Determination of the Role and Regulation of Matrix Metalloproteinase-25 During Mouse Secondary Palate Formation

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By

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ABSTRACT

Development of the secondary palate (SP) is a complex event despite the small area it encompasses. Problems with SP development can lead to a cleft palate, which is one of the most common birth disorders. The matrix metalloproteinases (MMPs) are required for proper SP development, but a functional role for any one of them remains unknown. MMP-25 is a candidate MMP to have a functional role in SP formation as genetic scans of the DNA of human cleft palate patients indicate a common mutation at a region upstream of the *Mmp-25* gene. The purpose of this thesis is to investigate gene expression of *Mmp-25* in the developing mouse SP, whether it has a functional role in mouse SP development and begin to identify factors potentially upstream of *Mmp-25* expression.

Mmp-25 mRNA and protein is found at all SP developmental stages in mice with highest expression at embryonic day (E) 13.5 when analyzed by quantitative real-time PCR and western blotting. Immunohistochemistry localizes MMP-25 protein primarily to the plasma membranes of palate shelf epithelial cells with secondary expression in apical mesenchymal cells. *Mmp-25* knockdown with siRNA in palatal cultures resulted in a significant decrease in palate shelf fusion and persistence of the medial edge epithelium *in vitro*. *Mmp-25* mRNA and protein levels are significantly decreased *in vitro* when cultured palate shelves are incubated in growth medium with 5 μg/ml of a TGFβ3-neutralizing antibody.

Mmp-25 gene expression is highest at E12.5 and E13.5, which corresponds to increasing palate shelf growth downward alongside the tongue. Immunohistochemistry

localized MMP-25 protein expression predominantly in the epithelium of the palate shelves, but also in areas of the mesenchyme that were immediately adjacent to the epithelium and apical in location. Knockdown of *Mmp-25* expression resulted in palate shelf fusion being impaired and significant medial edge epithelium remaining in contacted areas. Bioneutralization of TGFβ3 resulted in a significant decrease in *Mmp-25* gene expression. These data suggest a functional role for MMP-25 in mouse SP development by removing extra-cellular matrix barriers to increased palate shelf growth and place its expression downstream of TGF-β3 signaling. This is the first research to present a role for a single MMP in mouse SP development.

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List of Abbreviations

ANOVA	Analysis of Variance
BLASTn	Nucleotide Basic Local Alignment Search Tool
BM	Basement Membrane
BMP	Bone Morphogenetic Protein
cDNA	Complementary DNA
CL/P	Cleft Lip with or without Cleft Palate
CNCC	Cranial Neural Crest Cells
CP	Cleft Palate
E	Embryonic
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EPD	Eukaryotic Promoter Database
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FRS	Fibroblast Growth Factor Receptor Substrate
FNP	Frontonasal Prominence
GAPDH	Glyceraldehyde -3-Phosphate Dehydrogenase
GLI	Glioma-Associated Oncogene Homolog
GPI	Glycosyl-phosphatidyl Inositol
H&E	Hematoxylin and Eosin
HOX	Homeobox
IHC	Immunohistochemistry

kDa	Kilodalton
MANP	Mandibular Prominence
MAPK	Mitogen-Activated Protein Kinase
MAXP	Maxillary Prominence
MEE	Medial Edge Epithelium
MEOX	Mesenchyme Homeobox
MES	Midline Epithelial Seam
MFS	Mean Fusion Score
MMP	Matrix Metalloproteinase
mRNA	Messenger RNA
MS	Multiple Sclerosis
MT-MMP	Membrane-Type MMP
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PP	Primary Palate
PTCH	Patched
PVDF	Polyvinylidene Fluoride
qRT-PCR	Quantitative Real-Time PCR
RAS	Rat Sarcoma
RIPA	Radioimmunoprecipitation Assay
RPM	Revolutions per Minute
RQ	Relative Quantitation
RT-PCR	Reverse Transcriptase PCR

SHOX	Short Stature Homeobox
SBE	Smad Binding Element
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SHH	Sonic Hedgehog
siRNA	Small Interfering RNA
SMO	Smoothened
SNARE	Soluble N-Ethylmaleimide-Sensitive Factor Attachment Receptor
SP	Secondary Palate
TF	Transcription Factor
TβR	Transforming Growth Factor Beta Receptor
TGFβ3	Transforming Growth Factor Beta 3
TIMP	Tissue Inhibitor of Metalloproteinase
TRED	Transcriptional Regulatory Elements Database
TSS	Transcription Start Site
ZO	Zonula Occludens

Research Hypotheses

1. *Mmp-25* is constitutively expressed by the medial edge epithelium (MEE) during mouse secondary palate (SP) development and involved in palatal shelf fusion and midline epithelial seam (MES) degradation.
2. Knocking down *Mmp-25* gene expression results in delayed fusion of the palate shelves and persistence of the MEE.
3. *Mmp-25* is primarily regulated by the growth factor, transforming growth factor beta 3 (TGFβ3).

Research Objectives

The first goal was to characterize the temporal and spatial distribution of MMP-25 expression during the development of the SP in mice. Using quantitative real-time polymerase chain reaction (qRT-PCR), western blot analysis and immunohistochemistry (IHC) techniques, the expression profile of *Mmp-25* and its protein was determined in the developing mouse SP.

The second goal of this research was to investigate the role of *Mmp-25* during mouse SP development using small interfering RNA (siRNA) to knockdown gene expression. Establishing a role for *Mmp-25* during mouse palatogenesis could provide a starting point for MMP research in the SP.

The final goal of this research was to initiate an investigation into the transcriptional regulation of *Mmp-25* during palatogenesis. Through promoter analysis, *in vitro* palate cultures, qRT-PCR and western blots, some of the molecules controlling *Mmp-25* expression were determined.

1. Introduction

1.1 Introduction to Orofacial Clefts

1.1.1 Classifications and Anatomy

Orofacial clefts are birth disorders involving the nose, upper lip and palate. They are disfiguring and affect the speech, respiration and eating. Although they can be surgically repaired, these operations begin at a very young age and rehabilitation can continue into adulthood. Orofacial clefts can be broadly classed into two categories: cleft lip with or without cleft palate (CL/P) and cleft palate (CP) alone (Figure 1). Additional forms of orofacial clefts exist, but these are rare and usually represent a variation of CL/P (Gorlin et al., 2001). The palate, which separates the oropharynx (oral cavity) from the nasopharynx (nasal cavity), is comprised of two parts: a primary palate (PP) and SP. The PP is located anterior to the SP and consists of the philtrum (indentation) of the upper lip and a portion of the maxillary alveolus, in which the four maxillary incisors are set. The SP is just posterior to the PP and consists of an anterior ossified hard palate and posterior sub-mucous soft palate, which ends with the uvula (Gorlin et al., 2001). The two main types of orofacial clefting can be further sub-classified firstly into unilateral clefts, which affect one side of the face, or bilateral clefts, which affect both sides of the face; and secondly, unilateral or bilateral CL/P and unilateral or bilateral CP may be grouped into complete, incomplete or sub-mucous clefts, which would only affect the upper lip or soft palate respectively (Shprintzen, 2002).

1.1.2 Epidemiology

Orofacial clefts are some of the most common birth disorders in humans today. Although there are over 400 established syndromes that display CL/P or CP as a clinical

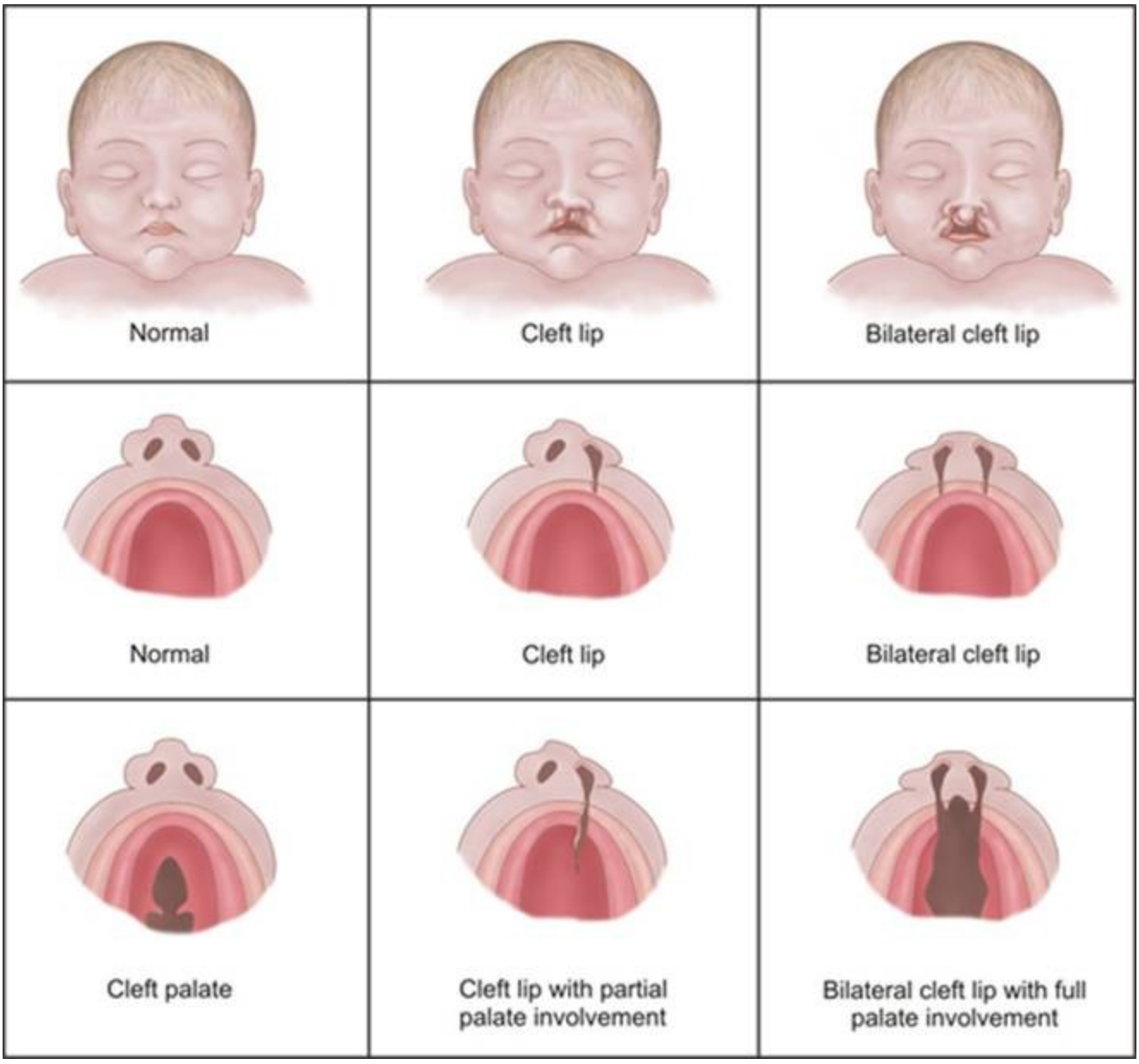


Figure 1: Illustration of orofacial clefting variety. Reprinted with permission from Children's Hospital of Wisconsin.

symptom, non-syndromic CL/P and CP are still more common (Shprintzen, 2002). Worldwide, CL/P occurs in approximately 0.2 to 2.3 births per 1000 and CP in 0.1 to 1.1 per 1000 births (Mitchell, 2009). There seem to be racial differences in the prevalence of CL/P. Those of Asian ancestry have the highest prevalence of CL/P including Native American Indians at a rate of approximately 3.6 births per 1000, Japanese at 2.1 births per 1000 and Chinese at 1.4 births per 1000. People of European ancestry have a CL/P frequency of approximately 0.7 to 1.3 births per 1000 while individuals of African ancestry have the lowest frequency of CL/P at 0.3 births per 1000 (Gorlin et al., 2001). Despite the variation in CL/P birth frequency between races, there does not seem to be such an association for CP. Those of European and African ancestry have CP in approximately 0.4 births per 1000 (Gorlin et al., 2001). In Canada, CL/P occurs in approximately 1.08 births in 1000 and 0.77 births in 1000 for CP (Health Canada, 2002). In Saskatchewan between 1994 and 2003 the prevalence of CL/P was approximately 0.95 births per 1000 while CP occurred among 0.59 births per 1000 (Winquist et al., 2005).

CL/P occurs more frequently in males, but CP is more common in females (Gorlin et al., 2001). Analyses of genetic studies have indicated CL/P has a hereditary association, but is not inherited in a classical, Mendelian fashion. Indeed a monozygotic twin only has a 25%-40% chance of developing CL/P if their other twin has it (Mitchell and Risch, 1992). A hereditary association for CP also appears to exist, but evidence is conflicting; one study indicates first-degree relatives stand only a three percent increased chance of developing CP if a parent or sibling also had it (Christensen and Mitchell, 1996) while another states a three percent increased chance if a sibling has a CP and a seven percent increased chance if a parent has a CP (Bernheim et al., 2006).

1.1.3 Environmental Risk Factors

Because development of CL/P and CP is a multi-factorial inheritance, there is a complex interaction between multiple genetic loci and environmental factors, thus many exogenous factors have been proposed as contributors to CL/P and CP. A variety of prescription drugs have been implicated in CL/P and CP.

Excess retinoic acid, the metabolite of vitamin A, has the strongest association with CP. Pregnant women who use isotretinoin (13-*cis*-retinoic acid) to treat acne in the first semester, when the palate is developing, are approximately 26 times more likely to have a child with a CP than is another pregnant woman from the general population (Mitchell, 2009). An *in vitro* study showed exposure to excessive retinoic acid at embryonic day (E) 10.0 inhibited growth of the palate shelves, which never made contact, and exposure at E12.0 resulted in normal palate shelves, but prevented fusion of the shelves to each other due to altered cell differentiation where the shelves make contact (Abbott et al., 1989).

Pregnant women who take phenytoin, phenobarbital, valproic acid or carbamazepine for any cause (epilepsy, depression, migraines, recurring pain) are at an increased risk of having a child with an orofacial cleft. These women are 7.8 and 3.6 times more likely to have children with CL/P or CP respectively (Mitchell, 2009). The usage of corticosteroids in general during the first trimester increases the chances of having a child with CL/P or CP by 4.3 and 5.3 times respectively (Mitchell, 2009) and usage of prednisone, a type of corticosteroid, has been estimated as increasing a pregnant woman's risk of having a child with an orofacial cleft by 3.4 times (Park-Wyllie et al., 2000). There is conflicting information linking the benzodiazepines to CL/P and CP. The

risk associated with the benzodiazepines has been estimated from no risk to a 1.8 times increased chance of an orofacial cleft (Mitchell, 2009).

A lack of folate/folic acid during the first trimester has been suggested as a causal trigger of CL/P and CP, however evidence to support this is inconclusive. One study found high dosages of folic acid (6 mg) prevented CL/P and CP (Czeizel et al., 1999); another suggested dosages of folic acid found in a typical multi-vitamin were enough to reduce risk of CL/P and CP, by 48% and 19% respectively, if women started taking the vitamins before getting pregnant or in the first month (Itikala et al., 2001). Yet another study reported no link between any sort of increased folate/folic acid intake and a reduction in CL/P and CP occurrences (Little et al., 2008).

Maternal smoking is another inconclusive causal factor in CL/P and CP. One study indicates a slight dose-dependent effect of maternal smoking in contributing to CL/P, but not CP, with light smokers (1-14 cigarettes/day) having an increased risk of 1.09 times, moderate smokers (15-24 cigarettes/day) at 1.84 times and heavy smokers (25+ cigarettes/day) at 1.85 times (Lieff et al., 1999). Another study found an increased risk of 1.34 and 1.22 times for CL/P and CP respectively (Little et al., 2004).

Finally, alcohol consumption as the palate is developing has an association with the development of CL/P, but not CP, however pregnant women must consume greater than five drinks per occasion at least once a week to be 3.4 times more likely than the general population to have a child with CL/P (Shaw and Lammer, 1999). Light and moderate drinkers had no increased risk of CL/P or CP. The association of so many environmental factors with CL/P and CP has to do with the development of the face and palate being early in the first trimester before many women realize they are pregnant.

1.2 Formation of the Secondary Palate

1.2.1 Branchial Arch Population

Craniofacial development begins with the migration of the cranial neural crest cells (CNCC) into the branchial arches. The CNCC are a set of pluripotent stem cells that arise from the neural fold boundary between the neural ectoderm (neural tube) and epidermis of the embryonic hindbrain segments, the rhombomeres (Sperber, 2002). Expression of *Distalless-5* and *Pax3* in the neural fold specifies the boundary between the neural tube and epidermis and prevent the cells in this region from becoming either neural tube or epidermis (Gilbert, 2006). At the same time Distalless-5 and Pax3 induce expression of *Slug* and *FoxD3*, which specifies cells in the neural fold to become CNCC and enables them to migrate ventro-laterally away from the dorsal regions of the rhombomeres (Gilbert, 2006). Migration and proliferation of the CNCC populates the branchial arches, which are embryonic folds of the future face and neck located ventrally to the rhombomeres (Figure 2; Sperber, 2002). The direction of migration of the CNCC into the branchial arches is initially determined by expression of the homeobox (HOX) genes, which are crucial in establishing the anterior-posterior axis of embryos, as the CNCC generally express the same HOX genes as the rhombomeres they originate from (Burdi, 2006). The main HOX genes involved in rhombomere, and thus CNCC and branchial arch, identity are *Hoxa2*, *-a3*, *-b2* and *-b3* (Trainor and Krumlauf, 2000). CNCC from rhombomeres one and two, as well as from the midbrain, populate the first branchial arch; CNCC from rhombomere four populate branchial arch two and CNCC from rhombomeres six to eight populate branchial arches three and four (Gilbert, 2006).

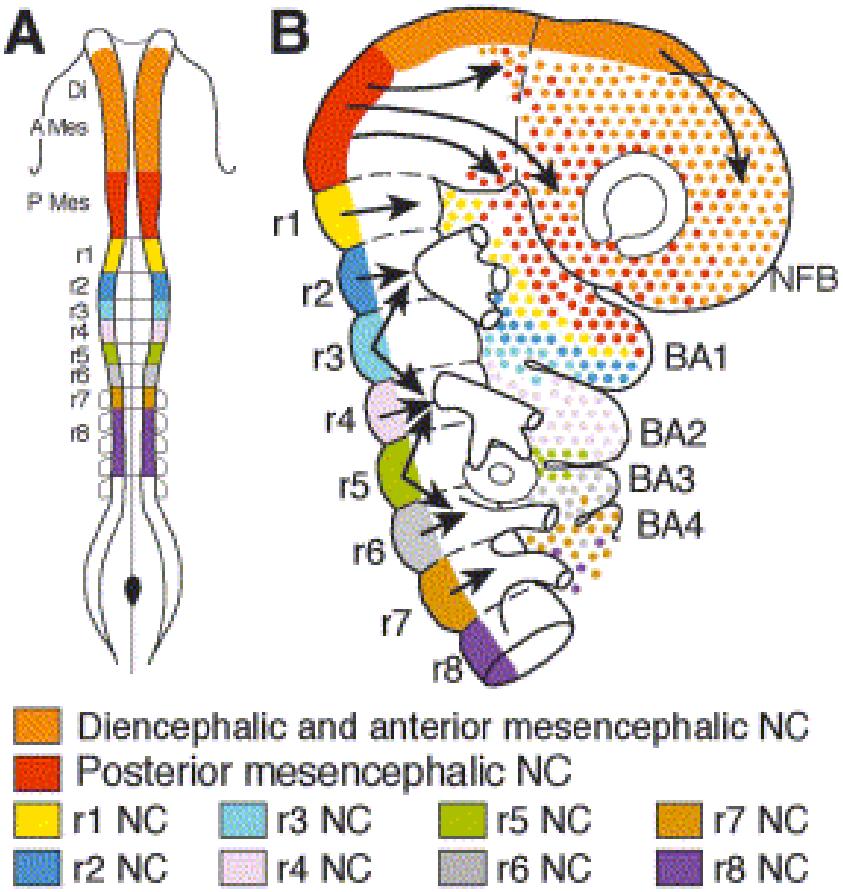


Figure 2: Direction of cranial neural crest cell migration from embryonic mesencephalon and rhombencephalon brain segments into the branchial arches. Reprinted with permission from Le Douarin, 2004.

CNCC from rhombomeres three and five do not contribute to craniofacial development (Burdi, 2006).

1.2.2 Development of the Face

Central to the developing face is the stomodeum, a pit that will be the future mouth (Burdi, 2006). Within the stomodeum lies the oropharyngeal membrane, which marks the boundary between the endoderm of the future throat and the ectoderm of the future mouth before undergoing apoptosis and degrading at 28 days thereby creating a continuous tube from mouth to gut (Sperber and Sperber, 2009; Burdi, 2006). Around the stomodeum are three primary prominences that will give rise to the face: the frontonasal (FNP), the maxillary (MAXP) and the mandibular (MANP; Sperber and Sperber, 2009). Although it is CNCC of branchial arch one that give rise to the MAXP and MANP, CNCC of the midbrain give rise to the FNP (Gilbert, 2006). The FNP lies atop the stomodeum, the MAXP on either side of it and the MANP below it (Figure 3; Sperber, 2002). The nose forms when nasal pits in the FNP on either side of the face are flanked by lateral and median nasal prominences that ring the nasal pits and then grow out from the face (Sperber, 2002). Growth and movement of the facial prominences is due to migration and proliferation of the cells within them (Burdi, 2006). As the FNP grows downward, the nasal pits are brought closer together until the median nasal prominences merge with each other and with the medial ends of the MAXP, which has been growing up and out towards the center of the face on either side of the primitive mouth (Sperber, 2002). Fusion of the median nasal prominences with the MAXP and fusion of the bilateral MAXP segments together results in the nose, upper jaw, upper lip, cheeks and PP being formed (Sperber and Sperber, 2009). The philtrum of the upper lip develops

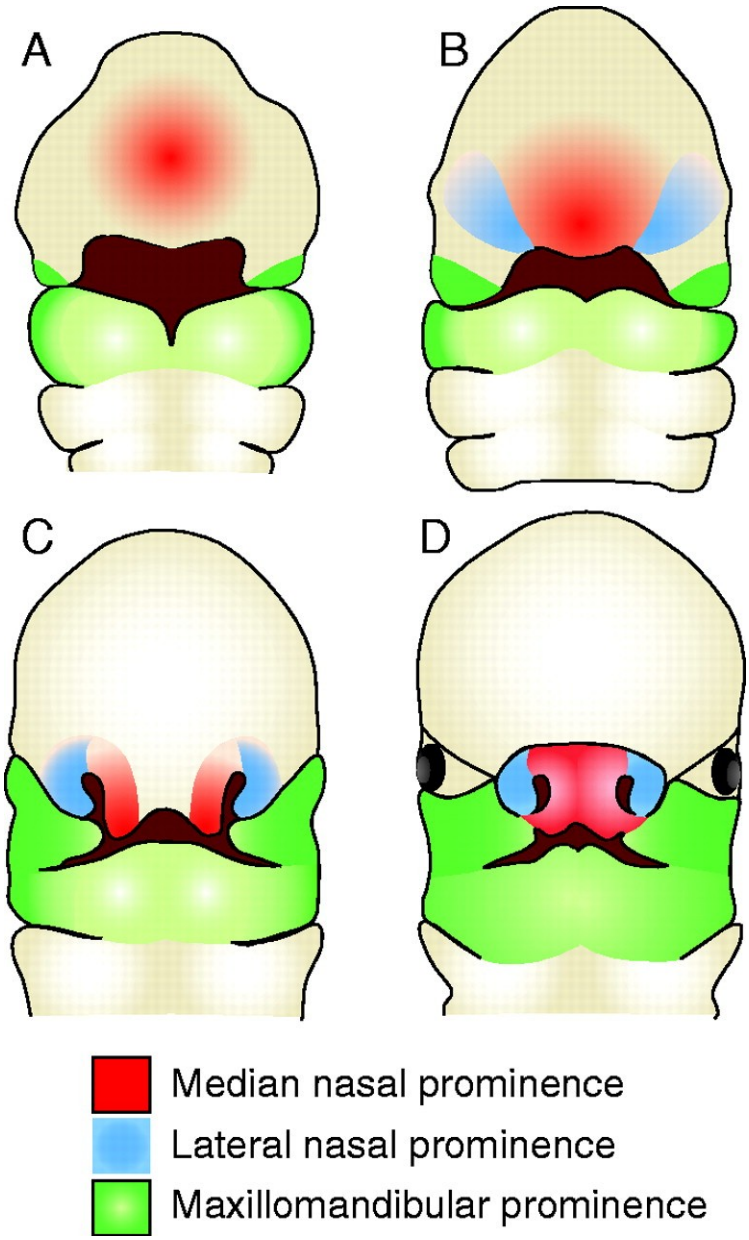


Figure 3: Illustration of embryonic facial prominences and progression of facial development. Reprinted with permission from Helms et al., 2005.

later due to preferential collagen deposition in the middle of the upper lip (Burdi, 2006). Movement of the eyes from a lateral to central position is due to the FNP narrowing as the nose develops and lateral expansion of the rest of the face (Sperber, 2002). The mouth opening narrows as the MAXP and MANP grow towards each other and takes on its definitive shape when the two jaw processes fuse (Sperber, 2002). Concurrent to the movement and fusion of the facial prominences is the migration and differentiation of CNCC from the first branchial arch that did not populate the FNP, MAXP or MANP. These cells will form the underlying muscles, skeleton, nerves, blood vessels and connective tissue of the face (Burdi, 2006).

1.2.3 Development of the Secondary Palate

Development of the SP, or palatogenesis, takes place between E11.5 and E16.0 in mice and is critical in gestation weeks six to nine in humans (Burdi, 2006). Development of the secondary hard palate and secondary soft palate is slightly different, however the similarities between mouse and human SP development make the mouse an excellent model animal with which to study palatogenesis. Initially, bilateral palatal processes bud from the MAXP within the stomodeum, forming palate shelves, and grow downward alongside the tongue (Figure 4; Dudas et al., 2007). Around the end of week eight in humans and E14.0 in mice, the tongue will drop due to an increase in stomadeum size and the palate shelves will reorient themselves horizontally above it (Sperber, 2002). The mechanism behind the sudden elevation of the palate shelves is an increase in fluid turgor, due to accumulation of hydrated hyaluronic acid, and asymmetrical proliferation in the mesenchyme (Sperber and Sperber, 2009). Once elevated the hard palate shelves will grow together and make contact not only with each other along their medial edges,

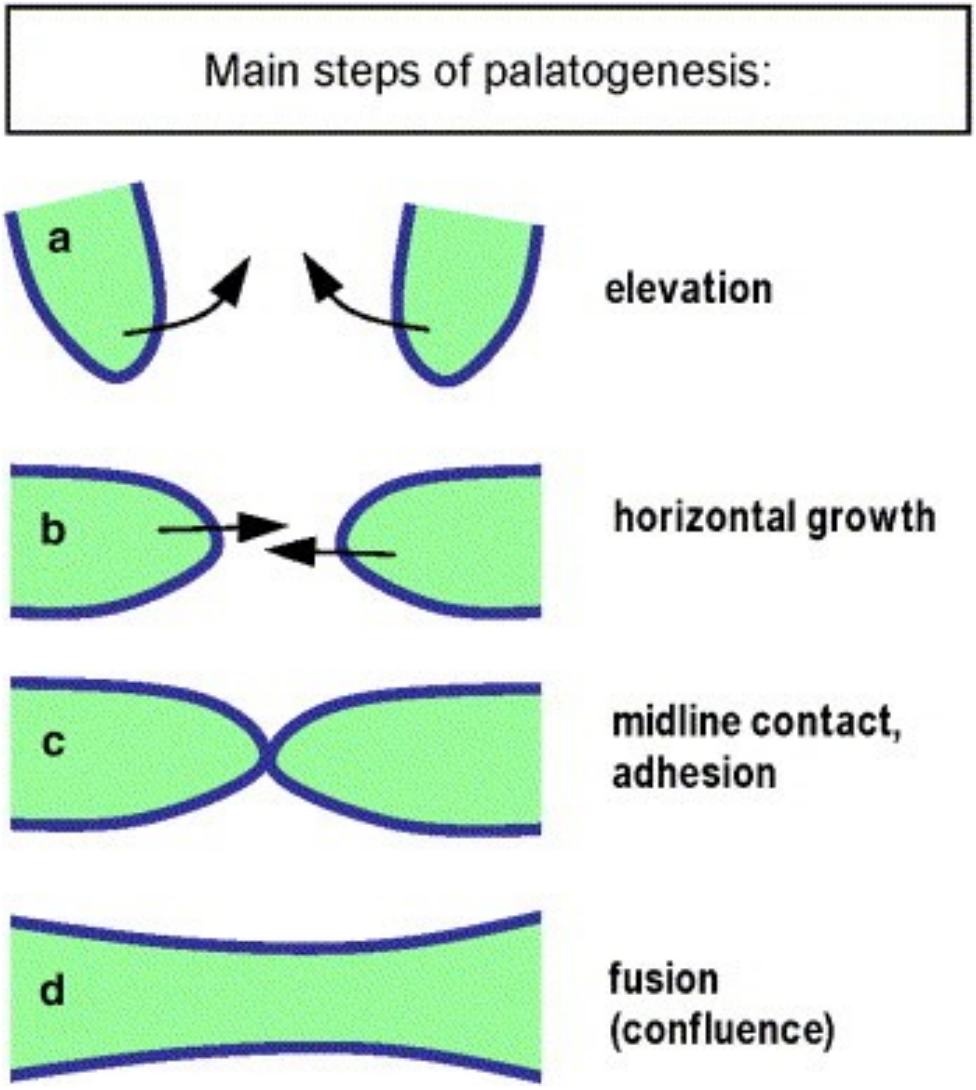


Figure 4: Illustration of the main stages in secondary palate development. Reprinted with permission from Dudas et al., 2007.

but also with the base of the nasal septum, which has been growing downward from the roof of the stomodeum, and the PP, which formed earlier as the median nasal prominences fused with the MAXP (Sperber, 2002). Upon making contact, the hard palate shelves fuse together via microvilli and proteoglycan attachment on the MEE (Burdi, 2006), which creates the MES. Fusion occurs in the medial area of the hard palate shelves first and proceeds in a zippering-like manner anteriorly and posteriorly simultaneously (Sperber and Sperber, 2009). Degradation of the MES and removal of the MEE between the fused hard palate shelves is the final critical step in successful palatogenesis. The epithelial cells of the MES may undergo apoptosis, migrate away from the seam or, least likely, undergo an epithelial-to-mesenchymal transition (Gibbins et al., 1999; Vaziri Sani et al., 2005; Xu et al., 2006; Cuervo and Covarrubias, 2004; Jin and Ding, 2006). Although evidence regarding the fate of the MES is still conflicting, apoptosis of the MEE cells seems to be the primary means through which the MES disappears (Dudas et al., 2007). Following degradation of the MES, there is increased proliferation in the mesenchyme of the SP to fill the gap left by the MEE. Shortly after fusion ossification begins in the anterior, hard SP from primary ossification centers located in the maxillae and palatine bones, but does not extend posteriorly into the sub-mucous, soft SP near the back of the throat (Sperber and Sperber, 2009). Formation of the soft SP occurs at the most posterior end of one of the lateral palatal processes while the uvula develops from the most posterior end of the other (Burdi, 2006). The two posterior ends do not fuse like the hard palate shelves, but rather undergo proliferation in the mesenchyme until the two posterior ends have grown together and merged (Burdi, 2006). Fusion of the anterior shelves together and merging of the soft SP with the uvula

posteriorly as well as fusion to the base of the nasal septum and PP creates a confluent SP.

1.2.4 Genetic Control of Secondary Palate Development

1.2.4.1 Transcription Factors

A variety of transcription factors (TFs) have been implicated as having crucial roles in SP development and many are homeobox genes involved in establishing the embryonic body pattern. Mouse *Lhx8* is a TF containing domains for interacting with DNA and regulatory interactions with other proteins (Zhao et al., 1999). *Lhx8* is expressed exclusively in the mesenchyme of the palate shelves throughout SP development with stronger expression near the tips of the palate shelves directly underneath the epithelium (Zhang et al., 2002). *Lhx8*$^{-/-}$ mice have a CP, which is due to the palate shelves never making contact or fusing after elevation, although they exhibit no other craniofacial defects (Zhao et al., 1999). *Lhx8* expression is detected in the mesenchyme of the tooth primordia and its continued expression is dependent on the overlying epithelium (Grigoriou et al., 1998) so *Lhx8* was proposed to be involved in regulating epithelial-mesenchymal interactions during SP development and in its absence a CP forms (Zhao et al., 1999). More recent research has suggested human *LHX8* plays a minor role in contributing to orofacial clefts (Vieira et al., 2005) and no further work has been done in mice.

Human *TBX22*, a TF with conserved DNA-binding T-Box domain, was first implicated in a syndromic form of X-linked CP when *TBX22* loss-of-function mutations were discovered in CP patients (Braybrook et al., 2001). Although linked to a syndromic form of CP caused by defects in tongue musculature, it was hypothesized *TBX22*, and its

mouse orthologue *Tbx22*, were involved in proper development of the SP and disruption of their expression could lead to isolated, or non-syndromic, CP (Braybrook et al., 2001; Braybrook et al., 2002). Expression of *Tbx22* is restricted to the mesenchyme of the palate shelves as they bud from the MAXP and is highest at E13.5, but *Tbx22* expression has been completely down-regulated by E14.5 once the palate shelves have elevated and begun to fuse along their medial edges (Braybrook et al., 2002; Bush et al., 2002). In addition to temporal regulation, expression of *Tbx22* appears to be spatially-regulated as its expression is minimal in the anterior SP, but high in the medial and posterior portions where it is downstream of the TF *Mn1* (Liu et al., 2008). The tightly-regulated nature of *Tbx22* expression coupled with its syntenic chromosomal location in the genomes of mice and humans has led to more recent genomic investigations in humans that established a significant role for *TBX22* in CP (Bush et al., 2002; Marçano et al., 2004).

Hoxa2 is a mouse homeobox transcription factor involved in establishing rhombomere, CNCC and branchial arch identity along the anterior-posterior axis (Trainor and Krumlauf, 2001). *Hoxa2*$^{-/-}$ mice also develop a CP; although the palate shelves grow, they do not elevate normally, however this is in addition to defects in the skull, ears and facial nerves so the CP is believed to be the secondary result of an antecedent defect (Gendron-Maguire et al., 1993; Rijli et al., 1993). Indeed, later work demonstrated that in *Hoxa2*$^{-/-}$ mice the hyoglossus muscle, responsible for depressing the tongue during SP development, does not form properly and thus the tongue represents a barrier to palate shelf contact in *Hoxa2*$^{-/-}$ mice and a CP forms (Barrow and Capecchi, 1999). The most recent work in the developing SP indicates additional roles for *Hoxa2* as the central figure in numerous signal transduction pathways and could represent novel ways *Hoxa2*

contributes to the formation of CP (Nazarali et al., 2000; Smith and Nazarali, unpublished data).

Msx1 is another homedomain TF involved in SP development. *Msx1*$^{-/-}$ mice have palate shelves that grow and elevate normally, but do not make contact resulting in a CP (Satokata and Maas, 1994). In addition to a CP, these mice also exhibit a complete lack of alveolae and teeth and have ear and skull malformations (Satokata and Maas, 1994). Expression of *Msx1* is strictly in the anterior palate shelf mesenchyme with strong expression at E12.5 and E13.5, but no results for later developmental stages (Zhang et al., 2002). CP in *Msx1*$^{-/-}$ mice is caused by decreased mesenchymal cell proliferation and a failure to grow together and make contact around E14.0 as *in vitro* palatal cultures showed palate shelves from *Msx1*$^{-/-}$ mice retained the ability to fuse normally (Zhang et al., 2002). Transgenic studies have shown *Msx1* is upstream of bone morphogenetic protein 4 (*Bmp4*) expression, which in turn up-regulates sonic hedgehog (*Shh*) and *Bmp2* resulting in increased cell proliferation in the palate shelves (Zhang et al., 2002). The close family member of *Msx1*, *Msx2*, does not appear to play a role in SP development. Though *Msx2* is expressed in a spatially over-lapping manner and is said to be functionally redundant to *Msx1*, it does not rescue the CP phenotype of *Msx1*$^{-/-}$ mice (Alappat et al., 2003).

There are regional differences in the expression of some TFs in SP development due to reciprocal interactions between cells in the anterior, medial and posterior portions of the palate shelves (Hilliard et al., 2005). *Msx1* expression is only found in the anterior palate shelves while expression of *Tbx22* and its inducer *Mn1* is located only in the posterior palate shelves, however regional differences in expression are not static.

Expression of the TFs short stature homeobox 2 (*Shox2*) and mesenchyme homeobox 2 (*Meox2*) alters considerably from E12.5 to E14.5; at E12.5 *Shox2* expression is found only in the anterior-most 25% of the palate shelves while *Meox2* expression covers approximately 70% of the palate shelf from the posterior end, but by E14.5 *Shox2* expression has expanded to cover 60% of the palate shelf and *Meox2* has regressed to the posterior-most 25% (Li and Ding, 2007). This anterior regulation of *Shox2* expression is responsible for *Shox2*$^{-/-}$ mice developing a very rare CP in which in the soft, posterior SP has formed normally, but the hard, anterior SP has a cleft (Yu et al., 2005). Expression of the TF *Osr2* is restricted to the medial regions of the palate shelves where they initially bud from the MAXP and is significantly down-regulated once the shelves elevate (Lan et al., 2004). *Osr2*$^{-/-}$ mice develop a CP due to reduced mesenchymal cell proliferation in the medial region, but not anterior or posterior regions, which contributes to a significant delay in shelf elevation and lack of contact afterward (Lan et al., 2004).

1.2.4.2 Growth Factors and Receptors

Growth factors have an integral role in SP development and the most important of these are the TGFβ family. There are three secreted members in the TGFβ family of growth factors, TGFβ1 to TGFβ3, which bind to the TGFβ receptor type 2 (TβR2) as ligands (Dünker and Krieglstein, 2000). This results in dimerization and phosphorylation between TβR2 and TβR1, which are receptor tyrosine kinases. The initial targets of phosphorylation from the activated TGFβRs are the SMAD proteins; SMAD2 or SMAD3 are phosphorylated, combine with SMAD4 and translocate to the nucleus resulting in alterations of target gene transcription (Dünker and Krieglstein, 2000). Of the three TGFβ members, TGFβ2 and TGFβ3 seem to affect SP development the most.

Mouse lines that have *tgfβ2* or *tgfβ3* knocked out have a CP. *Tgfβ2*$^{-/-}$ mice develop a CP that results from an inability of the palate shelves to elevate and reorient themselves horizontally above the tongue around E14.0 although this phenotype is just one of a wide variety of developmental defects (Sanford et al., 1997). Treatment of *in vitro* palatal cultures with exogenous TGFβ2 or TGFβ1 accelerates fusion between the palate shelves, although only *tgfβ2*$^{-/-}$ mice have a CP albeit with less than one-quarter of *tgfβ2*$^{-/-}$ mice displaying the CP (Gehris and Greene, 1992). Expression of *tgfβ1* and *tgfβ2* is found in the MEE and mesenchyme immediately adjacent to the MEE respectively and neither is expressed until the palate shelves have elevated above the tongue (Fitzpatrick et al., 1990). Both *in vivo* and *in vitro* work suggest *tgfβ1* and *tgfβ2* contribute to mouse SP development, but *tgfβ3* appears to contribute the most.

Tgfβ3$^{-/-}$ mice develop a CP at nearly 100% levels, although the mice develop different types of CP; some have clefting of the anterior portion of the SP, some have posterior SP clefting while others show a complete SP cleft (Proetzel et al., 1995). This in contrast to *tgfβ2*$^{-/-}$ mice, which develop the same, complete SP cleft, but display far less penetrance of the CP phenotype. Also, the CP in *tgfβ3*$^{-/-}$ mice is the only developmental defect displayed as problems with the airway may be secondary to the CP in *tgfβ3*$^{-/-}$ mice (Proetzel et al., 1995). Expression of *tgfβ3* is first found at approximately E13.5 in the palate shelf epithelium, but becomes more restricted to the MEE following palate shelf elevation before being eliminated once the palate shelves fuse (Fitzpatrick et al., 1990). The role of *tgfβ3* in SP development appears to be mediating fusion of the palate shelves as the shelves of *tgfβ3*$^{-/-}$ mice make contact with each other, but never fuse and the MEE remains at the MES while application of exogenous TGFβ3 completely rescued the

defective fusion (Kaartinen et al., 1997). Further analysis into the role of *tgfβ3* in palate shelf fusion showed just prior to fusion filopodia are formed from the outer cell surfaces of the MEE and appear to be coated with proteoglycans to aid in adhesion and fusion of the palate shelves, whereas these structures are lacking in the *tgfβ3*$^{-/-}$ mice. Addition of exogenous TGFβ3 to *in vitro* palatal cultures of *tgfβ3*$^{-/-}$ mice recovered development of the proteoglycan-covered filopodia (Taya et al., 1999). The principal proteoglycan induced by TGFβ3 on the filopodia was chondroitin sulfate, which is synthesized and secreted just prior to palate shelf fusion and spatially restricted to expression in the MEE (Gato et al., 2002).

The TGFβ3 signal transduction pathway incorporates SMAD2, SMAD3 and SMAD4 as intra-cellular mediators of gene transcription and knockdown of *Smad2* expression produces a phenotype similar to *tgfβ3*$^{-/-}$ mice (Cui et al., 2005) while over-expression of SMAD2 in the MEE of *tgfβ3*$^{-/-}$ mice partially recovers a wild-type phenotype (Shiomi et al., 2006). As TGFβ3 binds to a heterodimeric receptor complex of TβR1 and TβR2 it is no surprise that TβR2 is necessary for proper SP development in order to initiate the TGFβ3 pathway. Specific deletion of TβR2 in the CNCC prior to SP development results in a complete CP due to decreased proliferation of the palatal mesenchyme, however, unlike the *tgfβ3*$^{-/-}$ mice, these palate shelves are able to fuse when their medial edges contact (Ito et al., 2003). Specific deletion of TβR2 solely in the epithelium of the palate shelves causes an incomplete CP and persistence of the MES, which resembles the inability to fuse or degrade the MES of *tgfβ3*$^{-/-}$ mice (Xu et al., 2006). The role of TβR3, long believed to be non-signaling due to lack of a cytoplasmic domain, during SP development has been outlined recently. Expression of TβR3 in the

SP very closely mimics that of TGFβ3 both temporally and spatially and specific knockdown of TβR3 causes a significant delay in palate shelf fusion and decreases intra-cellular levels of phosphorylated SMAD2 (Nakajima et al., 2007), yet exactly what role TβR3 is playing in SP development remains unclear. Taken together it is clear the evidence to support a critical role for TGFβ3, its receptors and intra-cellular mediators, the SMADs, during SP development is very strong.

The fibroblast growth factor (FGF) family is another group with members required for proper SP development. Like the TGFβ3 signaling pathway, the FGF signaling pathway depends on dimerization of the FGF receptors (FGFRs) upon ligand binding, but then utilizes another signal transduction pathway. The activated FGFR tyrosine kinases phosphorylate fibroblast growth factor receptor substrate 2 (FRS2), which provides binding sites for proteins that initiate the rat sarcoma (RAS)-mitogen-activated protein kinase (MAPK) signal transduction pathway that will affect target gene transcription (Borland et al., 2001). Mice lacking the IIIb isoform of FGFR2 develop a CP, among various other developmental defects (De Moerlooze et al., 2000), as do *fgf18*$^{-/-}$ mice (Liu et al., 2002). A closer examination of the FGF family and its relation to SP development revealed *fgf10*$^{-/-}$ mice, in addition to *fgf18*$^{-/-}$ and *fgfr2b*$^{-/-}$ mice, also develop a CP and display reduced proliferation in both the epithelium and mesenchyme of the palate shelves that results in decreased shelf growth and precludes the shelves from elevating (Rice et al., 2004). The roles of *fgf10* and *fgfr2b* illustrate the importance of epithelial-mesenchymal interactions in proper SP development. Expression of *fgf10* and *fgfr2b* is predominantly found in the mesenchyme immediately adjacent to palatal epithelium and in the oral epithelium respectively (Rice et al., 2004). Activation of

epithelial FGFR2b by its ligand FGF10, produced in the mesenchyme, increases epithelial expression of other signaling molecules, like Sonic Hedgehog (SHH); these signaling molecules bind to and activate their receptors back in the palate shelf mesenchyme and modulate FGF control over cell proliferation (Rice et al., 2004).

SHH and members of the bone morphogenetic protein (BMP) family are crucial contributors to SP development, however their expression is dependent on TFs and growth factors such as MSX1, TGFβ3 and FGF10 (Zhang et al., 2002; Sasaki et al., 2007; Rice et al., 2004), with which they function in an auto-looping mechanism dependent on both molecules (Gritli-Linde, 2007). SHH is a paracrine signaling molecule that binds to its receptor patched (PTCH), which relieves repression of another membrane-localized protein smoothened (SMO); SMO is internalized from the membrane in a vesicle with a protein complex where it participates in a series of phosphorylation events that prevent degradation of the glioma-associated oncogene homolog (GLI) proteins, which then translocate to the nucleus and affect gene transcription (Chari and McDonnell, 2007). The BMPs are a member of the same superfamily as the TGFβs and their signal transduction pathway is very similar to that of the TGFβs. Binding of a BMP ligand to a type II receptor causes dimerization with a type I receptor that leads to phosphorylation and dimerization of SMAD1, SMAD5 or SMAD8 with SMAD4, which leads to altered gene transcription (von Bubnoff and Cho, 2001).

Expression of SHH is found in the developing SP at all stages, but localized to distinct areas of oral epithelium; its receptor, PTCH, is found in the mesenchyme immediately adjacent to the epithelium expressing SHH while the other downstream

proteins (SMO, GLIs) are expressed widely throughout the palate (Rice et al., 2006). Like FGF signaling, SHH signaling is an example of crucial epithelial-mesenchymal interactions in the SP while it forms. *Shh*$^{-/-}$ mice have a CP with palate shelves that fail to grow properly or elevate (Rice et al., 2004) and though SHH is produced by the oral epithelium of the palate shelves, following stimulation by mesenchymally-produced FGF10 (Rice et al., 2004), its signaling target is the mesenchyme, where it drives cell proliferation and palate shelf growth through SMO (Lan and Jiang, 2009). One of the transcriptional targets in the mesenchyme for SHH is *fgf10*, which completes the looping expression mechanism and will result in further *Shh* expression thereby driving cell proliferation in the mesenchyme of the palate shelves (Lan and Jiang, 2009).

While no CP phenotype has been reported for knockouts of the BMP ligands, knocking out the BMP receptor, *Bmpr1a*, in mice results in bilateral cleft lip with CP (Liu et al., 2005) and expression of *Bmp4* under the control of the *Msx1* promoter rescues the CP that develops in *Msx1*$^{-/-}$ mice (Zhang et al., 2002). BMP expression in the SP is part of another looping expression mechanism whereby the TF MSX1 upregulates expression of *Bmp2* and *Bmp4* in the anterior palate shelf mesenchyme and these two BMPs then act to upregulate *Msx1* expression (Zhang et al., 2002). Expression of *Bmp* expression is localized to palate shelf mesenchyme and the defects associated with impaired BMP signaling in the developing SP appear to be caused by reduced mesenchymal cell proliferation and impaired palate shelf growth as the palate shelves of *Bmpr1a*$^{-/-}$ mice could still fuse *in vitro* (Liu et al., 2005).

1.2.4.3 Juxtacrine Interactions

Not only do paracrine interactions such as SHH signaling have an integral role in SP development, but also juxtacrine interactions of the Notch signal transduction pathway in which ligand and receptor are on two opposing cells contacting each other. In this pathway one cell displays the NOTCH1 receptor on its surface while neighbouring cells express a ligand for the NOTCH1 receptor; binding of the ligand from one cell to the receptor on another triggers proteolysis of the receptor, which then interacts with various cytosolic and nuclear proteins to affect gene transcription (Jiang et al., 1998). Mice that have had *Jag2*, a ligand for NOTCH1, knocked out develop a CP and die shortly after birth (Jiang et al., 1998). Expression of both *Jag2* and *Notch1* is found in the oral epithelium of the palate shelves and appears to both prevent adhesion of the oral epithelium to the tongue epithelium and facilitate adhesion and fusion of the palate shelves (Casey et al., 2006). Mouse SP development is under the control of many TFs and signaling molecules acting in paracrine and juxtacrine manners, but there is another large family of proteins, which seem to play a role in SP development and are not TFs, growth factors or any type of signaling molecule; the matrix metalloproteinases.

1.3 The Matrix Metalloproteinases

1.3.1 Domain Structure

The matrix metalloproteinases (MMPs) are a family of extra-cellular matrix (ECM)-degrading enzymes, requiring a zinc atom at their catalytic center to be active, which are involved in developmental events as well as the progression of cancers and other diseases. There are currently 25 members of the MMP family who share various conserved domains (Figure 5). As the MMPs are membrane-localized or exocytosed from

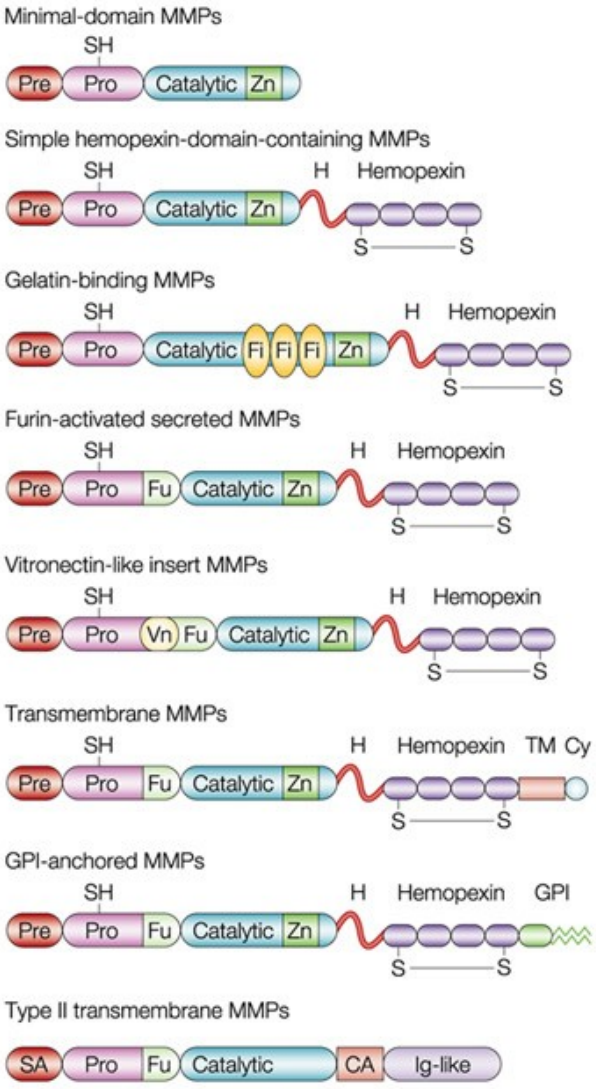


Figure 5: Schematic representation of matrix metalloproteinase (MMP) domain structure. Minimal domain: MMP-7, -26; simple hemopexin: MMP-1, -3, -8, -10, -12, -13, -18, -19, -20, -22, -27; gelatin-binding: MMP-2, -9; furin-activated secreted: MMP-11, -28; vitronectin-like insert: MMP-21; transmembrane: MMP-14, -15, -16, -24; GPI-anchored: MMP-17, -25; type II transmembrane: MMP-23. Reprinted with permission from Egeblad and Werb, 2002.

cells, all MMPs are initially synthesized with a signal sequence at their amino terminal end that will direct the nascent polypeptide into the secretory pathway for processing and

eventual export to the cell's exterior (Murphy and Nagase, 2008). This signal sequence is cleaved co-translationally once the polypeptide is in the endoplasmic reticulum. Since the MMPs are proteases, they are produced intra-cellularly as zymogens with a pro-domain that renders them catalytically inactive; following removal of the signal sequence, the pro-domain is at the amino terminal. All MMPs have a pro-domain and all but one have what is termed a "cysteine switch" in the pro-domain to maintain zymogen status (Nagase et al., 2006). Within the pro-domain the MMPs contain the highly conserved PR**C**GXPD cysteine switch amino acid sequence; the cysteine in the conserved sequence will coordinate with the zinc atom contained within the catalytic domain, which folds the pro-domain over top of the catalytic domain and prevents the zymogen from interacting with any substrates until the pro-domain has been removed (Nagase and Murphy, 2008). For 10 MMPs, removal of the pro-domain occurs intracellularly by a pro-protein convertase, most notably Furin, which cleaves the pro-domain from the rest of the zymogen at a conserved sequence of RXRXKR located at the junction between the catalytic and pro-domains (Yana et al., 2000; Nagase et al., 2006). The other 15 MMPs are activated extra-cellularly in cooperation with the MMPs activated intra-cellularly, which does not fully remove the pro-domain of the 15 MMPs, but rather begins the process by making a cleavage within the pro-domain that is finished auto-catalytically by the MMP being activated; this process has been well-characterized for MMP-2 (Morgunova et al., 1999; Sternlicht and Werb, 2001; Nie and Pie, 2003; Morrison and Overall, 2006). The catalytic domain of the MMPs is the most highly conserved domain in the family and to be considered an MMP, family members must display catalytic domain amino acid sequence homology to MMP-1 (Murphy and Nagase, 2008). All

MMPs contain a conserved **H**EXX**H**XXGXX**H** sequence that sits at the center of the catalytic domain and is vital to the enzymes' activity; it is the three Histidine residues that will coordinate the zinc atom at the active site with the assistance of, first, the cysteine residue in the cysteine switch of the pro-domain and, second, a water molecule once the enzyme has been activated (Nagase et al., 2006). All MMPs, with only three exceptions, contain a hemopexin-like domain at their carboxy terminal that is attached to the central catalytic domain through a flexible "hinge" region (Murphy and Nagase, 2008). The hemopexin-like domain affects MMP substrate specificity, proteolytic activity, pro-MMP activation and MMP inhibition (Sternlicht and Werb, 2001; Wang et al., 2004). In addition to all the previous structural features, there are seven MMPs classed as membrane-type MMPs (MT-MMPs). Whereas the other 18 MMPs end with the hemopexin-like or catalytic domains at their carboxy terminals and are simply secreted outside of the cell, these MMPs have either a trans-membrane domain that integrates them into the plasma membranes of cells or are attached to the exterior of the cell's plasma membrane through a glycosyl-phosphatidyl inositol (GPI) anchor that links the carboxy terminal of the MMP protein to the GPI group (Sohail et al., 2008).

1.3.2 Classifications and Substrate Specificity

The MMPs were once classified into sub-groups according to their function and primary substrates, but are now usually referred to by their numeric order. MMP-1, -8 and -13 are the collagenases and cleave collagen types I, II and III preferentially. MMP-2 and -9 are the gelatinases and cleave a wide variety of ECM and basement membrane proteins including denatured collagen from which they derive their name. MMP-3, -10 and -11 are stromelysins, which are similar to the collagenases, but can not cleave as

many substrates. MMP-12, -20 and -27 have not yet been grouped as stromelysins, but share all the same characteristics. The matrilysins (MMP-7 and -26) are also referred to as minimal domain MMPs as they contain only the conserved pro and catalytic domains and lack a hemopexin-like domain. In addition to ECM protein substrates, these MMPs can also activate pro-cytokines (Parks et al., 2004). The MT-MMPs are classed within themselves, but also in the larger family context. Thus MT1-MMP is MMP-14, MT2-MMP is MMP-15, MT3-MMP is MMP-16 and MT5-MMP is MMP-24. These are the integral trans-membrane MT-MMPs while MT4-MMP (MMP-17) and MT6-MMP (MMP-25) are the GPI-anchored MT-MMPs (Murphy and Nagase, 2008). In addition to various sub-groups, the MMPs can be broadly classed into two large categories: the MT-MMPs and the secreted MMPs.

1.3.3 Tissue Inhibitors of Metalloproteinases

The endogenous regulators of MMP activity are the tissue inhibitor of metalloproteinases (TIMPs), which paradoxically are involved not only in the inhibition of MMPs, but also their extra-cellular activation. There are four TIMPs that collectively can inhibit the entire MMP family in a 1:1 TIMP to MMP ratio; the amino terminal domain of TIMPs binds to the catalytic domain of the MMP while the TIMP carboxy terminal domain binds to the MMP hemopexin-like domain (Flannery, 2006). The TIMP amino domain acts like the pro-domain of MMP zymogens in that a critical cysteine residue in the amino domain displaces the water molecule coordinating with the zinc atom at the catalytic center, which covers the MMP active site and prevents any further catalysis (Nagase et al., 2006). In addition to their inhibitory actions, TIMPs can be utilized for the activation of some secreted MMPs in cooperation with membrane-

associated MMPs. By binding to the catalytic domain of a membrane-associated MMP with their amino domain and to the hemopexin-like domain of the secreted pro-MMP with their carboxy domain, the TIMPs hold the secreted pro-MMP in place while another nearby membrane-associated MMP activates it (Birkedal-Hansen et al., 1993; Morgunova et al., 1999; Van Den Steen et al., 2001; Bernardo and Fridman, 2003). In more recent years additional activities not related to the MMPs have been attached to the TIMPs. Mitogenic and anti-apoptotic properties have been described for TIMP-1 and -2 while a pro-apoptotic role has been outlined for TIMP-3 in addition to inhibiting the vascular endothelial growth factor signaling pathway and angiogenesis by binding to the growth factor's receptor (Murphy and Nagase, 2008).

1.3.4 Roles in Disease States

1.3.4.1 Autoimmune

Autoimmune diseases are characterized by the cells of the immune system attacking self targets within the body instead of proper pathogenic targets. The arthritides are common autoimmune diseases and as enzymes that degrade the ECM, which is the target of degradation in the arthritides, the MMPs have a role in the progression of both osteoarthritis and rheumatoid arthritis. The cartilage of joints is composed primarily of a proteoglycan called aggrecan, which can be hydrated in association with other proteoglycans, and type II collagen (Poole, 1999). Rheumatoid arthritis is a systemic, chronic inflammation of joints typically found in elderly populations whereas osteoarthritis tends to develop in joints that have been injured or worn down and can develop in younger individuals. In both cases there is a cytokine signaling cascade that initiates ECM breakdown by up-regulating expression of ECM proteinases, which

releases growth factors and pro-cytokines that further stimulate synthesis and secretion of ECM proteinases like the MMPs (Burrage et al., 2006). The predominant MMPs involved in the progression of the arthritides are MMP-1, -3 and -13 with conflicting evidence regarding a role for MMP-9 (Burrage et al., 2006; Naito et al., 1999; Ram et al., 2006). Over-expression of MMP-1, -3 and -13 occurs in synovial fibroblasts and cartilage cells within the joint due to cytokine stimulation, primarily IL-1 and TNF-α, which is produced by macrophages and the chondrocytes. The cytokine signal transduction pathway involves various kinases that will eventually up-regulate expression of MMP-1, -3, -13 and pro-TNF-α, which is activated outside of the cell and then acts in an autocrine and paracrine manner to further increase expression of itself and the three MMPs (Vincenti and Brinckerhoff, 2002; Nah et al., 2007). The targets of MMP-1, -3 and -13 in the arthritides are the ECM components aggrecan and type II collagen. All three MMPs cleave aggrecan at a specific site that removes a key domain and prevents aggrecan from binding hyaluronan anymore; this lack of binding decreases localized hydration (Takaishi et al., 2008). Type II collagen is the major collagen in joints and is efficiently cleaved, which leads to its denaturation at physiological temperature, by MMP-1 and -13 (Takaishi et al., 2008). Though the initiation of osteoarthritis and rheumatoid arthritis is different, localized over-expression of MMP-1, -3 and -13 by fibroblasts and chondrocytes in the joints leads to a vicious cycle in which key ECM components are continually degraded until the joint is no more.

Multiple sclerosis (MS) is another autoimmune disease in which there is emerging evidence to support a role for the MMPs. MS is a chronic disease characterized by a break down of the myelin protein sheath of nerves within the central nervous system.

There are two stages of MS: initial development and relapses, which occur since the body tries to re-myelinate damaged areas. Exactly how MS is initially triggered remains unclear, but the disease develops when T cells and macrophages of the immune system invade the central nervous system and begin degrading the myelin covering of nerves; this is a process that involves MMP-9 and, secondarily, MMP-2 as the immune system cells utilize these proteases to both gain access to the central nervous system and degrade myelin (Leppert et al., 1998; Sastre-Garriga et al., 2004). One study found expression of MMP-9 significantly increased in all patients with relapsing MS and in 57% of patients initially developing MS; they hypothesized that immune system cells infiltrating the central nervous system and causing the MS were the source of the increased MMP-9 expression (Leppert et al., 1998). Further work found expression of MMP-9 and -2 and their endogenous regulators, TIMP-1 and -2 respectively, in MS patients was correlated with severity of the disease at the time; when patients were in a recovery phase, MMP expression was low and TIMP expression was higher, however when patients were in a relapse state, MMP expression was high and TIMP expression low (Liuzzi et al., 2002; Benešová et al., 2009). Treatment of MS with interferon-beta resulted in sustained increased expression of TIMP-1 and a positive response to treatment. Although there was no sustained decrease in MMP-9 and -2 levels, the increase in TIMP-1 levels could have been responsible for the positive response to treatment by blunting the effects of MMP-9 and -2 (Comabella et al., 2009). Finally, polymorphisms within the promoters of MMP-9 and -2 were believed to be linked to MS and indicate a genetic susceptibility, but analysis indicated only one of two MMP-2 polymorphisms was correlated with a higher risk of

MS in women while there was no such association with MMP-9 polymorphisms (Benešová et al., 2008).

1.3.4.2 Cancer

Development of cancer is complex and can depend on tissue of origin, but two of the central characteristics of these diseases are the growth and metastasis of primary tumours. To facilitate growth and metastases, tumours heavily over-express some MMPs and many of the most common cancers are linked to MMP over-expression.

Lung cancer is the most common cancer in North America. Two MMPs, MMP-2 and -7, play a large role in the progression of lung cancer and tumours expressing these MMPs at higher levels indicates a poor prognosis (Liu et al., 2007; Liu et al., 2008; Leinonen et al., 2008). In lung cancer tumours MMP-7 expression is downstream of the Wnt/β-catenin pathway, which is hyper-active in lung cancer (Huang et al., 2008). Once active MMP-7 is released into the ECM of tumours it can locally enhance insulin-like growth factor signaling by cleaving insulin-like growth factor binding protein 3, which stimulates increased cell proliferation within the tumour (Miyamoto et al., 2004). Other MMP-7 substrates include type IV collagen and proteoglycans that comprise the bulk of the ECM, which enables MMP-7 to further aid increased cell proliferation within lung cancer tumours by removing ECM barriers to increased tumour growth (Ii et al., 2006). MMP-7 is also able to cleave the extra-cellular domain of E-Cadherin (Davies et al., 2001), which causes cells to lose contact with one another and leads to a cellular phenotype more conducive to invasion and metastasis in cancer, although it is still unclear if it plays this role in lung cancer. MMP-2 is involved in growth and metastasis of lung cancers through angiogenesis facilitation, which is a key requirement for tumours to

grow larger, and suppression of apoptosis. MMP-2 expression in lung cancer is downstream of the PI3K signaling pathway (Saito et al., 2006), which is very commonly altered in many cancers so it is constitutively active. In a lung cancer cell line MMP-2 expression knocked down with siRNA resulted in increased endothelial cell apoptosis and inhibited the formation of endothelial tubes, which are precursors to new blood vessels and essential for tumours to grow larger (Chetty et al., 2008). Knockdown of MMP-2 expression also stimulates apoptosis in lung cancer cells by preventing the cleavage of Fas ligands on the extra-cellular surface of the plasma membrane (Chetty et al., 2007).

Colon cancer is a disease in which the gelatinases, MMP-2 and -9, figure prominently. Over-expression of MMP-2 and -9 in colon cancer tumours indicates a poor prognosis in the disease (Roeb et al., 2001; Hilska et al., 2007; Šundov et al., 2008). The gelatinases are acting as ECM-degrading enzymes and aid colon cancer progression by facilitating angiogenesis events and metastases. Expression and secretion of the gelatinases is downstream of the MAPK pathway and depends on protein-protein interactions between integrins and effectors of the MAPK pathway. Association of src tyrosine kinase, very commonly constitutively active in colon cancer and membrane-localized, with the intra-cellular domain of α1-integrin results in recruitment and phosphorylation of downstream effector proteins that leads to an increase in both latent and active forms of MMP-2 and -9 in colon cancer cells (Van Slambrouck et al., 2007). Similarly, association of αvβ6-integrin and ERK1/2, a downstream effector of the MAPK pathway, at the plasma membrane results in the eventual up-regulation of MMP-2 and -9 expression in colon cancer cells (Gu et al., 2002; Wang et al., 2008). The gelatinases are

key players in tumour angiogenesis (Itoh et al., 1998; Bergers et al., 2000; Chetty et al., 2008), however direct evidence for an angiogenic role in colon cancer development has only been uncovered for MMP-9; in the ECM MMP-9 can cleave heparan sulphate, which releases vascular endothelial growth factor from ECM binding and facilitates angiogenesis in colon cancer tumours (Hawinkels et al., 2008). In addition to colon cancer tumour development, MMP-9 appears to play a specialized role in some metastases of colon cancer. *In vitro* and *in vivo* research indicated MMP-9 was critical in facilitating the spread of colon cancer cells to the lung by degrading ECM components that hindered the formation of locomotory apparatuses (Lubbe et al., 2006); however an examination of colon cancer metastasis to the liver indicated MMP-9 was not primarily responsible for these metastases, although it was still heavily involved in the progression of the colon cancer to an invasive phenotype (Illemann et al., 2006).

Breast cancer is the most common female-specific cancer in North America. Once again the gelatinases, MMP-2 and -9, as well as MMP-14, or MT1-MMP, are key players. Expression of MMP-2, -9 and -14 is positively correlated with aggressive and invasive breast cancer with lower levels of expression seen in earlier stage and less aggressive breast cancer (Figueira et al., 2009); Of the three MMPs, high MMP-14 expression indicates a poor prognosis and low long-term survival (Têtu et al., 2006; Jiang et al., 2006). As in colon cancer, the expression, secretion and activation of the gelatinases in breast cancer is downstream of the MAPK signal transduction pathway and dependent on phosphorylated ERK1/2 (Lu et al., 2008; Sun et al., 2009). Expression of the gelatinases is associated with vascular endothelial growth factor and angiogenesis in breast cancer tumours (Kurizaki et al., 1998). A common treatment for breast cancer is

the anti-estrogen drug, tamoxifen, which paradoxically increases levels of active MMP-2 and -9 in breast cancer tumours, but also increases levels of TIMP-1, -2 and endostatin, an inhibitor of angiogenesis (Nilsson et al., 2007). Endostatin is the product of proteolytically-cleaved type XVIII collagen, a component of the ECM basement membrane (Marneros and Olsen, 2005); it carries out its anti-angiogenic activities in endothelial cells by down-regulating the expression of genes involved in migration including the gelatinases, which are also involved in cleavage of type XVIII collagen and endostatin formation, but also by binding to the catalytic domain of active MMP-2 in the pericellular environment (Shichiri and Hirata, 2001; Bendrik et al., 2008; Lee et al., 2002). The pro-angiogenic role for the gelatinases in breast cancer progression is tempered by their production of the anti-angiogenic peptide fragment endostatin, which doubles back and inhibits their expression and activity (Lee et al., 2002; Nilsson et al., 2007; Lu et al., 2008; Bendrik et al., 2008). In addition to its pro-angiogenic role, MMP-2 is involved in metastasis of breast cancer tumours through digestion of endothelial basement membrane components that would otherwise prevent the entry of cancer cells into blood vessels (Kargozaran et al., 2007).

MMP-14 has dual roles in invasive breast cancer; first, it is a protease with substrates that include many key components of the ECM and basement membrane, which allows it to facilitate increased tumour growth and metastases and, second, it is an activator of pro-MMP-2 (Bernardo and Fridman, 2003), which is itself implicated in breast cancer progression. Expression of MMP-14 in breast cancer cells is downstream of β-catenin signaling and dependent on the improper cytosolic localization of a crucial tight junction protein called zonula occludens-1 (ZO-1), which allows un-phosphorylated β-

catenin to accumulate and affect downstream gene transcription (Polette et al., 2005). Once expressed, MMP-14 is characteristic of invasive, metastatic breast cancers (Jiang et al., 2006) and is delivered in vesicles to the leading edge of tumours via the soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) protein, Vamp7, where it degrades its ECM substrates (Steffen et al., 2008).

1.3.5 Roles in Normal Development

Much of the developmental work carried out by the MMPs enables cell proliferation and migration as the MMPs degrade ECM barriers. The MT-MMPs seem to play a more significant role in development than do the secreted MMPs; this is likely caused by their dual roles as proteases and activators of the secreted MMPs. One crucial developmental process that utilizes the MMPs is bone development. The majority of embryonic bone development occurs through endochondral ossification, where a cartilage template is laid down and subsequently replaced by a mineralized bone matrix; this matrix is then vascularized by capillaries and followed by bone-specific osteoclasts and osteoblasts, which proliferate and are responsible for establishing final bone development on the mineralized matrix as well as maintenance throughout life (Vu and Werb, 2000). The key MMPs for bone development are MMP-9, -13 and -14, or MT1-MMP.

MMP-9 is important for bone development by stimulating angiogenesis, through the capillaries invading the cartilage template, and apoptosis of the hypertrophic cartilage matrix, which allows a secondary wave of bone-specific cells to finalize development (Vu et al., 1998). MMP-9 expression is downstream of the Wnt/β-catenin signaling pathway in bone development as is that of vascular endothelial growth factor, a potent stimulator of angiogenesis (Tamamura et al., 2005). Vascular endothelial growth factor

trapped within the ECM is a target for active MMP-9 and once released it stimulates the recruitment of endothelial cells that will form capillaries and is a chemoattractant for osteoclasts. Passage of the endothelial and osteoclastic cells through the un-mineralized cartilage template is also mediated by the proteolytic capabilities of MMP-9 (Engsig et al., 2000). Apoptosis of the chondrocytes within the cartilage template is required concurrent with the migration of endothelial and osteoclastic cells in proper bone development. Resorbing chondrocytes highly express MMP-9, as do the invading endothelial and osteoclastic cells, and lack of MMP-9 results in decreased cartilage apoptosis and migration into the bone matrix (Takahara et al., 2004); the pro-apoptotic and pro-migration actions of MMP-9 can also be attributed to its cleavage of ECM downstream effectors that interact with additional ECM components (Ortega et al., 2005). The evidence to support a strong role for MMP-9 in bone development is strong and *Mmp9*$^{-/-}$ mice display developmental defects in embryonic bone development related to impaired apoptosis and angiogenesis, however these defects are eventually compensated for post-natally and *Mmp9*$^{-/-}$ mice are viable and normal about a month after birth (Vu et al., 1998).

Much like MMP-9, the role of MMP-13 in bone development regards degradation of the ECM in the un-mineralized cartilage template; expression of MMP-13 is high in terminally-differentiating cartilage and active MMP-13 is required to initiate the establishment of the mineralized bone matrix in place of the cartilaginous template (D'Angelo, et al., 2000). Two main components of the cartilage ECM, type II collagen and the proteoglycan aggrecan, and type I collagen in bone are substrates for MMP-13 and lack of MMP-13 expression in the cartilage template results in significantly higher

levels of surviving chondrocytes and reduced migration of bone-producing cells into the bone matrix (Stickens et al., 2004). Expression of MMP-13 is downstream of both the Wnt/β-catenin (Tamamura et al., 2005) and MAPK signaling pathways in the terminally-differentiating chondrocytes (Ronzière et al., 2005). Expression of MMP-13 via the MAPK pathway is dependent on α1β1-integrins, which are extra-cellular receptors for both type I and II collagen (Ronzière et al., 2005). Research indicates there is a switch from type II collagen to type I collagen in the cartilage template just prior to degradation that could signal to the α1β1-integrins and, subsequently, MAPK pathway to increase MMP-13 expression (Ronzière et al., 2005). Mice that are *Mmp13*$^{-/-}$ display a phenotype similar to *Mmp9*$^{-/-}$ mice; there is decreased chondrocyte apoptosis, vascularization and bone-specific cell migration, but post-natally these defects are eventually compensated for and *Mmp13*$^{-/-}$ mice are largely normal (Inada et al., 2004), however as adults they do have problems with repairing bone fractures and breaks (Behonick et al., 2007).

The most important MMP contributor to bone development is MMP-14 or MT1-MMP. Not only is it an activator of certain secreted MMPs, but it is also a peri-cellular protease with ECM substrates. Like MMP-9 and -13, MMP-14 facilitates vascularization of the cartilage template, which leads to mineralization and bone development, as well as delayed apoptosis of the terminally-differentiated chondrocytes and ossification (Zhou et al., 2000). Expression of MMP-14 in pre-osteoblasts is induced by the ECM components type I collagen and fibronectin binding to α2β1-integrin or α4/α5β1-integrin dimers respectively, which activate the MAPK signaling pathway; up-regulation of MMP-14 expression in the pre-osteoblasts results in mineralization of the cartilage template and ossification (Manduca et al., 2009). MMP-14 is also indispensable to immediate post-

natal bone development in which it mediates a type of non-endochondral bone development that resorbs and replaces the cartilage template with bone, but skips the mineralization of the cartilage step (Holmbeck et al., 2003). Unlike *Mmp9*$^{-/-}$ and *Mmp13*$^{-/-}$ mice, *Mmp14*$^{-/-}$ mice have profound skeletal defects that include skull malformations, decreased bone density after birth, osteoarthritis, disrupted vascularization of the cartilage template in future bone, delayed hypertrophic chondrocyte apoptosis and delayed ossification of the mineralized cartilage template by reduced osteoclast and osteoblast activity (Holmbeck et al., 1999). In addition to the bone defects, the *Mmp14*$^{-/-}$ mice never sexually mature and most die between 50 and 90 days (Holmbeck et al., 1999). Despite a strong role in bone development for MMP-9 and -13, their mouse knockout models were able to eventually compensate for the loss of those two MMPs; the loss of MMP-14 was obviously unable to be developmentally compensated for indicating a more important and non-redundant role for MMP-14 in bone development. The importance of the MMPs in bone development has been well characterized, however the role they play in SP development, which also involves ossification, remains unclear.

1.4 Matrix Metalloproteinases and Palatogenesis

Little is currently known about what role the MMPs play in SP development. An association between the MMPs and craniofacial morphogenesis was first noted in 1997; expression of MMP-2 was detailed throughout the developing mouse face (Iamaroon et al., 1997) while a functional role in mandibular, tongue and facial cartilage development was outlined for the MMPs as a family (Chin and Werb, 1997). Expression of the MMPs and TIMPs in SP development was first outlined in 1999 and then refined in 2000; MMP-2, -3, -7, -9, -13, TIMP-1 and -2 expression was found to be temporally regulated in the

developing mouse SP (Morris-Wiman et al., 1999) while further investigation determined expression of MMP-2, -3, -9, -13, TIMP-1, -2 and -3 was not only temporally regulated, but also spatially within the SP according to local concentration of their primary substrates (Morris-Wiman et al., 2000). A functional role for the MMP family in SP development was described in 2001 and confirmed in 2002; treatment of *in vitro* palatal cultures with a chemical inhibitor of the MMPs blocked fusion of the palate shelves and resulted in persistence of the MEE in contacted areas (Blavier et al., 2001; Brown et al., 2002). Early hypotheses, based on expression of the MMPs in the SP, focused on MMP-2, -3 and -9 as the MMPs with functional roles in SP development (Morris-Wiman et al., 2000; Brown et al., 2002), however *Mmp2*$^{-/-}$, *Mmp3*$^{-/-}$ and *Mmp9*$^{-/-}$ mouse models were fertile, viable and, most importantly, did not display a CP phenotype (Itoh et al., 1997; Shapiro, 1997; Vu et al., 1998). Which of the MMPs has a functional role in SP development remained unknown for years as no further research was done in the area since Blavier et al., 2001 and Brown et al., 2002, however recent work has implicated the MT-MMPs as being the primary MMPs in SP development. Mice with a double knockout of MT1- (MMP-14) and MT3-MMP (MMP-16) have a complete CP (Shi et al., 2008), but mice with a single knockout of either MT1- or MT3-MMP do not, although they do have other severe developmental defects (Shi et al., 2008; Holmbeck et al., 1999). These results demonstrate crucial, non-redundant functions for the MT-MMPs, however in SP development it appears MT1-MMP is able to compensate for MT3-MMP when it is knocked out so a role for a single MMP still remains unclear in SP development, although a new candidate is MT6-MMP or MMP-25.

1.5 Matrix Metalloproteinase-25

MMP-25 was the sixth MT-MMP cloned and the second GPI-anchored MT-MMP (Kojima et al., 2000), which means it is attached to the exterior of the cellular plasma membrane rather than embedded in it. MMP-25 contains the same conserved domains of the other MMPs: amino terminal signal sequence, pro-domain with RXRXKR activation sequence, catalytic domain, hinge region, hemopexin-like domain and a GPI-anchoring region at the carboxy terminal (Pei, 1999).

Mmp-25 was first cloned in 1999 from human leukocytes and was hypothesized to facilitate leukocyte invasion to sites of injury through proteolytic degradation of ECM and basement membrane components (Pei, 1999). Further investigation into leukocytes determined *Mmp-25* expression was restricted to the neutrophil sub-population, predominantly found in cytosolic granules and vesicles and MMP-25 was deployed by neutrophils when stimulated by cytokines (Kang et al., 2001); a more systemic look at *Mmp-25* expression found mRNA in all organs examined with highest expression in the heart and lungs (Nuttall et al., 2004).

ECM and basement membrane substrates of MMP-25 include gelatin, type IV collagen, fibrinogen, fibrin, fibronectin, chondroitin sulfide and dermatin sulfide (Kang et al., 2001; English et al., 2001) and its catalytic activities were inhibited strongest by TIMP-1, but also effectively by TIMP-2 and -3 (English et al., 2001); MMP-25 is also significantly inhibited via its hemopexin-like domain by clusterin, a protein that inhibits the other GPI-anchored MT-MMP, MMP-17, but does not do so for any other MMPs examined (Matsuda et al., 2003). Additional targets for active, membrane-localized MMP-25 could include activation of pro-MMP-2, however evidence for this is

conflicting (Kojima et al., 2000; Velasco et al., 2000; Nie and Pie, 2003; Sun et al., 2007).

Like other MMPs, *Mmp-25* is over-expressed in certain cancers. Expression of *Mmp-25* is very low in the brain normally, but significantly up-regulated in astrocytoma and glioblastoma brain tumours, likely indicating a proteolytic role in the development of these tumours *in vivo* (Velasco et al., 2000). Similarly, expression of *Mmp-25* is absent in the healthy colon, but is significantly increased in *in vivo* colon cancer and transfection of colon cancer cell lines with vectors expressing *Mmp-25* resulted in those cell lines acquiring an aggressive, invasive phenotype relative to controls (Sun et al., 2007). MMP-25 over-expression has also been linked to urothelial and prostate cancers (Sohail et al., 2008).

No role for MMP-25 in embryonic development has ever been described, however genomic scans of the DNA of human CP patients revealed consistent appearance of markers at a locus just upstream of what turned out to be the *Mmp-25* gene (Blanton et al., 2004). These researchers determined there was a significant enough association between *Mmp-25* and CP to justify a molecular investigation into the role of MMP-25 and SP development, which was the basis for this current study.

1.6 Implications for Research

CP is one of the most common birth defects today. As such its cumulative costs are large, both financially and socially. Of course direct financial estimates do not take into account peripheral costs such as traveling, accommodations or time off work during hospital stays. Over an individual's lifetime all of these costs will accumulate. Social costs can also accumulate. Because CP is better hidden from the world than CL/P, most studies

examining the psychological and social consequences of orofacial clefting have focused on CL/P. Yet with so many visits to medical specialists, including speech pathologists, even a young child with CP can not fail to understand they are different and might feel less attractive to the world because of their multiple surgeries and/or speech problems. So it is probable that patients with CP also suffer from psychological and social isolation, though it may not be as severe as those with CL/P, who are more noticeably dissimilar.

Research into *Mmp-25* in the SP will help researchers understand the formation of the SP better and enhance knowledge about the MMP family during tissue remodeling events. Combined with research into environmental contributing factors, this information will make combating CP easier as its origins will gradually become better understood, making available new treatment strategies that can be used in the prevention and therapy of CP in children.

2. Materials and Methods

2.1 Primer Design and Semi-Quantitative and Quantitative PCR

All mice were handled and research carried out in accordance with the University Committee on Animal Care and Supply. Total RNA isolations were done on embryonic CD-1 mouse SP tissue using the RNeasy® Mini Kit according to manufacturer's protocol (Qiagen, Mississauga, ON). RNA isolations were done on SP tissue corresponding to days E12 to E15. Reverse transcriptase PCR (RT-PCR) was carried out on total RNA isolations using the SuperScript™ RNase H-Reverse Transcriptase according to manufacturer's recommendations to generate embryonic SP complementary DNA (cDNA; Invitrogen, Carlsbad, CA).

Semi-Quantitative PCR primers were designed to recognize the 3′ end of mouse *Mmp-25* mRNA transcripts. The primers amplified a deoxynucleotide fragment of 295 base pairs (bp) from an embryonic murine SP cDNA pool if *Mmp-25* cDNA is present. Due to homology with other murine mRNAs, the *Mmp-25* mRNA sequence was aligned with these mRNAs and examined for regions of low homology using the online multiple sequence alignment software, ClustalW (Appendix A). The primers were then constructed and chosen using Primer3 (v.0.3.0) and NetPrimer software available online (Appendix A). Finally, primer sets were analyzed using the online nucleotide basic local alignment search tool (BLASTn) to ensure that only *Mmp-25* cDNA would be amplified during subsequent PCR (Appendix A). Final Semi-Quantitative PCR primer sets are displayed in Table 1.

Semi-Quantitative PCR was carried out using the EconoTaq™ DNA polymerase and 1x reaction buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100, 1.5 mM

Table 1: List of Semi-Quantitative PCR primer sets designed to amplify an oligodeoxynucleotide fragment using *Mmp-25* cDNA as a template.

Name of PCR Primer	5’ -> 3’ Primer Sequence
Mmp25a (Sense)	CGAAAACGACATCCAAAACC
Mmp25b (Antisense)	CCCTAACAGAAAGCAGACTCA

$MgCl_2$) according to manufacturer’s specifications (Lucigen, Middleton, WI). All Semi-Quantitative PCR used a final volume of 50 µl unless otherwise noted and was done on a MJ Research PTC-100 Thermal Cycler (Bio-Rad, Mississauga, ON). To amplify the 295 bp fragment from *Mmp-25* cDNA, cycling conditions were as follows: 1 cycle at 95ºC for 3 min followed by 30 cycles at 94ºC for 1 min, 55ºC for 1 min, 72ºC for 2 min completed by 1 cycle at 72ºC for 8 min. Semi-Quantitative PCR products were visualized using 1% agarose gel electrophoresis (100 ml) and 70 nanograms of ethidium bromide. The size of Semi-Quantitative PCR products was compared relative to the Ready-Load™ 1 Kb DNA Ladder (Invitrogen).

qRT-PCR was carried out using the E12 to E15 SP cDNA described above, an *Mmp-25*-specific TaqMan® Gene Expression Assay according to manufacturer’s protocol (Applied Biosystems, Foster City, CA) and a 7300 Real Time PCR System (Applied Biosystems). qRT-PCR was analyzed using the Sequence Detection Software (v. 1.4) supplied with the 7300 Real Time PCR System (Applied Biosystems). Relative quantitation (RQ) values for the SP were obtained in comparison to a β-actin control and standardized to an E15 test run. The β-actin control was validated on a standard curve to ensure measurements would only take place during the exponential phase and did not vary between runs. Statistical analysis on the RQ values developed by the Sequence Detection Software (v. 1.4) was carried out using GraphPad Prism for Windows (v. 5.01; GraphPad Software Inc., La Jolla, CA).

2.2 *In Vitro* Embryonic Mouse Palate Cultures

Pregnant CD-1 mice (E13) were killed by cervical dislocation after anaesthesia with Halothane® (MTC Pharmaceuticals, Cambridge, ON) and the embryos were aseptically removed in Hanks' Balanced Salts solution (Sigma-Aldrich, Oakville, ON). The SPs were aseptically dissected and palate shelf cultures set up as previously described (Shiomi et al., 2006; Nakajima et al., 2007) with two modifications: the addition of 1M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) to a final concentration of 20mM and addition of a penicillin-streptomycin antibiotic solution to a final concentration of 0.1% (Sigma-Aldrich). Briefly, the E13.0 palate shelves were excised and arranged in pairs with their medial edges in contact and in proper anterior-posterior orientation on Nucleopore Polycarbonate Track-Etch filters (8.0 μm pore; Whatman, Inc., Newton, MA). Up to three filters were floated in a 35mm tissue culture dish containing 1.4 mL of BGJb medium (Invitrogen), supplemented as detailed above, and incubated with 5% CO_2 at 37°C for 72 h. Following 72 hours incubation, 10 palatal cultures from each group (wild-type, scrambled siRNA control, *Mmp-25* siRNA) were immersed in freshly-prepared 4% paraformaldehyde for approximately 2 h then placed in 30% sucrose. The preserved palate cultures were embedded in Tissue-Tek® O.C.T. Compound (Sakura Finetek U.S.A., Inc., Torrance, CA), frozen and serial sections (8 μm) prepared. Serial sections were examined by myself in a non-blinded fashion and scored and the cumulative scores were averaged to obtain a mean fusion score (MFS) as previously described (Figure 6; Yu et al., 2008; Kang and Svoboda, 2002). The Kruskal-Wallis non-parametric statistical test was performed on the MFSs of the groups to determine significance where significance is indicated by $p < 0.05$. After scoring, palate

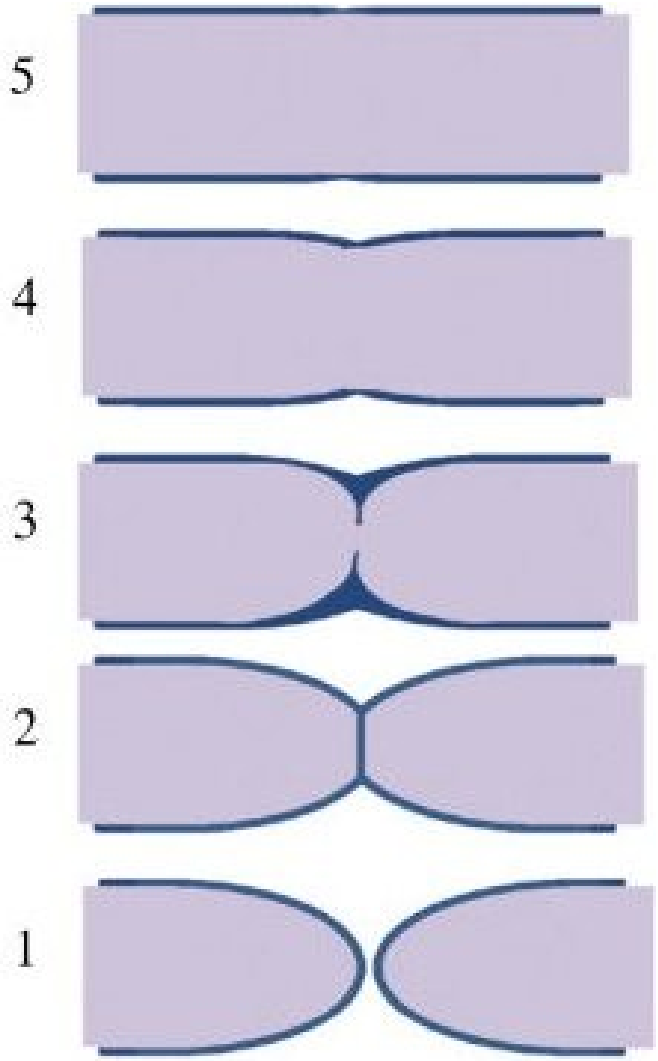


Figure 6: Standards used to obtain the mean fusion scores for the *in vitro* palatal cultures. Reprinted with permission from Yu et al., 2008.

sections were stained with hematoxylin and eosin (H&E) using the following modified protocol: 3 min. with hematoxylin followed by two changes of tap water, one change of

acid alcohol (0.5% hydrochloric acid in 95% ethanol), two changes of tap water, one change of distilled water, 1 min. with eosin, one change of 95% ethanol and mounting in 50% glycerol. The remaining palate cultures were pooled for either RNA or protein isolations as detailed in **2.1** and **2.3** respectively.

2.3 Western Blot Analysis with an MMP-25 Antibody

All mice were handled and research carried out in accordance with the University Committee on Animal Care and Supply. Embryos from CD-1 mice were aseptically removed at stages E12 through E15 and placed in 1x phosphate-buffered saline (PBS). Embryos from each stage of SP development (ie: E12 to E15) had their SPs dissected out and preserved at -20^{o}C in 1.5 ml eppendorf tubes for protein isolations, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses. The lungs, kidneys, liver, heart, spleen and brain were dissected from the adult female CD-1 mouse, following removal of the embryos, to use as MMP-25 positive control organs during Western blot analysis (Nuttall et al., 2004; Bernal et al., 2005). To isolate proteins, thawed tissue was homogenized in 150 µl of modified radioimmunoprecipitation assay (RIPA) buffer (5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM TRIS pH 8.0, 0.1% sodium deoxycholate, 0.01% SDS, 1% Triton X-100, 150 mM sodium chloride, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin) using a mechanical homogenizer at 4^{o}C. Homogenized samples were centrifuged at 4^{o}C and 11,000 revolutions per minute (RPM) for 15 min. The supernatant was transferred to a new 1.5 ml eppendorf tube and protein concentration was quantified using the *DC* Protein Assay kit (Bio-Rad). To prepare for SDS-PAGE, 2x SDS gel-loading buffer (100 mM TRIS pH 6.8, 4.0% SDS, 0.2% bromophenol blue, 20.0% glycerol, 200 mM β-mercaptoethanol) was added to each

sample to give a final 1x concentration (Sambrook and Russell, 2001a). Samples were then boiled at 100°C for 10 min to denature proteins and could be stored at -20°C until future SDS-PAGE or used immediately.

SDS-PAGE gel preparation and SDS-PAGE was carried out according to established protocol (Sambrook and Russell, 2001b) with 1.5 mm thick stacking gels containing 4% polyacrylamide and 1.5 mm thick separating gels 10% polyacrylamide. From 10 µg to 25 µg total protein was loaded onto the polyacrylamide gels in addition to 10 µl of the Kaleidoscope™ Precision Plus Protein™ Standard (Bio-Rad). SDS-PAGE was run at room temperature for approximately 1.5 h at 100 volts. Following SDS-PAGE, gels were either stained using 0.05% Coomassie Brilliant Blue or utilized in a Western blot transfer. Polyacrylamide gels were stained in 0.05% Coomassie Brilliant Blue according to established protocol (Sambrook and Russell, 2001c).

For Western blot analysis, a Novex Mini-Cell electrophoresis tank containing an XCell II™ blot module was utilized in the transfer (Invitrogen). Proteins separated by SDS-PAGE were transferred to a PolyScreen® polyvinylidene fluoride (PVDF) transfer membrane (PerkinElmer, Waltham, MA). The XCell II™ blot module was filled, from cathode to anode, as follows: two blotting pads, filter paper, SDS-PAGE gel, PVDF transfer membrane, filter paper and two more blotting pads. Blotting pads and filter paper were soaked in 1x transfer buffer (24 mM Tris base, 192 mM glycine, 20% methanol) prior to assembly in the blot module. The PVDF transfer membrane was soaked in 100% methanol for 30 sec, rinsed twice in deionized water and soaked in 1x transfer buffer for at least 5 min prior to assembly in the blot module. Transfer of protein on to a PVDF membrane ran for approximately 2 h at 30 volts. The inner compartment of the blot

module was filled with 1x transfer buffer while the outer reservoir contained 650 ml of deionized water. Following the transfer, the PVDF transfer membrane was blocked at 4°C in 200 ml of 3% skim milk overnight on an Orbitron Rotator II (Boekel Industries Inc., Feasterville, PA).

Subsequent to blocking the PVDF transfer membrane, Western blot analysis was performed. Following removal of the transfer membrane from the blocking solution, no washing steps occurred. The transfer membrane was rotated using a model 2000 Micro Hybridization Incubator (SciGene Corporation, Sunnyvale, CA) at room temperature with 3 ml of 3% skim milk containing the primary antibody in a 15 ml BD Falcon™ conical tube (BD Biosciences, Canaan, CT) for approximately 1.5 h. Primary antibody titres varied depending on the protein being probed and antibody being used. To assess MMP-25 levels, an anti-MMP-25 rabbit polyclonal antibody with an amino terminal epitope was utilized (1:2000; Santa Cruz Biotechnology Inc., Santa Cruz, CA). As a loading control, transfer membranes were probed with an anti-β-actin (1:1000; Santa Cruz Biotechnology Inc.) or anti-glyceraldehyde-3-phosphate dehydrogenase primary antibody (GAPDH; 1:20,000; Sigma-Aldrich). After incubation with the primary antibody solution, the transfer membrane was washed three times with 0.08% Tween-20 (Polyoxyethylene-Sorbitan Monolaurate) in 1x PBS for 15 min. each. Following the wash steps, the transfer membrane was rotated at room temperature for 1.5 h with 3 ml of 3% skim milk containing a goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:2500; Bio-Rad) as the primary antibody was. After incubation with the secondary antibody, the transfer membrane was again washed three times for 15 min. each with 0.08% Tween 20 in 1x PBS. Subsequent to this, the transfer membrane was

soaked for 30 seconds in 2 ml of a 1:1 mixture of enhanced luminol reagent and oxidizing reagent from the Western Lightning™ Chemiluminescence Reagent Plus kit (PerkinElmer). After contact with the chemiluminescent reagent, the transfer membrane was lightly blotted on a piece of filter paper, enclosed in plastic wrap and secured with adhesive tape into one corner of an X-Omatic® cassette (Kodak, Toronto, ON). In a dark room, the transfer membrane was exposed to X-Omat™ Blue XB-1 X-ray film (Kodak) for various time periods to best enhance specific binding bands and minimize background non-specific binding. Following exposure to the chemiluminescent transfer membrane, the X-ray film was developed at 32°C in an Optimax® X-ray film developer (Protec®, Oberstenfeld, Germany).

2.4 IHC with an MMP-25 Antibody

Embryos from CD-1 mice were aseptically removed at stages E12 through E15 and placed in 1x PBS. Heads from each stage were removed by cutting through the neck with micro-dissecting scissors to include the mandible and placed in freshly-prepared 4% paraformaldehyde at 4°C. E12 heads were maintained in 4% paraformaldehyde at 4°C for approximately 16 h; E13 heads for 18-20 h; E14 heads for 24 h and E15 heads for 24 h. Following preservation in 4% paraformaldehyde, the heads were placed in 30% sucrose at 4°C and could be maintained for several weeks by changing the 30% sucrose every week. Embryonic heads were embedded in Tissue-Tek® O.C.T. Compound (Sakura Finetek U.S.A., Inc.), wrapped in tinfoil to prevent drying and frozen overnight at -20°C. Coronal sections were cut (8 µm) and placed on glass microscope cover-slips covered in 0.5% gelatin (Sigma-Aldrich). IHC with an primary anti-MMP-25 antibody (1:50; Santa Cruz Biotechnology Inc.) was carried out as follows: two washes in 1x PBS for 30 min

each followed by a 20 sec exposure to pepsin digest-all solution (Zymed, San Francisco, CA), which was washed off by immersion in the 1x PBS for approximately three more minutes; blocking in blocking solution (3% skim milk, 0.1% Triton X-100 in 1x PBS) at room temperature for approximately 1.5 h; incubation with 100 µl of the primary antibody, made up in 1x PBS, for 2 h at room temperature followed by overnight at 4^{o}C; two washes in 1x PBS for approximately 5 min each; incubation with 100 µl of the Alexa Fluor® 594 goat anti-rabbit IgG secondary antibody (1:400; Molecular Probes, Eugene, OR), made up in 1x PBS, for approximately 2 h at room temperature in the dark; 30 µl of Hoechst nuclear stain for 15 min at room temperature in the dark; two washes in 1x PBS for approximately 5 min each; mounting onto glass microscope slides using ProLong® Gold antifade reagent (Invitrogen). IHC was evaluated by observing sections on an Olympus BX40 fluorescent microscope (Olympus America Inc., Center Valley, PA) at an emission wavelength of 613 nanometers. Images were taken using Image-Pro Plus (v. 6.2; MediaCybernetics, Inc., Bethesday, MD). Determination of non-specific staining was assessed by substituting 100 µl of 1x PBS in lieu of 100 µl of the primary antibody solution. All preceding and subsequent steps were carried out as described above.

2.5 *Mmp-25* Gene Knockdown using *Mmp-25* siRNA

A role for *Mmp-25* during SP development was determined using *Mmp-25*-specific siRNA to degrade *Mmp-25* mRNA transcripts and knockdown gene expression. *Mmp-25*-specific siRNA was added to the *in vitro* palate cultures described above in **2.2** at a final concentration of 500 nanomolar (nM; Shiomi et al., 2006). Gene knockdown cultures and analysis was carried out as previously mentioned in **2.2**. Stealth™ RNAi *Mmp-25* siRNA (Invitrogen) and *Trans*IT –TKO® transfection reagent (0.2%; Mirus Bio,

Madison, WI) were utilized. To assess non-specific siRNA effects on the *in vitro* palate cultures Stealth™ RNAi Negative Universal Control siRNA (Invitrogen) was added to some control cultures at a final concentration of 500 nM to match the *Mmp-25* siRNA.

2.6 Characterization of *Mmp-25* Gene Regulation

To investigate the regulation of *Mmp-25*, a promoter analysis was completed using available online software such as the Eukaryotic Promoter Database (EPD), the Homeodomain Resource and the Transcriptional Regulatory Element Database (TRED) (Appendix A). The DNA-binding sequences of common transcription factors were retrieved using the TRED and Homeodomain Resource and the *Mmp-25* promoter sequence from -1000 to +100 base pairs relative to the transcription start site (TSS) was retrieved from the EPD. The *Mmp-25* proximal promoter was examined by entering the consensus DNA-binding sequences of various transcription factors into the EPD. This examination of indicated *Mmp-25* could be regulated by Hoxa2 and TGFβ3, through its intracellular mediators Smad2/3 and Smad4.

To determine if the transcription factor, Hoxa2, is involved in the transcriptional regulation of *Mmp-25*, qRT-PCR was carried out on cDNA from wild-type and *Hoxa2*$^{-/-}$ E12.5 to E15.5 SPs according to manufacturer's protocol (Applied Biosystems) as described above. Statistical analysis of the RQ values obtained from qRT-PCR was carried out using Student's t-test ($p < 0.05$) on GraphPad Prism for Windows (v. 5.01; GraphPad Software Inc.).

Mmp-25 promoter analysis indicated *Mmp-25* could be regulated by the growth factor, TGFβ3. Since TGFβ3 is a well-characterized molecule that regulates many developmentally-important genes, this growth factor could be the chief regulator of

Mmp-25 expression and is one of my research hypotheses. To assess whether TGFβ3 affects *Mmp-25* transcription, a TGFβ3-neutralizing antibody (R&D Systems, Minneapolis, MN) was added to the *in vitro* SP cultures described above at final concentrations of 5 μg/ml, 10 μg/ml and 25 μg/ml, thereby eliminating this secreted growth factor from the cultures, with one alteration: SP cultures were incubated for 24 h, not 72 h (Yu et al., 2008). Following completion of organ cultures, qRT-PCR and Western blots were done to determine the effect of TGFβ3 on *Mmp-25* mRNA and protein levels respectively. To ensure an alteration in *Mmp-25* expression could be attributed to a drop in TGFβ3 levels, western blot analysis was carried out on the same PVDF membrane as the MMP-25 blot using an anti-phospho-Smad1 antibody (Cell Signaling Technology, Danvers, MA; 1:500).

2.7 Statistical Analysis

All statistical analysis performed on qRT-PCR data groups was either Student's t-test with $P < 0.05$ or one-way analysis of variance (ANOVA) with $P < 0.05$ also indicating significance. Statistical analysis of the *in vitro* palatal culture MFSs was carried out using the non-parametric Kruskal-Wallis test with $P < 0.05$ indicating significance. A non-parametric test was carried out on the palatal cultures MFSs as scoring did not follow a normal distribution due to rankings (Figure 6).

3. Results

3.1 Primer Design and Semi-Quantitative and Quantitative PCR

Semi-Quantitative PCR primers were designed to amplify a 295 bp deoxynucleotide fragment using the 3′ end of *Mmp-25* cDNA as a template. Semi-Quantitative PCR analysis of *Mmp-25* cDNA using the Mmp25a and Mmp25b primers (Table 1) indicates that *Mmp-25* mRNA is expressed at all stages of SP development, from E12 through E15 (Figure 7). Semi-Quantitative PCR does not properly show if *Mmp-25* mRNA expression during SP development is constitutive or if there might be up- and down-regulation of *Mmp-25* so qRT-PCR was done.

To determine if *Mmp-25* mRNA expression is constitutive or variable during SP development, qRT-PCR was carried out. Further examination using qRT-PCR indicates there is a significant down-regulation in *Mmp-25* mRNA expression at E15.5 relative to E13.5 using one-way ANOVA ($P < 0.05$; Figure 8A). As an ECM-degrading enzyme, a role for MMP-25 in SP development could be breaking down ECM barriers during palate shelf growth. By E15.5 the palate shelves have fused and the MES is largely degraded thus maintaining increased expression of *Mmp-25* is no longer necessary.

3.2 Western Blot Analysis with an MMP-25 Antibody

Total protein from E12.5 to E15.5 SP protein isolations was separated via SDS-PAGE and probed for MMP-25 protein presence. Western blot analysis indicates MMP-25 exists at approximately 55 kilodaltons (kDa) in the mouse SP throughout palatogenesis (Figure 8B). This is in agreement with previous reports of MMP-25 at 57 kDa in white blood cells and a colon cancer cell line (Kang et al., 2001; Kuula et al.,

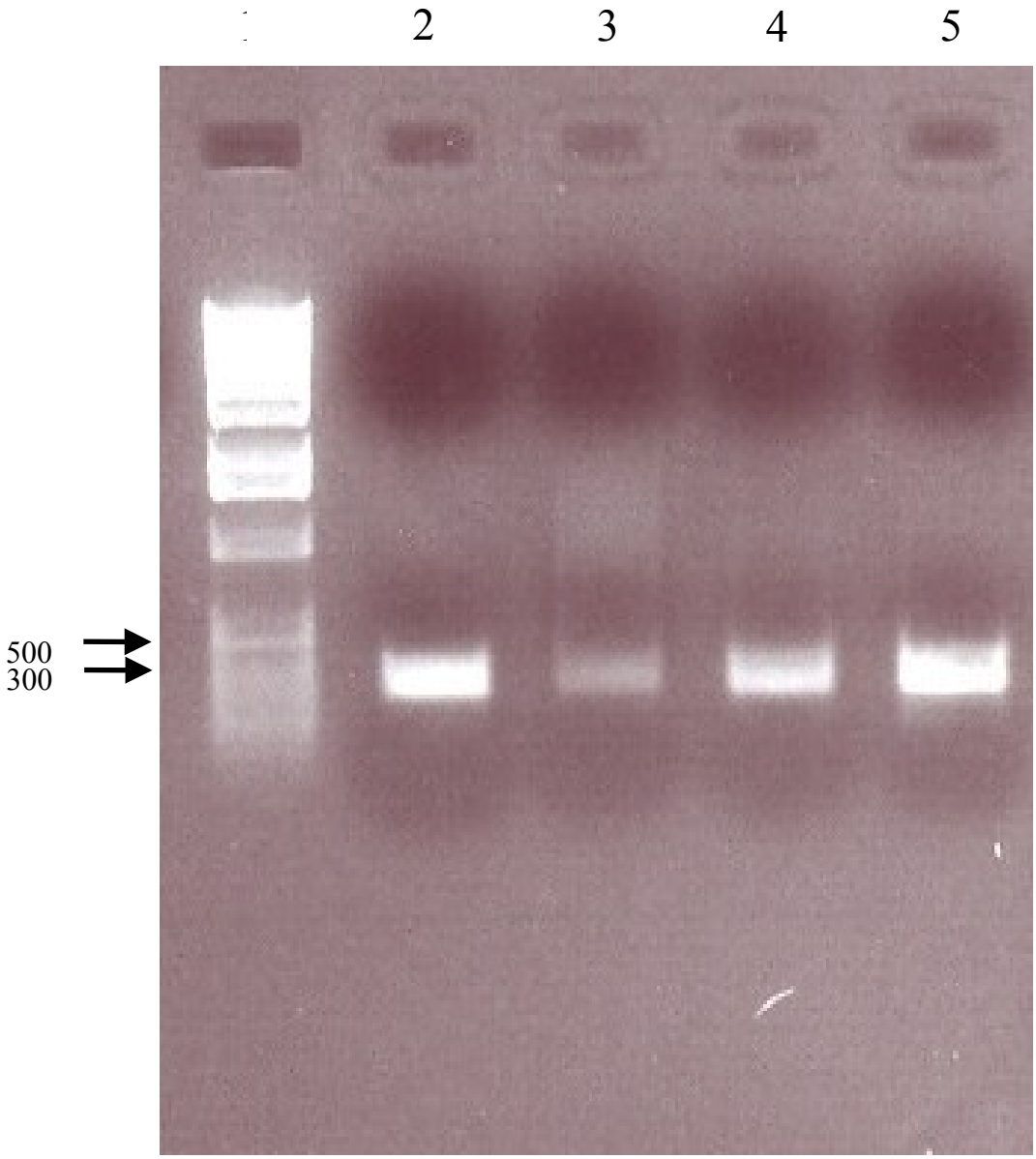


Figure 7: Polymerase chain reaction (PCR) and a 1% agarose gel confirm that *Mmp-25* messenger RNA (mRNA) is expressed in the mouse secondary palate (SP) from embryonic (E) days 12.5 through 15.5. Total RNA isolations were performed on mouse SP tissue from E12.5 to E15.5. Isolated RNA was reverse-transcribed into complementary DNA (cDNA). PCR was performed using the E12.5 through E15.5 cDNA as template. Expected fragment size was 295 base pairs if *Mmp-25* mRNA had been present and reverse-transcribed. Lane 1 represents 0.5 µg of Ready-Load™ 1 Kb DNA Ladder used to approximate the sizes of DNA bands on the gel (Invitrogen). Lane 2 represents E12.5. Lane 3 represents E13.5. Lane 4 represents E14.5. Lane 5 represents E15.5.

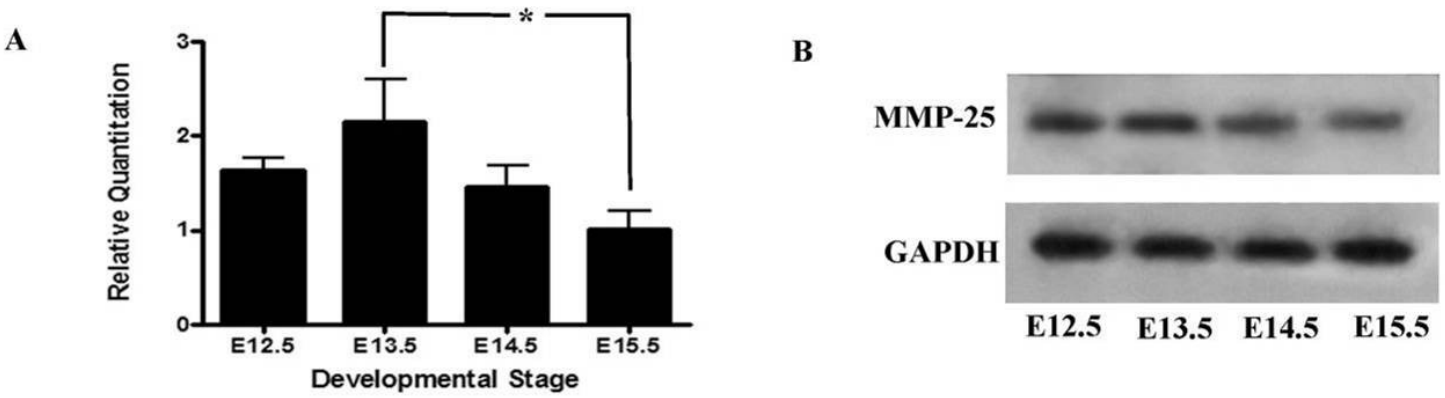


Figure 8: *Mmp-25* mRNA and protein expression. (A) quantitative real-time PCR displays significantly higher *Mmp-25* mRNA expression at embryonic day (E) 13.5 relative to E15.5 using one-way ANOVA ($P < 0.05$). N = 4 for each stage with four replicates within each N; error bars indicate standard error of the mean. (B) Western blot analysis shows MMP-25 protein levels parallel mRNA levels with higher protein expression at E12.5 and 13.5 when compared to E15.5. Approximately 20 µg of total protein was separated using 10% sodium dodecyl sulfate poly-acrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane and probed with an anti-MMP-25 antibody (Santa Cruz; 1:1000). An anti-GAPDH antibody (Sigma-Aldrich; 1:15,000) was used to assess loading equivalency.

2008; Sun et al., 2007). Western blots using an anti-GAPDH antibody (Sigma-Aldrich) indicate equivalent amounts of protein were loaded for MMP-25 Western blots.

3.3 Immunohistochemistry with an MMP-25 Antibody

IHC was carried out on 8 μm-thick coronal sections made from E12.5 to E15.5 mouse heads as detailed above. At all stages examined, MMP-25 expression is strong in the epithelium of the palate shelves and degrading MEE of later stages with secondary expression in the mesenchyme immediately adjacent to epithelium at E12.5 and E13.5 before becoming more dispersed in the mesenchyme at E14.5 and E15.5 (Figure 9). Expression of MMP-25 in the epithelium and mesenchyme at E12.5 and E13.5 appears to be strongest in the apical, or tip, area of the growing palate shelves; at E14.5 expression in the palate shelf epithelium seems strongest in the MEE and mesenchyme expression is less restricted to the apical portion of the palate shelves; at E15.5, as the MES is degrading, epithelial expression of MMP-25 is strongest in the oral epithelium with mesenchyme expression becoming stronger and more scattered throughout the SP.

3.4 *Mmp-25* Gene Knockdown using *Mmp-25* siRNA

To obtain a more objective measure of the *Mmp-25* siRNA effects, serial sections (8 μm) of preserved and frozen palatal cultures were generated. Every 8th section was examined by light microscopy and up to 15 sections were examined in total. Each section was given a score based on the completeness of fusion and quantity of remaining epithelium where 1 indicates no contact between the palate shelves and 5 indicates complete fusion (Kang and Svoboda, 2002). For each palatal culture thus examined the scores were combined and averaged to obtain a mean fusion score (MFS) and these MFSs

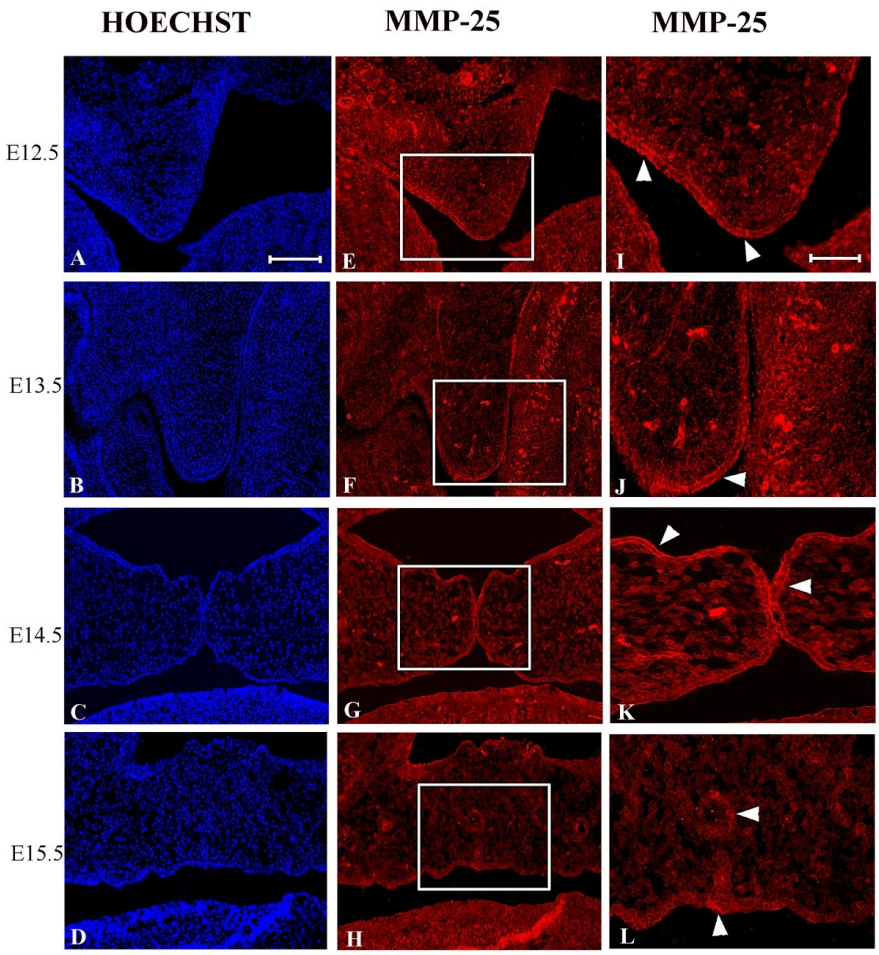


Figure 9: Immunohistochemical analysis of mouse secondary palate coronal sections from embryonic day (E) 12.5 to 15.5 with an MMP-25 antibody (Santa Cruz; 1:50). (A-D) Hoechst nuclear staining; scale bar = 10 μm. (E-H) Immunofluoresence shows MMP-25 is strongly expressed in the epithelium of the palate shelves throughout development. Expression in the mesenchyme is secondary to the epithelium and restricted to the apical area of the palate shelves at E12.5 and 13.5 before becoming more widespread at E14.5 and 15.5. (I-L) Increased magnification view of the boxes in E-H. Arrowheads indicate areas of high MMP-25 expression; scale bar = 5 μm

could themselves be averaged for an entire group. The results of the *in vitro* palatal cultures treated with 500 nM *Mmp-25*-specific siRNA are summarized in Table 2.

Table 2: Summary of results for *in vitro* palate cultures treated with *Mmp-25* siRNA

Condition	Total	MFS
Wild-type	10	4.14
Scrambled control siRNA	10	4.13
500 nM *Mmp-25* siRNA	10	2.50

The wild-type control palatal cultures (n = 10) examined had a cumulative MFS of 4.14 while the scrambled duplex siRNA negative control group (n = 10) had a MFS of 4.13. The palatal culture group treated with 500 nM *Mmp-25* siRNA (n = 10) had a MFS of 2.50, which is a significant decrease based on the Kruskal-Wallis test ($P < 0.05$). H&E staining showed wild-type palate cultures largely fused normally and had little to no MEE remaining after 72 h incubation (Figure 10A). Cultures treated with 500 nM control siRNA also fused normally and little remained of the MES after 72 h incubation. Cultures treated with 500 nM *Mmp-25* siRNA exhibited significantly decreased palate shelf fusion and persistence of the MEE after 72 h incubation. To verify the changes in the cultures could be attributed to a loss of *Mmp-25* expression, qRT-PCR and western blot analysis was performed on cDNA and protein from treated cultures and compared to the wild-type and negative control siRNA groups. Treatment with 500 nM *Mmp-25* siRNA resulted in significantly decreased levels of both *Mmp-25* mRNA (Figure 10D) and protein (Figure 10E). These results indicate a functional role for *Mmp-25* in mouse secondary palate formation.

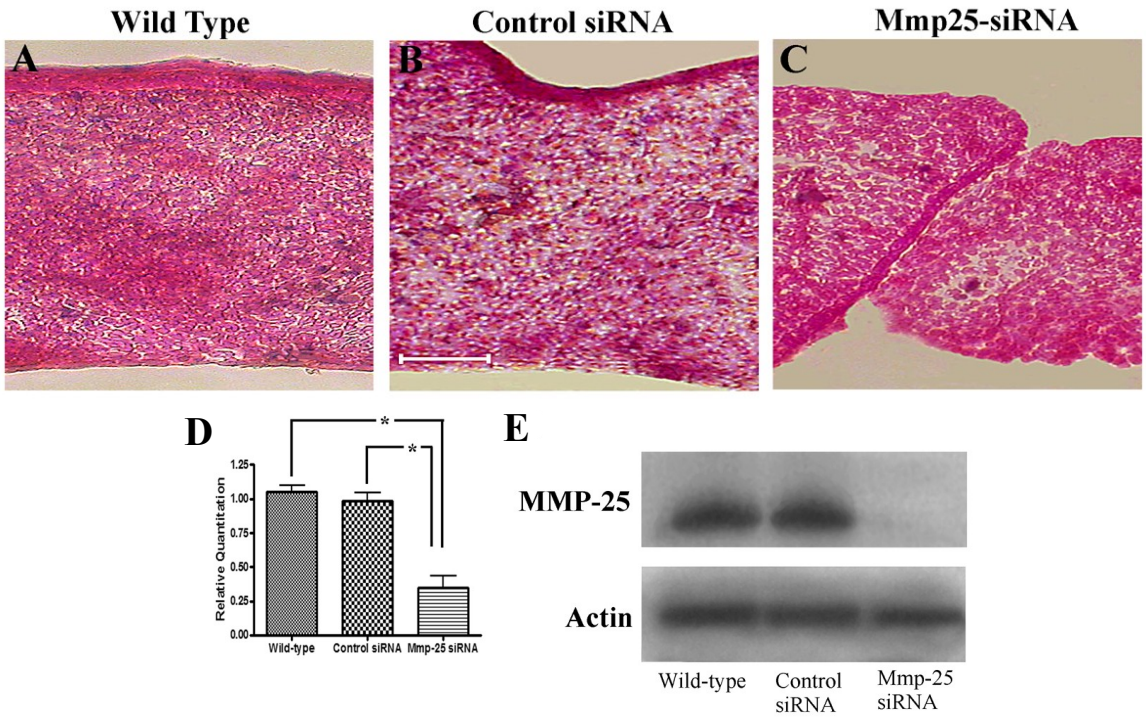


Figure 10: (A-C) Representative hematoxylin and eosin-stained E13.0 secondary palate sections (8 μm) following 72 h incubation. (A-B) Wild-type and control siRNA palate shelves fused normally; scale bar = 10 μm. (C) Palatal cultures treated with 500 nM *Mmp-25* siRNA displayed significantly decreased fusion and persistence of the medial edge epithelium. (D-E) Confirmation of *Mmp-25* expression knockdown in palatal cultures. (D) Quantitative real-time PCR confirms a significant *Mmp-25* mRNA knockdown in palatal cultures treated with 500 nM *Mmp-25* siRNA using one-way analysis of variance ($P < 0.05$); $n = 4$ for each group with four replicates per n. Error bars indicate standard error of the mean. (E) Western blot analysis demonstrates absence of MMP-25 protein in palatal cultures treated with 500 nM *Mmp-25* siRNA. Approximately 10 μg of total protein was separated via sodium dodecyl sulfate poly-acrylamide gel electrophoresis and probed with an anti-MMP-25 antibody (Santa Cruz; 1:1000). An anti-actin antibody was used to assess loading equivalence (Santa Cruz; 1:1000).

3.5 Characterization of *Mmp-25* Gene Regulation

Preliminary *Mmp-25* promoter analysis using the TRED, Homeodomain Resource and EPD indicates putative Smad-binding element (SBE) and Hoxa2 binding sequences in the proximal promoter of *Mmp-25* (Figure 11). Smad2/3 and Smad4 have been previously described to preferentially bind to the sequences 5'-GTCT-3' and 5'-CAGA-3' (Zawel et al., 1998; Jonk et al., 1998; Dennler et al., 1998). A Hoxa2 consensus binding sequence consisting of 5'-(T/A)GAT(T/G)GA(T/A)(G/C)(G/T/A)-3' has been previously described (Lampe et al., 2004).

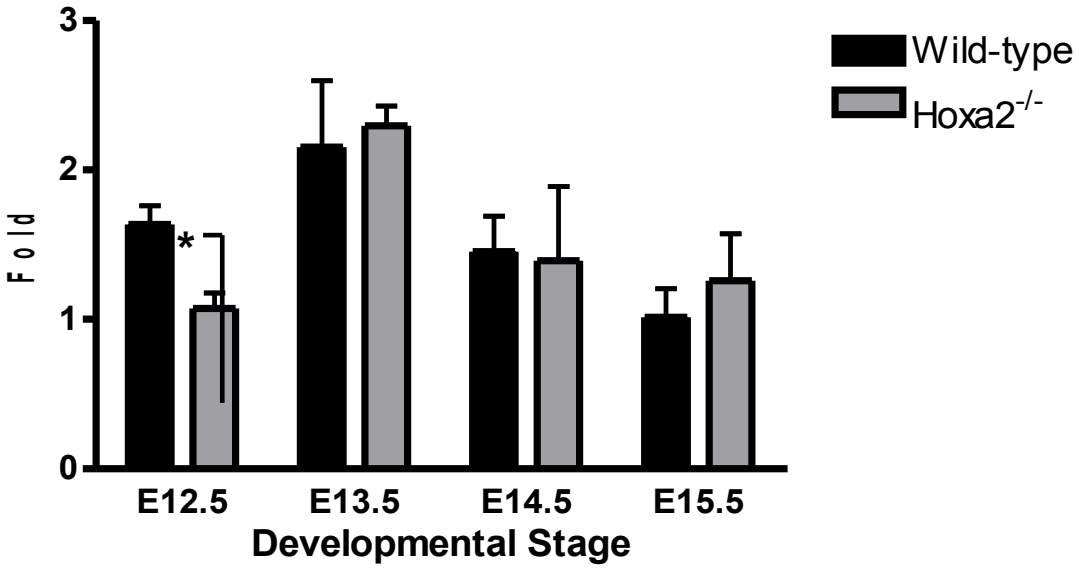
The *Mmp-25* proximal promoter contains a 5'-AGATGGGAACA-3' sequence from deoxynucleotide bases -472 to -482 that could be a binding sequence for Hoxa2. *Mmp-25* mRNA expression from E12.5 to E15.5 in the SPs of wild-type and *Hoxa2*$^{-/-}$ mice was analyzed using qRT-PCR. At E12.5 there is a significant down-regulation of *Mmp-25* mRNA expression in *Hoxa2*$^{-/-}$ mice, relative to the wild-type mice, before *Mmp-25* mRNA expression recovers (Figure 12). This significant difference between wild-type and *Hoxa2*$^{-/-}$ gene expression at E12.5 in the mouse SP is seen with several other genes (Smith and Nazarali, unpublished data).

Mmp-25 proximal promoter analysis reveals numerous repeats of 5'-GTCT-3' and 5'-CAGA-3', which are described as being SBEs (Figure 11; Zawel et al., 1998; Jonk et al., 1998; Dennler et al., 1998). As the Smad proteins are intra-cellular mediators of TGFβ3 signaling, *Mmp-25* could be transcriptionally regulated by TGFβ3. Treatment of *in vitro* palatal cultures with 5μg/ml, 10μg/ml or 25μg/ml of a TGFβ3-neutralizing

antibody revealed that *Mmp-25* is TGFβ3-regulated. *Mmp-25* mRNA is significantly decreased relative to wild-type levels when treated with 5μg/ml of neutralizing antibody

Figure 11: The *Mmp-25* proximal promoter sequence from -1000 to +100 base pairs relative to the transcription start site. Possible Smad-binding element sequences are highlighted in yellow and green; a possible Hoxa2 binding sequence is highlighted in grey. The transcription start site is underlined and bolded.

Figure 12: Quantitative real-time PCR of *Mmp-25* mRNA expression in the mouse



secondary palate of wild-type and Hoxa2-/- mice. *Mmp-25* mRNA levels are significantly decreased at embryonic day 12.5 in the Hoxa2-/- mice using Student's t-test ($P < 0.05$). N = 4 for all groups with four replicates per N; error bars indicate standard error of the mean as confirmed by qRT-PCR (Figure 13A); a sharp decrease in MMP-25 protein levels is also observed relative to wild-type (Figure 13B). There was no further decrease in *Mmp-25* mRNA or protein levels between the 5μg/ml, 10μg/ml or 25μg/ml treated groups. To assess the efficacy of the TGFβ3-neutralizing antibody treatments, western blot analysis was done on protein samples from all groups with an antibody against phospho-Smad1. Levels of phospho-Smad1 decreased as the concentration of the TGFβ3-

neutralizing antibody increased (Figure 13C). These results indicate *Mmp-25* expression is downstream of the growth factor, TGFβ3.

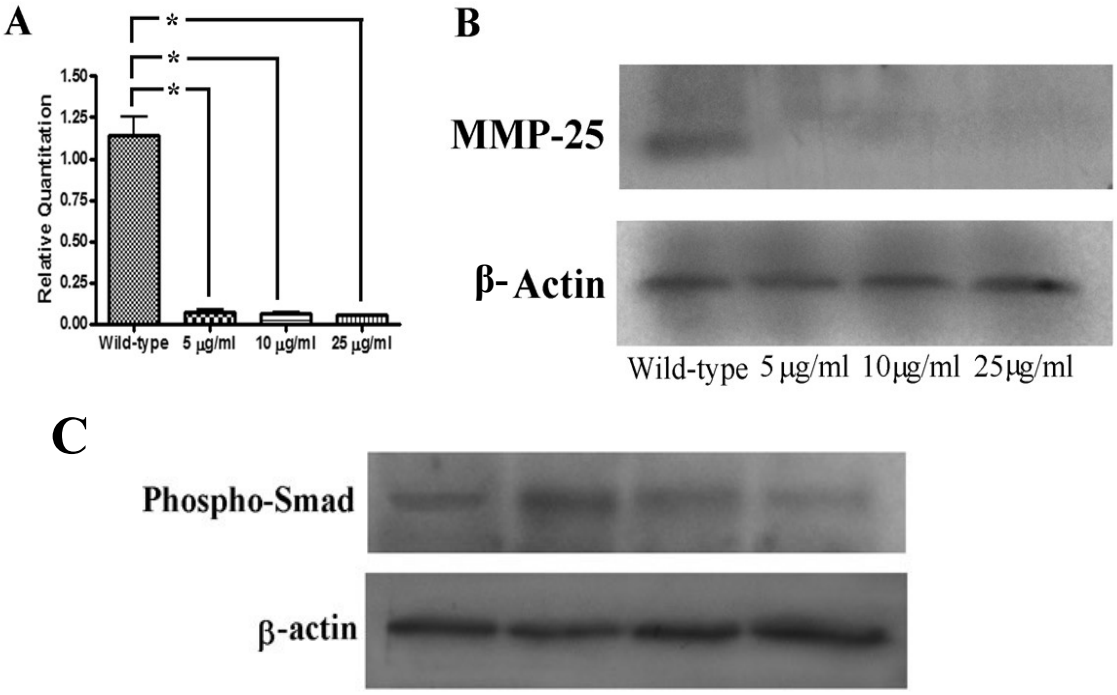

A
*
*
*
Relative Quantitation
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Wild-type
5 μg/ml
10 μg/ml
25 μg/ml
B
MMP-25
β-Actin
Wild-type 5 μg/ml 10μg/ml 25μg/ml
C
Phospho-Smad
β-actin

Figure 13: (A-B) Quantitative real-time PCR (qRT-PCR) and western blot analysis confirm *Mmp-25* mRNA and protein levels decrease after treatment with a TGFβ3-neutralizing antibody in embryonic day (E) 13.0 *in vitro* palatal cultures following 24 h incubation. (A) qRT-PCR shows a significant decrease in *Mmp-25* mRNA in *in vitro* palatal cultures using one-way analysis of variance following TGFβ3-neutralizing antibody treatment ($P < 0.05$). $N = 4$ for all groups with four replicates per N; error bars indicate standard error of the mean. (B) Western blot analysis shows a decrease in MMP-25 protein that parallels the drop in *Mmp-25* mRNA expression. Approximately 10 μg of total protein was separated via sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) and probed with an anti-MMP-25 antibody (Santa Cruz; 1:1000). An anti-β-actin antibody was used to assess loading equivalence (Santa Cruz; 1:1000). (C) Western blot analysis against phospho-Smad1 shows a TGFβ3-neutralizing antibody concentration-dependent effect, demonstrating the drop in *Mmp-25* mRNA and protein expression is due to a lack of TGFβ3 signaling. Approximately 10 μg of total protein was separated via SDS-PAGE and probed with an anti-phospho-Smad1 antibody (Cell Signaling Technology; 1:500). An anti-β-actin antibody was used to assess loading equivalence (Santa Cruz; 1:1000).

4. Discussion

4.1 *Mmp-25* mRNA and protein is expressed in the developing SP at all stages and has a functional role in mouse secondary palate development

Mmp-25 mRNA expression in the embryonic mouse SP at all developmental stages was confirmed through qRT-PCR (Figure 8A). Expression of *Mmp-25* mRNA rises from E12.5 to E13.5, where its expression is strongest, before decreasing at E14.5

and even further by E15.5. *Mmp-25* mRNA levels are significantly decreased at E15.5 relative to E12.5 and E13.5 ($P < 0.05$). At E12.5 and E13.5 the palate shelves are budding from the maxilla and growing downward alongside the tongue. At E15.5 the palate shelves have fused and the MES is degrading as the SP finishes forming. The increased expression of *Mmp-25* at E12.5 and E13.5 suggests a role for MMP-25 in promoting palate shelf growth as a slight decline in *Mmp-25* expression is seen as the shelves are fusing (E14.5) and a further significant decline as the MES is degrading (E15.5). Western blot analysis indicates MMP-25 protein expression levels parallel mRNA levels in the embryonic mouse SP (Figure 8B). MMP-25 appears as a band of approximately 55 kDa, which corresponds to previously published reports of a 57 kDa molecular weight for MMP-25 (Kang et al., 2001; Kuula et al., 2008; Sun et al., 2007).

Immunohistochemical staining indicates MMP-25 protein localizes more strongly to the epithelium of the palate shelves rather than the underlying mesenchyme (Figure 9). At E12.5 and E13.5 the strongest immunofluoresence can be detected in the epithelium of the palate shelves with secondary expression in portions of the mesenchyme immediately adjacent to the epithelium. These two stages represent continued palate shelf growth alongside the tongue before the shelves elevate and make contact along their medial edges. Secondary expression of MMP-25 protein in the mesenchyme appears to be much more apical on the leading edge of palate shelf growth rather than deeper into the interior of the palate shelf (Figure 9I, J). At E14.5 the palate shelves are fusing along their medial edges and immunofluoresence shows strong MMP-25 expression in the epithelium of the palate shelves, but secondary expression in the underlying mesenchyme appears much more scattered as opposed to the focused mesenchymal expression at E12.5 and E13.5

(Figure 9G, K). It is possible that this decrease in focused mesenchymal expression in the apical palate shelf is due either to the focus moving from growth to fusion in SP development at E14.5 or to the new horizontal orientation of the palate shelves, which still require some growth at E14.0 to make contact after elevation prior to fusion. At E15.5 the MES is degrading and there is little epithelium left between the two palate shelves. MMP-25 immunofluoresence at this stage is noticeably weaker than that from E12.5 to E14.5, but continues to be primarily epithelial as evidenced by the immunofluoresence of the oral epithelium and a degrading remnant of the MES (Figure 9H, L). This drop in MMP-25 immunofluoresence at E15.5 coincides with a confirmed drop in *Mmp-25* mRNA and protein levels, however mesenchymal expression appears to be stronger at E15.5 than any other stage and mesenchymal expression is more dispersed rather than localized to apical areas adjacent to epithelium as from stages E12.5 and E13.5 (Figure 9L). At all stages MMP-25 immunofluoresence in the SP localized to the area around the plasma membranes of the epithelial and mesenchymal cells, which is expected as MMP-25 is a MT-MMP (Kojima et al., 2000). The data collected supports the hypothesis that MMP-25 is expressed in the MEE, but seems to disagree with the hypothesis of MMP-25 having a role during degradation of the MES as IHC results point to decreased MMP-25 expression during this stage; however protein expression of MMP-25 is not significantly decreased at E14.5 relative to the highest levels at E13.5 so MMP-25 could still be involved in fusion of the palate shelves. The data indicating strongest MMP-25 protein expression in apical areas of palate shelf growth from E12.5 and E13.5 suggest MMP-25 plays a role in facilitating this growth by removing ECM barriers to increased cell proliferation and movement and, possibly, by activating pro-MMPs that

could do the same (Nie and Pei, 2003). Location and timing of the strongest immunofluorescence would suggest MMP-25 might be acting through its traditional role as a protease and removing ECM barriers to increased palate shelf growth.

To determine if MMP-25 is playing a functional role during mouse SP formation, *Mmp-25* mRNA expression was knocked down *in vitro* using *Mmp-25* siRNA and whole SP cultures as previously described (Shiomi et al., 2006; Nakajima et al., 2007). Addition of Stealth™ RNAi *Mmp-25* siRNA (Invitrogen) to a final concentration of 500 nM resulted in decreased palate shelf fusion and persistence of the MEE *in vitro* as evidenced by representative hematoxlyin and eosin-stained palatal sections (Figure 10A-C). Whereas the wild-type and negative control siRNA palatal cultures fused normally, the palatal cultures treated with *Mmp-25* siRNA seemed largely unable to progress to the fusion stage even though they were contacting one another. Interestingly, the phenotype of the *Mmp-25* siRNA-treated palatal cultures (Figure 10C) after 72 h incubation is very similar to the phenotype of other *in vitro* palatal cultures treated with a general chemical inhibitor of the MMPs (Blavier et al., 2001). To obtain a more objective measure of the *Mmp-25* siRNA effects, serial sections (8 μm) of preserved and frozen palatal cultures were generated. Every 8th section was examined by light microscopy and up to 15 sections were examined in total. Each section was given a score based on the completeness of fusion and quantity of remaining epithelium where 1 indicates no contact between the palate shelves and 5 indicates complete fusion (Kang and Svoboda, 2002). For each palatal culture thus examined the scores were combined and averaged to obtain a mean fusion score (MFS) and these MFSs could themselves be averaged for an entire group. The results of the *in vitro* palatal cultures are displayed in Table 2. The wild-type

control palatal cultures (n = 10) examined had a cumulative MFS of 4.14 while the scrambled duplex siRNA negative control group (n = 10) had a MFS of 4.13. This puts both groups solidly in the fusion category and shows absolutely minimal non-specific siRNA or transfection reagent effects. The palatal culture group treated with 500 nM *Mmp-25* siRNA (n = 10) had a MFS of 2.50, which is a significant decrease based on the Kruskal-Wallis test ($P < 0.05$). To ensure the drop in MFS could be attributed to an *Mmp-25* mRNA and subsequent protein knockdown, quantitative real-time PCR and western blot analysis was carried out on total RNA and protein isolated from the remaining palatal cultures of each group. The *Mmp-25* siRNA did knockdown both *Mmp-25* mRNA and protein levels efficiently. A significant decrease in *Mmp-25* mRNA levels in the *Mmp-25* siRNA-treated group was found relative to the control groups using qRT-PCR (Figure 10D; $P < 0.05$) and western blot analysis displayed a sharp reduction in MMP-25 protein levels in the treated group when compared to the control groups (Fig. 10E). Taken together, these data suggest MMP-25 does have a functional role in mouse SP development and validate my hypothesis that *Mmp-25* knockdown would significantly inhibit palate shelf fusion and cause the MEE to persist.

Overall, little is still known about MMP-25 developmentally or the relationship between the MMPs and palatogenesis. Expression of *Mmp-25* is highest at stages corresponding to palate shelf growth and immunofluoresence localizes this expression to apical epithelial and mesenchymal areas of the palate shelves. Between the epithelial and mesenchymal portions of the palate shelves lies the basement membrane (BM), in addition to the ECM surrounding these cells in all other dimensions. Cell proliferation and migration in the palate shelves is impossible with cells solidly adhering to the BM.

The primary structural component of the BM is type IV collagen, which is unique to the BM and provides a scaffold for other proteins within the BM (Kühn, 1995); MMP-25 cleaves type IV collagen very efficiently (English et al., 2001), but also fibrin and fibronectin, which are important BM proteins (Kang et al., 2001). Fibrin binds to and is assembled primarily by type IV collagen (Jones and Gabriel, 1988) so not only can MMP-25 cleave fibrin, but loss of type IV collagen through MMP-25 cleavage will further weaken the BM through loss of an anchoring point for fibrin assembly. Fibronectin is a key BM protein that binds to α5β1-integrin embedded in the plasma membranes of epithelial cells in apical areas of the palate shelves (Martìnez-Sanz et al., 2008). It is possible MMP-25 targets fibronectin for degradation in the apical epithelium during palate shelf growth or expression of *Mmp-25* depends on fibronectin/integrin binding. Expression of MMP-13 and MMP-14 is dependent on α1β1-integrin in endochondral development (Ronzière et al., 2005) and α5β1-integrin in joint cartilage formation (Forsyth et al., 2002) while MMP-14 expression is downstream of both fibronectin and α4/α5β1-integrin dimers (Manduca et al., 2009), but nothing is currently known about the role of integrins or fibronectin in modulating expression of *Mmp-25* in general or in SP formation. The strongest evidence to support a role for MMP-25 in facilitating palate shelf growth comes from MT-MMP knockout mouse models.

Mmp-14$^{-/-}$ (MT1-MMP) mice have significant developmental defects related to a decrease in skeletal cell proliferation caused by an inability of bone-specific cells to reach sites of proliferation (Holmbeck et al., 1999; Zhou et al., 2000). *Mmp-16*$^{-/-}$ (MT3-MMP) mice are largely normal, but *Mmp-16*$^{-/-}$ mice that are also *Mmp-14*$^{-/-}$ exhibit significant skeletal defects similar to those of the *Mmp-14*$^{-/-}$ mice; the double knockout mice also

develop a complete CP, which does not occur in the single *Mmp-14*$^{-/-}$ mice (Shi et al., 2008). Improper SP development in the double knockout mice was the result of impaired palate shelf growth due to a decrease in mesenchymal cell proliferation, although the tiny palate shelves elevated normally (Shi et al., 2008). This is significant evidence to support my contention that the MT-MMPs are indispensable to palate shelf growth by removing ECM barriers to proliferation. A lack of CP in *Mmp-14*$^{-/-}$ mice could be due to MMP-25 functionally substituting for MMP-14 in the SP just as MMP-14 can substitute for MMP-16 in SP development (Shi et al., 2008).

4.2 *Mmp-25* is a downstream transcriptional target of TGFβ3

It has been known for some time that the secreted growth factor, TGFβ3, is vital to the successful formation of the mouse SP (Kaartinen et al., 1997; Taya et al., 1999). TGFβ3 exerts biological activity through its receptors, which are tyrosine kinases, and intra-cellular mediators, the Smad proteins, which translocate to the nucleus affecting gene transcription following phosphorylation (Chai et al., 2003). A brief examination of the proximal promoter sequence of *Mmp-25* obtained from the EPD displayed repeating sequences of 5′-GTCT-3′ and 5′-CAGA-3′, which have been demonstrated to be sites of preferential binding by the Smad proteins (Dennler et al., 1998; Jonk et al., 1998; Zawel et al., 1998). Because of their existence just upstream of the *Mmp-25* transcription start site, it was hypothesized *Mmp-25* could be a transcriptional target of TGFβ3 signaling. A TGFβ3-neutralizing antibody (R&D Systems) was added to *in vitro* palatal cultures to final concentrations of 0 μg/ml (wild-type control), 5 μg/ml, 10 μg/ml and 25 μg/ml and incubated for 24 h. Following incubation, qRT-PCR and western blot analysis was

carried out on total pooled RNA and protein isolated from each group. Preliminary analysis into a link between TGFβ3 and *Mmp-25* showed *Mmp-25* mRNA and protein levels are significantly decreased ($P < 0.05$) following 24 h incubation with 5 μg/ml of a TGFβ3-neutralizing antibody (Figure 13A,B). There was no significant difference in *Mmp-25* mRNA or protein levels between the groups treated with different concentrations of the TGFβ3-neutralizing antibody. To make sure the decrease in *Mmp-25* mRNA and protein could be attributed to a loss of TGFβ3 activity in the cultures, western blot analysis was carried out on total protein isolated from the four groups with a phospho-Smad1 antibody. Levels of phospho-Smad1 were inversely correlated with the concentration of the TGFβ3-neutralizing antibody; as the bioneutralizing antibody concentration went up, phospho-Smad1 levels went down (Figure 13C). A link between the MMPs and TGFβ3 is not without precedent; MMP-13 expression has been demonstrated to be downstream of TGFβ3 (Blavier et al., 2001) as has expression of the gelatinases, MMP-2 and -9 (Greene et al., 2003). Expression of MMP-9 can also be suppressed by TGFβ3 via a Smad7/NF-κB-dependent pathway (Ogawa et al., 2004). Mice, which are *Tgfβ3*$^{-/-}$, have smaller palate shelves that elevate, but never grow together and make contact along their medial edges (Kaartinen et al., 1997). These data suggest MMP-25 is a direct transcriptional target for TGFβ3 in the mouse SP and support my final hypothesis that *Mmp-25* would be transcriptionally regulated by TGFβ3.

5. Future Directions

The development of an *Mmp-25*$^{-/-}$ mouse will be a huge step toward uncovering the role of MMP-25 in mouse embryonic development. It is certainly possible that *Mmp-25*$^{-/-}$ mice will have similar, post-natal lethal developmental defects that *Mmp-14*$^{-/-}$ mice

display. Regarding SP development, generation of *Mmp-25*$^{-/-}$ mice will allow an *in vivo* examination of the role of MMP-25 in palate shelf growth and whether alternate MT-MMPs can functionally substitute partially or wholly for MMP-25 in SP development. This is work not possible with an *in vitro* palatal culture system. Another possible avenue is an identification of MMP-25 substrates in the mouse SP. Is MMP-25 cleaving type IV collagen, fibronectin or fibrin in the BM? These are known substrates in other tissues, but may not be in the SP. Histological analysis and comparison of the developing SP between wild-type and *Mmp-25*$^{-/-}$ mice could determine whether MMP-25 is cleaving these substrates.

MMP-25 is efficiently inhibited by TIMP-2 and -3 and strongly inhibited by TIMP-1 (English et al., 2001). Does increased *Mmp-25* expression during SP development positively correlate with increased expression of one or more of the TIMPs? Is there any manner of temporal or spatial regulation in TIMP expression in the SP as it forms? Future work regarding MMP-25 in the SP could be directed at the relationship between these TIMPs and MMP-25 during SP development.

An association between *Mmp-25* gene transcription and TGFβ3 has been established. This link could be further strengthened by work demonstrating direct association between Smad2/3 and Smad4 and the *Mmp-25* promoter such as chromatin immunoprecipitation or electrophoretic mobility shift assays. A more in-depth examination of the *Mmp-25* promoter could identify additional regulatory molecules. Cloning of the *Mmp-25* promoter and insertion into an expression construct upstream of a reporter gene could help verify if the molecules identified in the promoter examination are actually controlling *Mmp-25* expression.

6. Conclusions

MMP-25 mRNA and protein is expressed in the developing mouse SP and protein expression largely localizes to the epithelium of the palate shelves. Expression of MMP-25 mRNA and protein is highest at E13.5 and there is a significant decrease in mRNA levels by E15.5. Immunohistochemical analysis of MMP-25 protein expression has shown MMP-25 protein is most abundant in the epithelium of the palate shelves with secondary expression in the underlying mesenchyme. Expression in the mesenchyme occurs in apical areas adjacent to the over-lying epithelium at E12.5 and E13.5, but becomes much more scattered by E14.5 and E15.5.

Through use of palatal cultures, it has been shown treatment with an MMP-25-specific siRNA decreases palate shelf fusion and results in significant persistence of MEE *in vitro*. This is the first evidence to support a functional role for a single MMP in mouse SP development. This *in vitro* palatal culture work coupled with the immunohistochemical and real-time PCR data suggests MMP-25 plays a key role in palate shelf growth at E12.5 and E13.5 by removing ECM barriers that would hinder cell proliferation and movement. The substrates of MMP-25 include type IV collagen, fibronectin and fibrin, which are all components of the BM and barriers to cell proliferation and movement; immunohistochemical analysis of MMP-25 protein expression in colorectal tissue sections shows MMP-25 is strongly expressed on the leading edge of tumours and positively correlates to an invasive cancer. These results indicate MMP-25 protein expression in the developing mouse SP is also strongest on the apical, or leading, edge. Most compellingly, double knock-out of MMP-14 (MT1-MMP) and MMP-16 (MT3-MMP) results in a cleft palate in mice due to impaired growth of the

palate shelves, which implies a role for proteolytic enzymes in mouse SP development even though there were no palate defects reported for the single gene knock-out of MMP-16 or MMP-14. These results indicate MMP-25 is a key proteolytic enzyme among the MMPs during mouse SP formation.

In addition to the results on MMP-25 gene expression and functional importance during mouse SP development, a link between MMP-25 and TGF-β3 in the developing mouse SP has been established; this is the first research to detail the transcriptional regulation of *Mmp-25*, however it is preliminary and warrants further investigation. Treatment of whole palatal cultures with a TGF-β3-neutralizing antibody in the culture medium resulted in a significant decrease in MMP-25 mRNA and protein. Given the steep decrease in both mRNA and protein levels, it would seem that MMP-25 is a direct target of TGF-β3 during mouse SP development.

Overall, nothing is known about the role of *Mmp-25* in embryonic development. This work represents the first research into a developmental role for MMP-25, in this case the mouse SP. These results are compelling and need further research to more clearly outline the role of MMP-25 in SP development.

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Appendix A

PCR Primer Design and Analysis Software

Primer3: http://frodo.wi.mit.edu/
NetPrimer: http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html

Multiple Sequence Alignment Software

ClustalW: http://www.ebi.ac.uk/clustalw/
BLASTn: http://www.ncbi.nlm.nih.gov/BLAST/

Promoter Analysis Software

Eukaryotic Promoter Database: http://www.epd.isb-sib.ch/

Homeodomain Resource: http://research.nhgri.nih.gov/homeodomain/
TRED: http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home

Appendix B

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On Mar 31, 2009, at 12:15 PM, Graham Brown wrote:

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>
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> cleft palate research. I saw the excellent Figure 1 from your paper
> "New insights into craniofacial morphogenesis" published in
> Development
> in 2005 and was wondering if I might be able to use it in my thesis to
> illustrate development of the face and head.
>
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Hi Graham,

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Good luck with your thesis!

-Renee

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Renee Prink
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On 3/25/09 3:55 PM, "Pam Spankowski" <pspankowski@chw.org> wrote:

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From: Graham Brown
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