblasts present at the growing osteogenic fronts of calvariae bones (Liu *et al.,* 1999). These results suggest that *msx2* plays a crucial role in the balance of proliferating osteoblasts and differentiating osteocytes in the developing intramembranous calvariae. Due to its involvement in the developmental pathway of these neural crest derived intramembranous bones, *msx2* was hypothesized to be involved in the expansion and growth of scleral ossicle condensations and scleral ossicles.

In order to determine if *msx2* was involved in the growth and expansion of scleral ossicles, HH stages 35 and later were investigated. Expression of *msx2* was not found at HH stage 35 during induction, or at later stages (HH 38 through HH 40) when condensation growth was occurring (Table 7). *Msx2* expression was shown in other areas of the HH 40 embryos such as the mandible and feathers (Figure 12B). These results suggest differences in the molecular signals involved in the growth of specific intramembranous bones such as calvariae and scleral ossicles.

5.0 Conclusions

Bone development is a highly regulated, step-wise process, which involves a number of tissues and hierarchal signalling events. In order to understand the process of intramembranous bone development, different intramembranous bones must be studied and compared. Although a variety of information exists pertaining to the development of calvariae (the intramembranous bones of the skull), in comparison very little information exists regarding the development of scleral ossicles (intramembranous bones of the eye). This study succeeded in unravelling more of the molecular pathway involved in the development of scleral ossicles, which has been a 30 year mystery until the research performed by our lab.

Firstly, the *BMP* family of genes is suggested to be crucial in the induction and subsequent development of scleral ossicles. Through local inhibition of the BMP family of genes via exogenous application of noggin, scleral ossicle formation was inhibited (Figure 16). This result suggests that scleral ossicles are unable to form without *BMPs*. Despite gaining the understanding that *BMPs* are crucial for the development of scleral ossicles, it is still uncertain how the *BMP* signalling is occurring and what specific *BMPs* are involved. Based on the literature, *BMP 2, 4,* and *7* are most likely. However, based on the previous real-time PCR and *in situ* hybridization evidence from the Franz-Odendaal lab (Franz-Odendaal, 2008), *BMP 2* and *4* have a minor role in ossicle development; *BMP7* is the next most likely candidate. Three possible pathways are suggested. First, *BMPs* might act via an epithelial mesenchymal interaction between the papillae and the underlying mesenchyme. Second, BMPs might be signalling in the mesenchyme alone

and could be responsible for the recruitment of mesenchymal cells into scleral condensations (Figure 16). Finally, *BMPs* interact via an epithelial mesenchymal interaction with the RPE and the overlying scleral mesenchyme. Due to the large distance between the bead placement under the epithelium and the RPE (beneath the scleral cartilage), this pathway is not likely. Further investigation is required in order to better understand the role that the *BMPs* play during the development of scleral ossicles.

Through inhibition of a scleral ossicle, observations were made on the ability of the ring of ossicles to compensate for a missing ossicle. The possibility for compensation of scleral ossicles shown in this study, demonstrates a potential evolutionary reason for why the scleral papillae and ossicles are patterned in such a sequential way. The delay between the development of groups of papillae/ossicles could allow the sclerotic ring to compensate for the loss of an ossicle or potential injury. This is a vital adaptation because the eyes are such an important organ for most vertebrates and scleral ossicles are important for accommodation in the eye. It would follow, that there would be mechanisms in place to ensure that the development of the eye occurs in a way that can consistently produce a functional eye, despite potential disruptions to the developmental system.

Through *in situ hybridization* a variety of genes were investigated for their involvement in the development of scleral ossicles. Firstly it was demonstrated that *shh* is likely the only member of the Hedgehog family involved in the induction of scleral ossicles. *Indian hedgehog* could also have been playing a role during this induction

however my research demonstrated that *indian hedgehog* is not present during the induction of scleral ossicles (Figure 16).

A potential role for *shh* during the induction of scleral ossicles has been suggested. The *in situ* hybridization expression pattern of the Hedgehog receptor *ptc* was determined and both an epithelial and mesenchymal component were discovered. This suggested that *shh* is likely acting in an autocrine fashion, signalling to the papillae likely for maintenance and proliferation. Also, *shh* is likely working as a paracrine factor, signalling via epithelial-mesenchymal interaction to the underlying scleral mesenchyme in order to assist in the formation of scleral condensations (Figure 16).

Finally, other candidate genes such as *scleraxis* and *msx2* were investigated via *in situ* hybridization. It is suggested that contrary to the important role *msx2* plays during the development of calvaria, *msx2* does not play a role in the development of scleral ossicles. In contrast, we were not predicting the involvement of *scleraxis,* since its presence is usually shown in systems not related to bone development. However, *scleraxis* expression was shown during HH stage 35 and 36 in the papillae (Figure 16). This result suggests that *scleraxis* is likely not involved in the inductive signalling events of scleral ossicle development. However, *scleraxis* is suggested to play a role in the support of mature papillae; possibly in conjunction with *tenascin* (unpublished, Franz-Odendaal).

In summary, this research has provided much more information regarding the molecular pathways involved in scleral ossicle development (Figure 16), and therefore has also contributed to the understanding of intramembranous bone development, in general. To better understand the complete developmental pathway involved in scleral

ossicles the following research should be completed, which was outside the scope of my thesis. Our lab is currently performing a comparative microarray experiment to further investigate the development of scleral ossicles. Further *in situ* hybridization experiments should be performed, specifically with *BMP7,* to attempt to highlight the presence of a specific member of the BMP family. As well, *in situ* hybridization experiments could be performed to locate the receptors of the BMP family in order to determine which tissues the BMPs are acting on. Finally, other gene families should be investigated such as the fibroblast growth factor (FGF) family. All of this information will help to complete the story of the molecular pathways involved in scleral ossicle development.

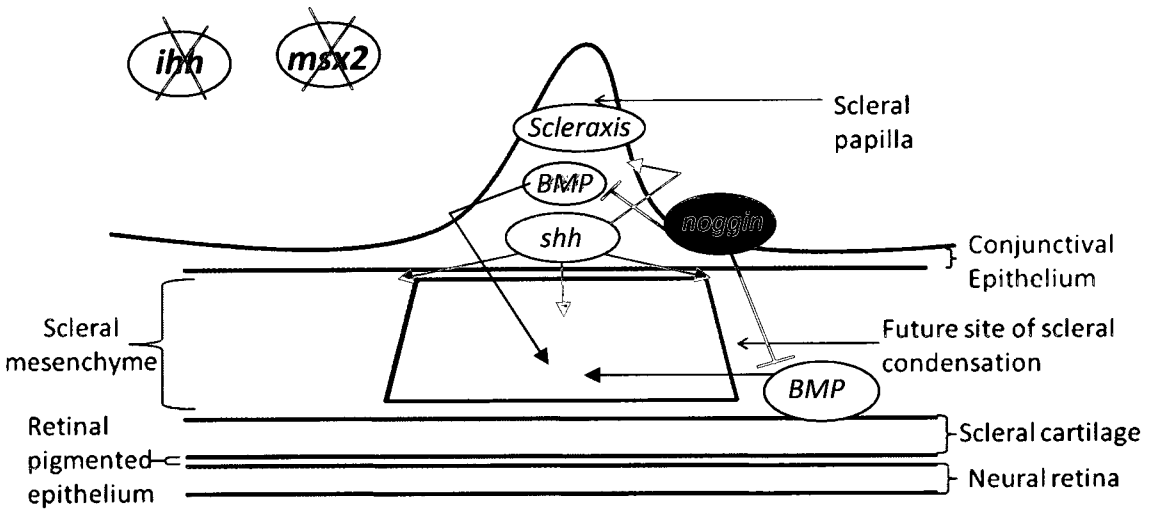

Figure 16: Suggested molecular pathway involved in the development of scleral ossicles. Potential signalling events from the *BMP* family of genes are indicated in blue. Inhibitory effects on the BMP family via noggin bead implantation at the epithelium shown in red. *Shh* autocrine (to the papilla) and paracrine (to the mesenchyme) signalling demonstrated by orange arrows based on the *in situ* hybridization expression of the Hedgehog receptor *ptc. Scleraxis* is found in the papillae (purple) for support of the papilla. *Indian hedgehog and* msx2 expression were not found and therefore are likely not involved in this developmental pathway (shown by red X's).

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Appendix A:

LB/ampicillin plates

6.25 g of Luria-Bertani (LB) Broth (Difco, 244620)

3.75 g of agar (Difco, 214530).

250 ml of dH2O.

- Heat until dissolved and then autoclave.
- After autoclaving and the solution has cooled, add 0.001% ampicillin (Sigma, A6140).
- Stirred well and then pour the solution into sterile petri-dishes and allow to cool until solid.
- Parafilm dishes closed and store the plates at 4°C until ready for use.

Appendix B.

Plasmid isolation protocol

- Place 2 ml from each overnight culture into individual eppendorf tubes. Spin these tubes for one minute, twice to obtain a large pellet. Remove supernatant.
- Add 250 ul of cold buffer pH8 to each sample and re-suspend pellet in the buffer.
- Add 250 µl cold lysis solution and mix. Place on ice for 5 minutes.
- Add 250 µl of cold 1M potassium acetate and mix the solution until cloudy. Place on ice for 5minutes.
- Centrifuge for five minutes and collect the supernatant in a fresh tube.
- Add 200 µl of the bottom layer of phenol-chloroform to the supernatant and mix for 30 seconds.
- Centrifuge the sample for five minutes and collect the top layer of solution with a pipette in a new tube.
- Add an equal volume (approximately 650 µl) of isopropanol, mix briefly and let stand for two minutes at room temp.
- Centrifuge for five minutes and discard the supernatant. Add 200 µl of cold 95% ethanol and invert the tube gently.
- Centrifuge for five minutes and remove the supernatant. Allow the final pellet to dry completely at room temperature before adding 30 µl dH2O. Store the sample at -20°C.

Plasmid isolation recipes:

Cold Buffer (made fresh) 1 ml

- 50mM TrisCl
- 10mM EDTA (Sigma, E5134)
- 100µg/ml Ribonuclease A (Sigma – R4642)

Lysis solution (made fresh) 1 ml

- 100 µl of 2 M NaOH
- 100 µl 10% SDS (Sigma, L4390)
- 800µl distilled water)

Appendix C

Standard RNA labelling reaction:

This reaction is done using a prepared kit (Roche – 11175025910)

- Add 1 µg of the purified DNA sample to a RNase free tube. Then, add enough DepC treated water to the vile for a total volume of 13 µl.
- Add the following reagents to the DNA sample:
    - 2 µl of 10X NTP labelling mixture
    - 2µl of 10X transcription
    - 1 µl of protector RNase inhibitor
    - 2 µl of RNA polymerase (T7 or SP6)
- Mix the reagents and DNA sample gently and then place them at 37°C for 2 hours.
- Add 2 µl of DNaseI to the tube and place back in the 37°C incubator for 15 minutes.
- Stop the reaction by adding 2 µl of 0.2 M EDTA (pH 8)
- Place 1 µl of the now labelled RNA into a new vial. Store the rest of the labelled RNA probe at -20°C.

Preparation of probe dilutions for the labelling detection protocol

- Using RNA Dilution buffer, dilute the 1 µl of labelled RNA from the previous step so that the final concentration is 10 ng/µl.
    - Dilution buffer: Mix in a sterile RNase free tube, DepC treated water, 20X SSC, and formaldehyde in the ratio of 5:3:2 respectively.
- Using the dilution buffer and the following table, prepare 5 dilutions of the previous RNA solution.

Tube	RNA (µl)	From Tube #	RNA dilution buffer (µl)	Dilution	Final Concentration
1	-	From previous step	-	-	10ng/µl
2	2	1	18	1:10	1ng/µl
3	2	2	198	1:100	10pg/µl
4	15	3	35	1:3:3	3pg/µl
5	5	3	45	1:10	1pg/µl

- Proceed to the DIG high prime DNA labelling and detection protocol

Appendix D

DIG high prime DNA labelling and detection protocol

- Place 1µl of each RNA probe dilution to the membrane and fix the nucleic acids to the membrane by baking them for 30 minutes at 120°C in a sterile glass dish.
- Transfer membrane to a plastic petridish with approximately 20 ml of Maleic acid buffer (enough to cover the membrane). Incubate at 20°C with shaking for two minutes.
- Incubate at 20°C in blocking solution with shaking for 20 minutes.
- Wash for five minutes in TBST at 20°C with shaking
- Incubate at 20°C in 10 ml of antibody solution for 30 minutes with shaking.
- Wash with washing buffer at 20°C with shaking for 15 minutes, twice.
- Equilibrate for five minutes in detection buffer at 20°C with shaking.
- Incubate at room temperature in the dark in 10ml of freshly prepared colour substrate solution, no shaking.
- Observe colour reaction over a 30 minute -12 hour period.
- To stop the reaction, place the membrane in TE-buffer (pH8) at 20°C with shaking for five minutes.
- Store the membrane in a ziplock bag at 4°C.

DIG high prime DNA labelling and detection recipes

Washing Buffer pH7.5	100 ml
- 0.1M Maleic acid (Fischer Scientific, 064402)	1.16 g
- 0.15M NaCl (Sigma, S3014)	0.876 g
- 0.3% Tween20 (Sigma, P9416)	0.3 ml
- 0.01% DepC treated water	

Maleic acid buffer pH7.5	100 ml
- 0.1M Maleic acid	1.16 g
- 0.15M NaCl	0.876 g
- 0.01% DepC treated water	

Detection buffer	100 ml
- 0.1M Tris-HCl	10 ml of 1M stock
- 0.1M NaCl	0.5844 g
- 0.01% DepC treated water	

TE-buffer pH8.0	100 ml
- 10mM Tris-HCl	1 ml of 1M stock
- 1mM EDTA	0.2 ml of 0.5M stock
- 0.01% DepC treated water	

Blocking solution (made fresh)	100 ml
- 2% Sheep serum (Sigma, S2263)	2 ml
- 3% Instant skim milk powder (Carnation Brand)	3 g
- 1X TBST 0.01% DepC treated	98 ml

Antibody solution (made fresh)	10 ml
- 1:5000 Anti-Digoxigenin –AP (Roche, 12930020)	2 µl stock
- 1X TBST 0.01% DepC treated	10 ml

Colour substrate solution (made fresh)	10 ml
- Sigma Fast tablet, NBT-BCIP (Sigma B5655)	1 Tablet
- Distilled water	10 ml

- Shake vigorously (in the dark) until dissolved.

Appendix E

Whole-mount *in situ* hybridization protocol

Day 1: Collect embryos for storage

- After staging, fix embryos in 4% Paraformaldehyde in 1X PBST made in DepC treated water.
- Wash twice for 10 minutes wash in 1X PBST on ice

Dehydrate embryos through MeOH series on ice as follows:

15 minutes in 25:75 MeOH/1X PBST

15 minutes in 50:50 MeOH/PBS

15 minutes in 75:25 MeOH/depc H2O

Stored in 100% MeOH overnight at -20C in 50ml tube

- Bisect heads

Rehydrate embryos through MeOH series on ice as follows:

15 minutes in 75:25 MeOH/depc H2O on ice

15 minutes in 50:50 MeOH/PBS on ice

15 minutes in 25:75 MeOH/1X PBST on ice

- Wash twice for 10 minutes in 1x PBST at room temperature with shaking in 50 ml tube
- Bleach for half hour in 10% Hydrogen Peroxide/1x PBST at room temperature with shaking.
- Wash three times for 10 minutes in 1x PBST at room temperature with shaking
- Store at -20°C until ready to continue with the protocol.

Day 2: Hybridization Step

- Wash three times for 10 minutes in 1x PBST at room temperature with shaking
- 30 minutes in 50ug/mL Proteinase K in 1XPBST at room temperature with shaking
- Fix heads for 20 minutes in 4% PFA/PBST and 0.25% glutaraldehyde/PBST (50:50) at room temperature

- Wash twice for 10 minutes in 10X PBST at room temperature with shaking in 50 ml tube
- Hybridisation step: add 0.5 ul probe to 10 mL of 1X Prehyb (with Yeast tRNA and heparin freshly added) and incubated at 60°C overnight.

Day 3: Antibody Step

- Wash three times for 20 minutes in Wash I (pre-warm to 60°C) at 60°C with shaking
- Wash three times for 20 minutes in Wash II (pre-warm to 60°C) at 60°C with shaking
- Wash twice for 10 minutes in TBST at room temperature with shaking
- Block for 1 hour in 20% heat inactivated sheep serum/TBST at room temperature with shaking
- **Pre-absorb antibody with chicken powder**:
    a. Combine 1 ml 20% inactivated serum/TBST and 1ul of Anti-Digoxigrnin-AP (Roche 12930021) and a tiny amount of embryo powder in an eppendorf tube.
    b. Shake tube.
    c. Let the powder settle for five minutes on ice.
    d. Remove the antibody and add it to the rest of the 20% inactivated serum/TBST to make the correct dilution (1 µl Presorbed α-DIG-AP in 10 ml with 20% Inactivated Serum/TBST)
- Placed embryos in the pre absorbed antibody solution overnight at 4°C with shaking

Day 4: Background reduction

Wash Embryos:

- Wash three times for 30 minutes in TBST at room temperature with shaking
- Wash three times for 1 hour in TBST+ 2mM Levamisole at 4°C with shaking
- One overnight wash in TBST + 2mM Levamisole at 4°C with shaking

Day 5: Colour Detection

- Wash twice for 20 minutes in NTMT + 2mM Levamisole at room temperature with shaking
- Dissolve one Sigma fast NBT-BCIP tablet in 10 mL of 10% PVA in NTMT.
- Incubate embryos in dark at room temperature with the NBT-BCIP solution until developed (usually between 24 and 36 hours)
- Wash twice for 10 minutes in PBST + 5mM EDTA at room temperature
- Postfix with 4% PFA for 20 minutes at RT, no shaking
- Wash twice for 10 minutes in 1XPBST at room temperature, no shaking

Dehydrate embryos in graded methanol

1. 15 minutes in 25:75 MeOH/1X PBST on ice (i.e. 25% methanol)
2. 15 minutes in 50:50 MeOH/PBS on ice (i.e. 50% methanol)
3. 15 minutes in 75:25 MeOH/depc H2O on ice (i.e. 70% methanol)

- Leech background from embryos in 90% MeOH O/N at 4°C no shaking

Day 6: Storage

- Place in 50:50 glycerol:water, store at 4°C

***In situ* hybridization recipes**

PBST	500 ml 10X
137mM NaCl	137 ml 5M
2.7mM KCl	3.37 ml 4M
4.3mM Na_2HPO_4	21.5 ml 1M
1.4mM KH_2PO_4	7 ml 1M
0.1% Tween-20(Sigma P9416)	5 ml
0.01% DepC treated	

Proteinase K stock (10mg/ml)	1 ml

10 mg Proteinase K (Sigma P2308) in 1 ml 1X PBST

Prehyb	500 ml 1X
50% Formamide	250 ml of 100% stock (ISC BioExpress 0606)
5X SSC	125 ml 20X stock (Sigma S6639)
1% SDS	50 ml 10% stock (Sigma L4390)

0.01% DepC treated

50µg/mL yeast tRNA (Roche 70132220) - add fresh

50µg/mL heparin (Sigma H3393) - add fresh

Wash I	200 ml 1X
50% Formamide	100 ml
4X SSC	40 ml 20X stock
1% SDS	20 ml 20% stock

0.01% DepC treated

Wash II	500 ml
50% Formamide	250 ml stock
2X SSC	50 ml stock

0.01% DepC treated

TBST	500 ml 10X
140mM NaCl	140 ml 5M
2.7mM KCl	3.37 ml 4M
2.5mM Tris-Cl ph 7.5	125 ml 1M
0.1% Tween-20	0.5 ml
0.01% DepC treated	

NTMT	250 ml 1X
100mM NaCl	5 ml 5M
100mM Tris-Cl pH 9.5	25 ml 1M
20mM MgCl2	5 ml 1M
0.1% Tween-20	250 µl
0.01% DepC treated H20	215 ml

10% Hydrogen Peroxide/PBST (make fresh) 75 ml

25 ml 30% Hydrogen Peroxide (Sigma H1009) + 50 ml 10x PBST

4% PFA/PBST + 0.25% glutaraldehyde/PBST (50:50) 10 ml

50 µl 25% glutaraldehyde (Sigma G5882) in 5 ml PBST + 5 ml 4% PFA/PBST

20% sheep serum/TBST 20 ml

4 ml sheep serum + 16 ml TBST

Heat inactivated for 30 minutes at 60°C

Chick Powder

Collect four 4-5 day embryos (HH stage 23-26) in a minimum amount of PBS.
Add 4 volumes of cold acetone and incubate on ice for 30 minutes.
Spin at 10000 RPM for 10 minutes and pour off the acetone.
Pour the embryos out onto clean filter paper and cut or crush them into a powder.
Store in the fridge

NBT/BCIP in 10%PVA in NTMT 10ml

1 g Polyvinyl Alcohol (Sigma 348406) in 10 ml NTMT

When 10% PVA in NTMT is cooled to room temperature; dissolve 1 tablet of Sigma fast NBT-BCIP per 10 ml 10% PVA