DECORIN AND BIGLYCAN EXPRESSION AND DISTRIBUTION DURING PALATAL SHELF ADHESION

A Thesis

by

ISRA M. IBRAHIM

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee,	Kathy Svoboda
Committee Members,	Louis-Bruno Ruest
	Xiaofang Wang
Head of Department,	Paul Dechow

May 2015

Major Subject: Oral Biology

Copyright 2015 Isra M. Ibrahim

ABSTRACT

Understanding the molecular events in palate development is a prerequisite to more effective treatments of cleft palate. The secondary palate in humans and mice forms from shelves of mesenchyme covered with medial edge epithelium (MEE). These shelves adhere to form the midline epithelial seam (MES). MES cells then proceed through epithelial to mesenchymal transition (EMT) and/or apoptosis to yield a fused palate. Adhesion of opposing MEE is a crucial event whose alteration causes cleft palate. Previous studies showed that chondroitin sulphate proteoglycans (CSPG) on the apical surfaces of MEE was an important factor in palatal shelf adhesion. In this study we investigated decorin and biglycan, being expressed in numerous craniofacial tissues, as potential proteoglycans involved in palatal shelf adhesion.

We used a laser capture microdissection (LCM) technique to collect MEE cells and real-time polymerase chain reaction, to determine mRNA levels of decorin and biglycan that correctly reflect changes in gene expression during various stages of palatal shelf fusion (Embryonic days 13.5, 14.0 and 14.5). Both decorin and biglycan were expressed on the apical surface as well as between the MEE cells. We found that biglycan protein and mRNA levels peaked as the palatal shelves adhered. Decorin on the other hand was less abundant on the surface and had reduced mRNA levels that might be due to the regulatory effects of TGFβ. Nevertheless, the temporal expression of both decorin and biglycan on the apical surface of MEE was suggestive of an important role in palatal adhesion.

DEDICATION

This Thesis is dedicated to my husband and friend Mohamed for all the support and love and also to my wonderful children Muneeb and Mumin.

I also dedicate this work and to my parents and sisters for the constant encouragement and prayers.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my committee chair and primary advisor Dr Kathy Svoboda for all the guidance in this research and my future academic life. I would like also to thank my committee members Dr Louis-Bruno Ruest and Dr Xiaofang Wang for their valuable feedback.

Special thanks to Dr Phillip Kramer, Dr Lynne Opperman and Dr Jay Groppe. I would like also to thank all who helped me making this research possible including my dear friend Maria Serrano, Lee Jordan, Rene, Chi Ma, Yan Jing, Shaun Logan, Crystal Stinson, Connie Tillberg and Priyam Jani.

Last but not the least, I would also like to convey my appreciation to Priscilla Hooks and Gerald Hill for their great help in the ARU. Thanks also go to my friends from the Graduate student association and Muslim student association for making my time at Baylor College of Dentistry, Texas A&M University a great one.

NOMENCLATURE

BGN	Biglycan
CDC	Centers for Disease Control and Prevention
CSPG	Chondroitin sulphate proteoglycan
DCN	Decorin
E 13.5	Embryonic day 13.5
EMT	Epithelial to mesenchymal transformation
ECM	Extracellular matrix
GAG	Glycosaminoglycan
GD	Gestational day
LCM	Laser capture microdissection
LLR	leucine-rich repeats
MEE	Medial edge epithelia
MES	Midline epithelia seam
mRNA	Messenger RNA
SLRP	Small leucine-rich proteoglycan
TGF-β	Transforming growth factor β

TABLE OF CONTENTS

Page

ABSTRACT ii

DEDICATION iii

ACKNOWLEDGEMENTS iv

NOMENCLATURE v

TABLE OF CONTENTS vi

LIST OF FIGURES vii

LIST OF TABLES viii

CHAPTER I INTRODUCTION 1

CHAPTER II MATERIALS AND METHODS 7

Animals and dissection 7
Cryoembedding and cryosectioning 7
Staining and laser capture microdissection 8
Isolation and characterization of RNA from LCM-procured cells 8
Real-time PCR analysis 8
Decorin and biglycan immunohistochemistry 9

CHAPTER III RESULTS 10

Decorin and biglycan quantitative real-rime PCR 10
Expression pattern of DCN and BGN in the palatal shelf epithelia 12

CHAPTER IV DISCUSSION AND CONCLUSION 17

REFERENCES 24

LIST OF FIGURES

Page

Figure 1 E14.5 palatal shelves immunolabeled with anti-CSPG monoclonal antibody.. ..3

Figure 2 Biglycan mRNA relative expression during far, close and fused palatal shelves......11

Figure 3 Decorin mRNA relative expression during far, close and fused palatal shelves......12

Figure 4 Confocal images of anti-BGN and anti-DCN immunolabeled far coronal palate sections......13

Figure 5 Confocal images of anti-BGN and anti-DCN immunolabeled approaching palate sections.. 14

Figure 6 Confocal images of anti-BGN and anti-DCN immunolabeled close palate sections 16

Figure 7 Confocal image of anti-BGN immunolabeled close palate section without the nuclear counterstaining. 16

LIST OF TABLES

Page

Table 1 Classes and structure of small leucine-rich repeat proteoglycan (SLRP) family members.. 4

CHAPTER I

INTRODUCTION

Cleft lip and cleft palate are among the most common craniofacial birth defects. The Centers for Disease Control and Prevention (CDC) estimates that each year in the United States, about 2,650 babies are born with a cleft palate and 4,440 babies are born with a cleft lip with or without cleft palate [1]. The management of cleft lip/palate requires an integrative multidisciplinary approach involving surgery, extensive dental treatment and speech therapy. The treatment thus, has a substantially elevated medical care costs.

The process of palatogenesis is similar in mice and human beings. The process starts at E11.5 (week 6-7 of human fetal development) when the secondary palate arises as paired outgrowths form the maxillary process. They initially grow vertically and undergo palatal shelf elevation above the dorsum of the tongue (E13.5). After elevation, the paired palatal shelves grow towards the midline (E.14.0). The medial edge epithelium (MEE) that covers the shelf tip adheres forming the midline epithelial seam (MES) (E 14.5, week 9-10 of human fetal development). This is followed by disappearance of the MES by epithelial to mesenchymal transition (EMT) and/or apoptosis to allow mesenchymal confluence (E15.5) [2]. By E17 (week 12 of human fetal development) the palatine bone has formed and the development of the palate is complete. Adhesion of MEE of the opposing palatal shelves is a critical step, failure of which will result in cleft palate.

Immediately before adhesion changes occur in the superficial MEE cells to favor the contact of palatal shelves. It becomes multilayered [3]. In addition its most apical cells bulge and develop filopodia and microvilli [4, 5]. Furthermore, its apical extracellular matrix composition varies by synthesizing adhesion associated molecules such as fibronectin, vinculin [6] and chondroitin sulphate proteoglycans [7]. Even though palatal shelf adhesion is an essential event whose alteration causes cleft palate, it was investigated by only few studies [6].

The formation of the secondary palate is a complex process in which many factors are involved: transcription factors, cytokines, growth factors, and extracellular matrix molecules including proteoglycans [8]. Proteoglycans (PGs) are molecules composed of a specific core protein covalently linked to glycosaminoglycan (GAG) chains. The point of attachment is a Serine residue to which the glycosaminoglycan is joined through a tetra saccharide bridge to the core protein. They commonly mediate the interactions of ECM components with growth factors and cytokines[9]. Chondroitin sulphate proteoglycans (CSPG) are involved in important cell processes, such as cell adhesion, cell growth, receptor binding, cell migration and interaction with other extracellular matrix molecules. A previous study demonstrated that a CSPG coat on the apical surfaces of MEE is an important factor in palatal shelf adhesion [10]. Not only does it appear on the apical surface of MEE cell immediately before palatal shelves contact (Figure 1) [7], but also a reduction in CSPG alters palatal shelve adhesion [7]. In addition, CSPG expression is controlled by TGF-β_3, a growth factor that plays an important role in triggering palate shelf adhesion and fusion [7, 11]. Because the

monoclonal antibody used in that study was an anti-chondroitin sulphate antibody, the core protein associated with CSPG on the apical surfaces of MEE was not identified.

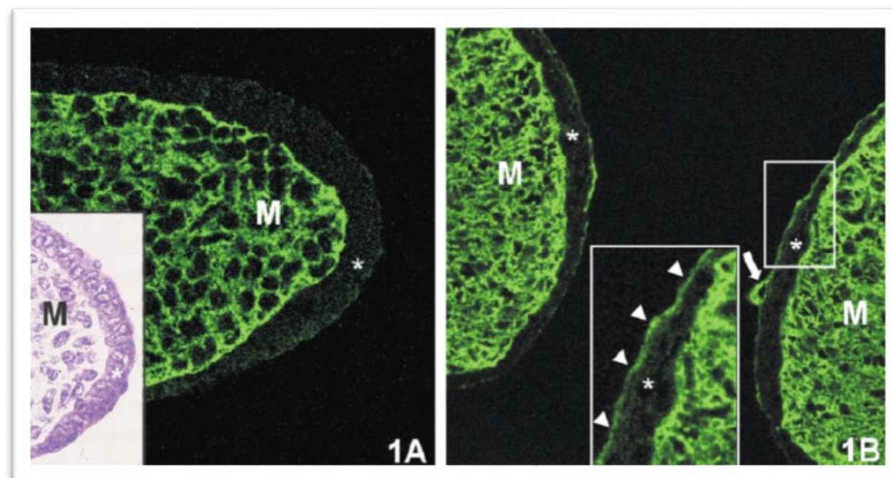

Figure 1 E14.5 palatal shelves immunolabeled with anti-CSPG monoclonal antibody. (A) Palatal shelves far from each other. (B) As palatal shelves approach each other. Source: Gato, A. et al, 2002 [7].

Small leucine-rich repeat proteoglycans (SLRPs) is a family of structurally related proteoglycans. Members of this family are characterized by a small protein core, consisting predominately of leucine-rich repeats (LRR). The LRR motif mediates

protein-protein interactions and is accountable for the many interactions that define their function [12] . The family is divided to five classes depending on genomic organization and protein structure (Table 1).

Table 1 Classes and structure of small leucine-rich repeat proteoglycan (SLRP) family members

Class	SLRP member	Number of LRR	GAG type
I	Decorin, Biglycan, Asporin, ECM2	12	CS/DS
II	Fibromodulin, Lumican, PRELP, Keratocan Osteomoduli	12	KS
III	Epiphycan, Opticin, Osteoglycin	8	DS & KS
IV	Chondroadherin, Nyctalopin, Tsukushi	12	Not Examined
V	Podocan, Podocan-like protein	20	Not Examined

CS, chondroitin sulfate; DS, dermatan sulfate; KS, keratan sulfate. (Dellett et al, 2012).

Biglycan and decorin belong to class 1 of this family and have either one (decorin) or two (biglycan) small chondroitin/dermatan sulphate side chains. The expression of decorin and biglycan overlaps in both skeletal and non-skeletal tissues [13]. In the craniofacial region, biglycan and decorin are present in various tissues including cranial sutures [13], teeth [14], periodontal tissue [15, 16], temporomandibular joint [17], palate epithelia and mesenchyme during palatogenesis [8]. Decorin null mice show an abnormal skin fragility due to reduced tensile strength [18].The collagen fibers of the null animal are both loosely packed and exhibit irregular contours. Biglycan null animals show reduced bone mass and osteoporosis-like phenotype [19]. BGN/DCN double deficient mice have a further severe phenotype in both long bones and skin [20] compared to the wild type or single deficient mice, suggestive of functional compensation. The double knockout models the phenotype of Ehlers-Danlos syndrome in humans. It is an inherited disease that affects connective tissue and characterized by reduced tensile strength and integrity of skin, joints, and other structures. The progeroid variant of the disease results from mutation of the enzyme necessary for the posttranslational glycosylation of biglycan and decorin, resulting in the secretion of abnormal protein cores [21].

Both decorin and biglycan have a broad binding repertoire, they can bind other ECM molecules such as collagens [18, 22] where they have an organizing function on the assembly of the extracellular matrix. Furthermore, they are important players in cell signaling capable of affecting cellular functions such as proliferation, differentiation, adhesion and migration [23].They can bind and modify the bioactivity of growth factors,

particularly those that belong to the transforming growth factor-β ligand super family [16, 24, 25]. Despite their presence in the palatal tissue during palatogenesis, nothing is known about their function and role in palatal shelf adhesion. Our observations lead us to hypothesize that decorin and biglycan, in participation with other proteoglycans, play an essential role in palatal shelf adhesion.

To investigate this hypothesis we utilized Laser Capture Microdissection (LCM). The use of LCM allows to successfully isolate medial edge epithelia (MEE) cells of the fetal palatal shelves [26, 27]. We processed LCM–procured MEE cells for isolation of BGN and DCN mRNA during various stages of palate development for quantitative analysis using real-time PCR. The sensitivity and accuracy of the technique had been verified [26-28]. Furthermore, we performed immunohistochemical analysis to evaluate the distribution of BGN and DCN in the developing palates.

In this study, we were able to identify a function for BGN and DCN in the palatal adhesion process. Our results provide evidence that they are expressed in a time-based manner in the palatal epithelia during development.

CHAPTER II

MATERIALS AND METHODS

Animals and dissection

Timed-pregnant CD-1 mice (Charles Rivers Laboratories,Inc) were used for these studies. Pregnant mice of the appropriate gestation age (GDs 13.5-14.5) were euthanized by Isoflurane inhalation followed by cervical dislocation. Fetuses were removed by caesarian section and placed into cold phosphate buffer saline (PBS). Under sterile conditions, fetuses' mandibles were removed. To preserve RNA quality all procedures were performed as quickly as possible. Fetuses intended for immunohistochemical analysis were fixed in Carnoy's fixative for one hour and allowed to sink in 30% sucrose overnight.

Cryoembedding and cryosectioning

Fetuses were mounted in OCT embedding medium (Tissue-Tek), snap frozen using liquid nitrogen and stored at -80°C until sectioning. Before sectioning, the blocks were equilibrated to the cryostat temperature (-20°C) for 15 min. Fetuses were sectioned in the coronal plane to a thickness of 8 μm and mounted onto HistoBond slides for immunohistochemical analysis or PEN membrane slides (Leica) for LCM. PEN membrane Slides were stored at -80°C in slide boxes with Drierite dessicant.

Staining and laser capture microdissection

Immediately before performing LCM, sections were stained with Histogene LCM Frozen Section Staining Kit (Arcturus) to identify the palatal epithelia, following manufacture's protocol. Stained dehydrated sections were then immediately processed for LCM. Using the built-in software of the Leica LMD 7000 (Leica Microsystems) cells of the palatal MEE were laser-captured and allowed to drop into a sterile PCR collecting tube cap containing 50µl of lysis buffer.

Isolation and characterization of RNA from LCM-procured cells

LCM-procured MEE cell from an average of 20-25 sections at a single stage of differentiation were pooled. Total RNA was extracted using RNAqueous Micro kit (Ambion/Life Technologies), sample were treated with DNase I to remove genomic DNA contamination. Extracted RNA quality and quantity was evaluated using NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific).

Real-time PCR analysis

To synthesize complementary DNA (cDNA) from RNA, the iScript™ Reverse

Transcription Supermix for RT-qPCR (Bio-Rad Laboratiries, Inc.) was used. Due to the low number of cells in the MEE collected by LCM, a preamplification step was used following cDNA synthesis and before real-time PCR. A matched pool of BGN, DCN and GAPDH PrimePCR preamplification assays (Bio-Rad Laboratiries, Inc.) combined with SsoAdvanced™ PreAmp Supermix (Bio-Rad Laboratiries, Inc.) was used for this

step, as described by the manufacturer. Real-time PCR was undertaken on CFX96 Optics Module with the gene specific primer sets (Bio-Rad Laboratiries, Inc.). Data was analyzed using Bio-Rad CFX Manager 3.1 software.

Bgn: Unique Assay ID: qMmuCID0014565.

DCN: Unique Assay ID: qMmuCID0039628.

GAPDH: Unique Assay ID: qMmuCED0027497.

Decorin and biglycan immunohistochemistry

The specificity of the rabbit antisera against mouse DCN (LF-113) and BGN (LF-159) used in this study was established [29]. The antibodies were kindly provided by Dr. Larry W. Fisher (National Institute of Dental Research, NIH, MD). Sections were washed briefly in 0.1 M sodium acetate-0.1 M Tris-HCl buffer (pH 7.3). To enhance signal strength sections were then exposed for 60 min at 37ºC to 0.42 U/ml protease-free chondroitinase ABC from *Proteus vulgaris* (Sigma-Aldrich Co. LLC). Sections were incubated with the primary antibody overnight with a dilution of 1:200. Sections were then reincubated for two hours with goat anti-rabbit IgG secondary antibody, Alexa Fluor 488 conjugate (1:500 dilution). TO-PRO (Life Technologies) was used for nuclear counter staining. Samples were analyzed using Leica TCS SP II Confocal Microscope.

CHAPTER III

RESULTS

Decorin and biglycan quantitative real-time PCR

During palate formation, the palates elevate horizontally at E13.5. By E14.0 the palatal shelves juxtapose. Finally by E14.5 they fuse and the midline edge epithelium covering each of the palatal shelves forms the midline epithelia seam. We laser captured the MEE and MES and compared the relative expression (normalized with Gapdh) of decorin and biglycan mRNA during these three stages (far, close and fused palatal shelves). When palatal shelves were far BGN expression level was low. As palatal shelves approached each other, there was a significant increase of more than 6 fold of relative BGN expression when compared to far palatal shelves. This was followed by a significant down regulation of BGN mRNA when the palatal shelves fused. Even though it was down regulated at this stage, it was still significantly upregulated by 2 fold when compared to far palatal shelves (Figure 2).

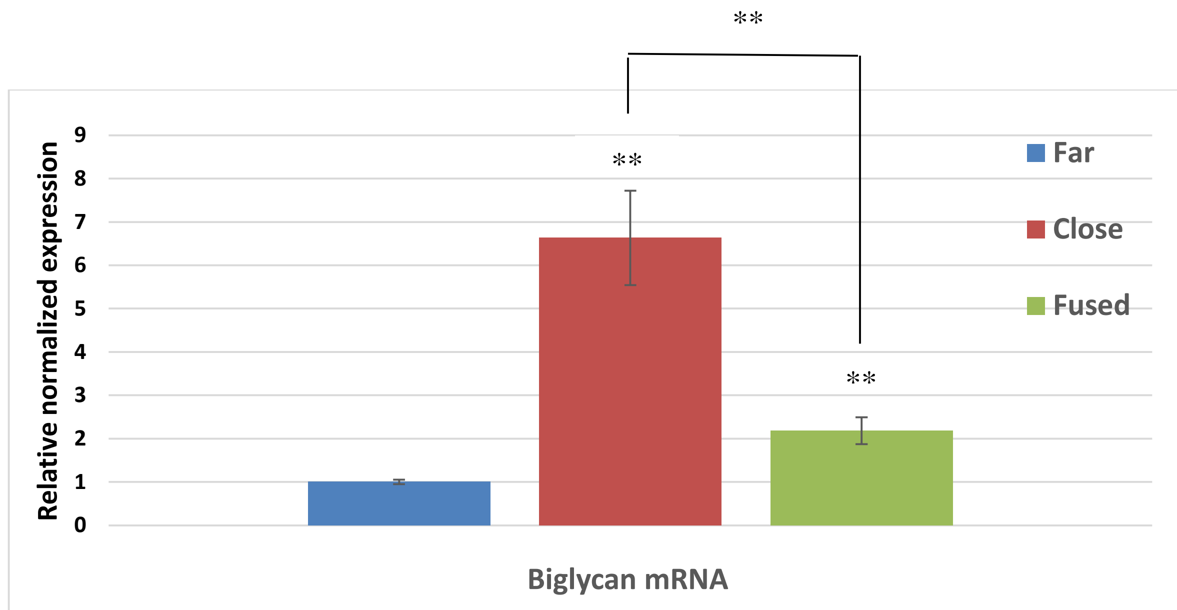

Figure 2 Biglycan mRNA relative expression during far, close and fused palatal shelves. The real-time PCR data, normalized with Gapdh are presented as mean ± SEM; *P<0.05; **P<0.01, compared to far palatal shelves.

DCN mRNA expression levels were different from that of BGN. When compared to far palatal shelves, there was a significant down regulation of 2.5 and 5 fold of DCN mRNA in the close and fused palatal shelves respectively. In addition, there was a significant down regulation of around 2 fold of DCN mRNA in fused palatal shelves when compared to close ones (Figure 3). Because of the low levels of DCN and to

ascertain that we have the accurate RT-PCR settings, we added a positive control. DCN is highly expressed in the meninges around E13.5 [30].

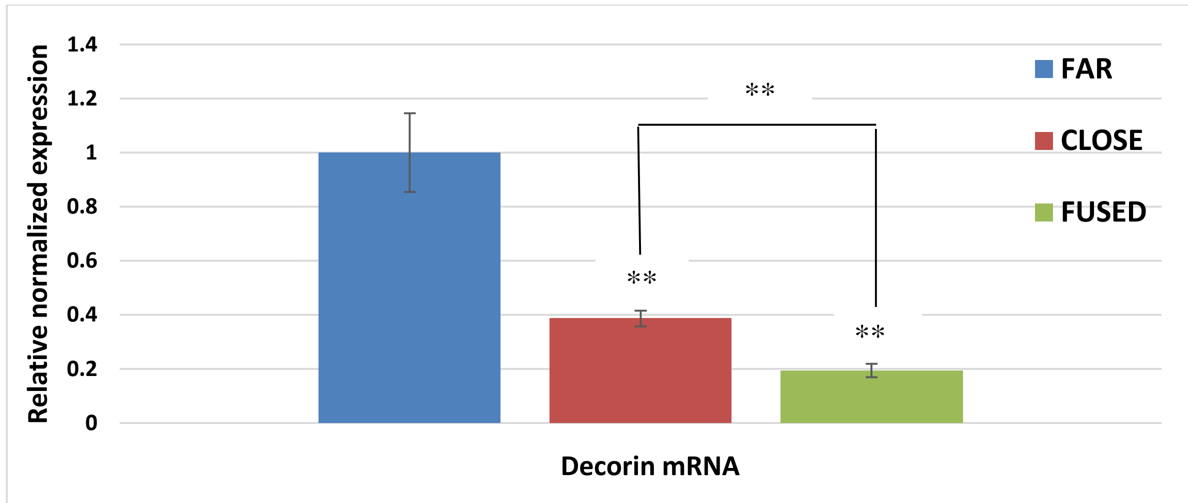

Figure 3 Decorin mRNA relative expression during far, close and fused palatal shelves. The real-time PCR data, normalized with Gapdh are presented as mean ± SEM; $*P<0.05$; $**P<0.01$, compared to far palatal shelves.

Expression pattern of DCN and BGN in the palatal shelve epithelia

We performed immunohistochemical analysis of DCN and BGN expression on the palatal shelves epithelia on various stages of development. Both BGN and DCN were not detected on the palatal shelves medial edge epithelia when the palatal shelves were vertical (data not shown). At E13.5 the palatal shelves have elevated but are still far from each other, expression BGN is noticeable between the MEE cells but is not detected in MEE cells immunolabeled for DCN (Figure 4)

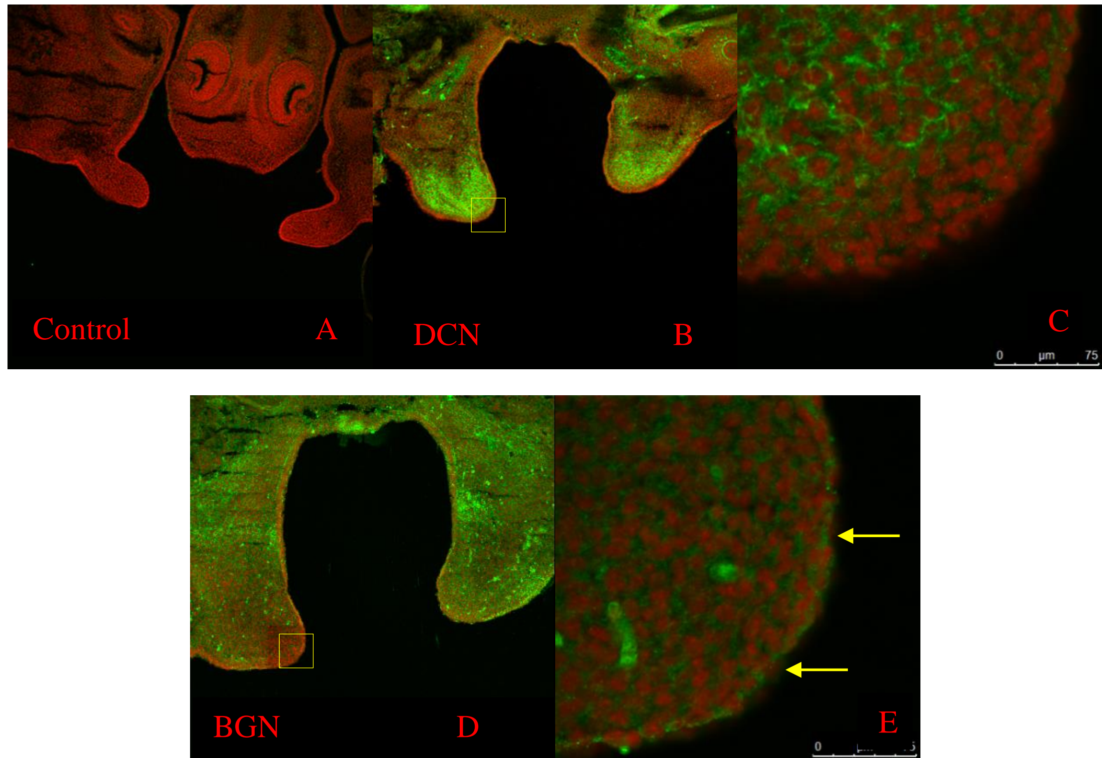

Figure 4 Confocal images of anti-BGN and anti-DCN immunolabeled far coronal palate sections. (A, B and D) Images of immunolabeled far palatal shelves for control, DCN and BGN respectively. (C and E) Higher magnification for the boxed area in B and D

As the palatal shelves approach each other a thin layer of BGN and DCN is noticed around and on the apical surface of the medial edge epithelia (Figure 5). BGN expression is more intense than DCN at this stage.

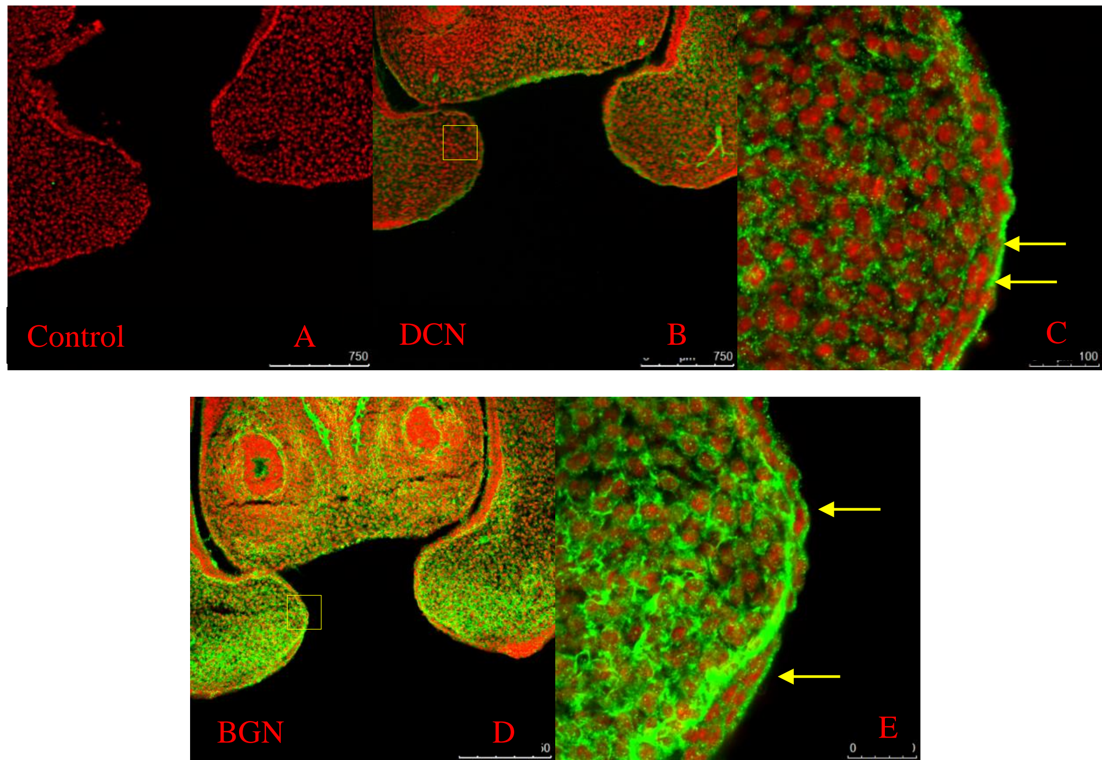

Figure 5 Confocal images of anti-BGN and anti-DCN immunolabeled approaching palate sections. Expression of BGN and DCN intensifies in the MEE of approaching palatal shelves. (A, B and D) Images of immunolabeled palatal shelves for control, DCN and BGN respectively. (C and E) The boxed area is shown at a higher magnification in which BGN and DCN are detected in the apical surface of MEE (arrows).

Palatal shelves at point of close contact show abundant expression of BGN and DCN in between and on the surface of MEE (Figure 6). The bulging MEE cells become covered with BGN to the point of contact (Figure 7). As the palatal shelves fuse and

MES is formed there is abundant expression of both DCN and BGN between the MES cells. The expression of the latter being more intense (Figure 8).

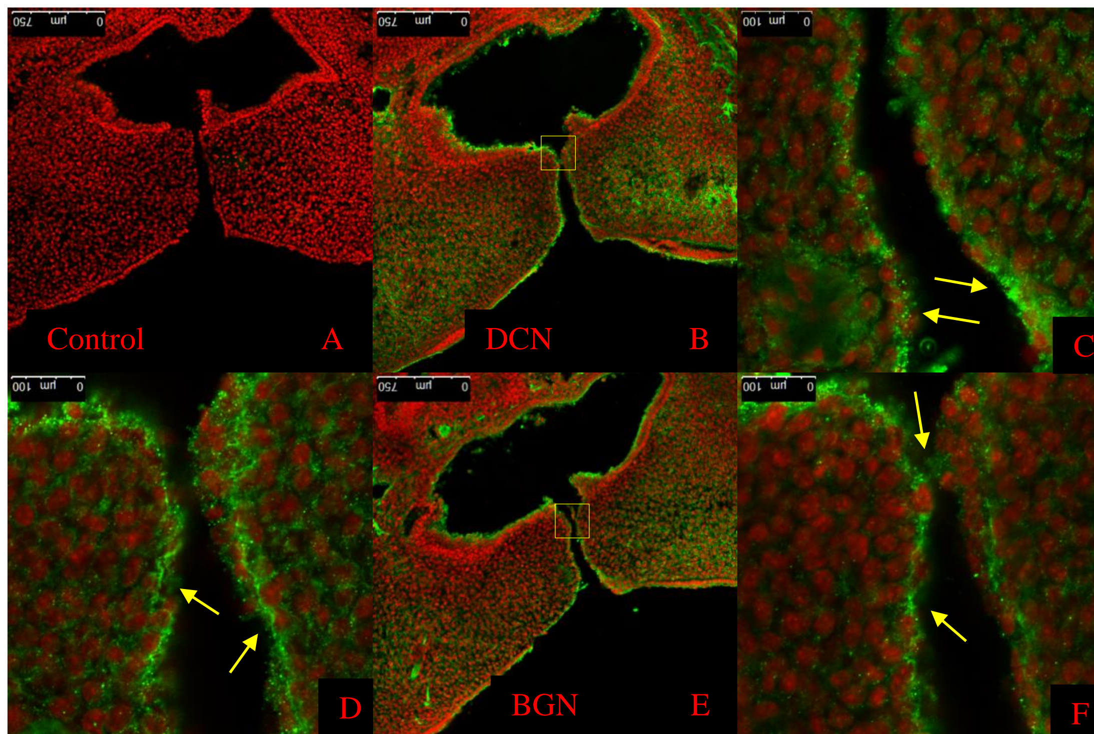

Figure 6 Confocal images of anti-BGN and anti-DCN immunolabeled close palate sections (A, B, E) Images of immunolabeled palatal shelves for control, DCN and BGN respectively. (C) The boxed area in B is shown at a higher magnification. Arrows point to apically expressed DCN. (D and F) boxed area in E shown at a higher magnification. Arrows point to apically expressed BGN.

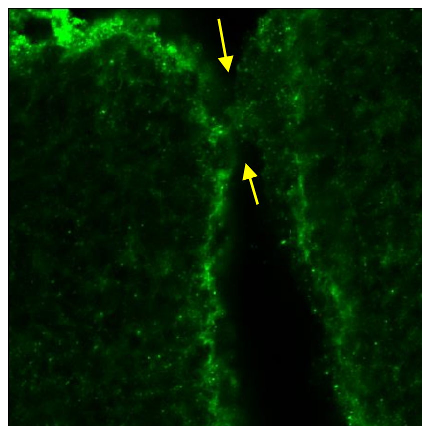

Figure 7 Confocal image of anti-BGN immunolabeled close palate section without the nuclear counterstaining. Abundant accumulation of BGN on the apical surface of the MEE to the point of contact (arrows)

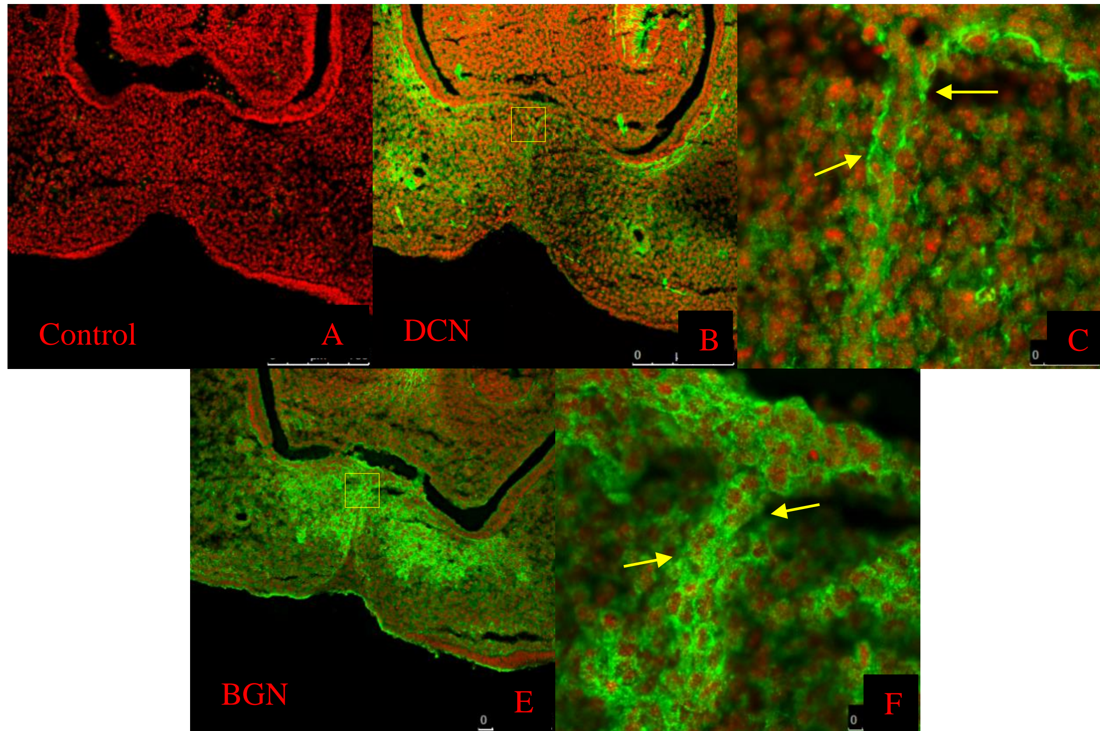

Figure 8 Confocal images of anti-BGN and anti-DCN immunolabeled fused palate sections. (A, B, D) Images of immunolabeled palatal shelves for control, DCN and BGN respectively. (C and E) the boxed area in B and D is shown at a higher magnification. Arrows point to DCN and BGN localization between the MES cells.

CHAPTER IV

DISCUSSION AND CONCLUSION

Lately, many genes have been studied for their role in palate formation, with most concentrating on the fate and disappearance of the MES. However, few studies focused on genes involved in the attachment of opposing palatal shelves. It had been established previously that expression of chondroitin sulphate proteoglycans on the apical surface of MEE cells is a key factor in palatal shelf adhesion [7].

In this study, we used the highly sensitive quantitative gene expression of LCM procured MEE / MES cells and immunohistochemistry analysis to demonstrate the expression of decorin and biglycan during palatal shelf adhesion. Both decorin and biglycan are localized between and on the surface of the MEE when palatal shelves start to adhere each other. This expression is intensified when the shelves are very close and more accumulation of the two proteoglycans surrounds the bulging MEE cells. Furthermore, the expression continues between the MES cells after fusion. However, biglycan was more intensely immunolabeled during all stages. The expression pattern on the surface is similar to that reported in a previous study [7], where they demonstrated the presence of a CSPG coat on the apical surface of the MEE as the palatal shelves adhere to each other, suggestive that DCN and BGN could be part of that glycolytic coat. However, in contrast to their study, our results show that both DCN and BGN were also expressed between the MEE cells in the cell-cell junction area. Decorin and biglycan, depending on the tissue, can have glycosaminoglycan (GAG) chains of either

chondroitin sulfate or dermatan sulphate [31]. This difference might be attributed to the anti-chondroitin sulphate monoclonal antibody CS-56 used in the previous study. It is reported to be specific for the GAG portion of native chondroitin, not dermatan, sulfate proteoglycan and hence might have not detected DCN or BGN.

The mRNA expression of DCN and BGN levels were dissimilar. Biglycan showed up regulation that had a peak when palatal shelves were very close. Decorin, on the other hand, was down regulated as the palates approached and when the palates fused and the MES was formed. Though structurally very similar, their mRNA expression patterns are suggestive of difference in regulation in palatogenesis. This is consistent with the only other study that investigated the expression of decorin and biglycan during palatogenesis [8]. Their results also demonstrated a difference in the regulation of both genes in response to retinoic-acid (RA) treatment.

Transforming growth factor beta (TGFβs) signaling is essential for normal palatogenesis. All three isoforms play a role in the process, but two members are expressed in the MEE: $TGF\beta_1$ and $TGF\beta_3$. In the mouse palate $TGF\beta_3$ is evident in the MEE as early as E.13.5 this expression increases as palatal shelves develop and ends when the MES disappears [32]. $TGF\beta_3$ knockout mice develop cleft palate, with an incidence of 100% [33]. In the knockout mice, the palatal shelves develop, elevate, and meet in the midline as in the wild type, but shortly afterwards they separate resulting in cleft palate [29]. Not only is TGFβ3 required for the adhesion and intercalation of the opposing MEE [3], but is inductive of the changes that occur in the MEE before fusion [4]. $TGF\beta_1$ expression is first observed at E14 in the MEE and in some isolated

mesenchymal palatal cells. The expression increases in both locations and disappears in the MES at the time of MES disappearance, persisting in the palatal mesenchyme in those areas of future ossification [32]. Several studies have shown that $TGF\beta_1$ has an active role in palatal fusion and that interaction between it and $TGF\beta_3$ occurs in the developing palate [11, 32, 34]. The binding of proteoglycans, including decorin and biglycan, to growth factors has recently been well recognized [35, 36]. Both decorin and biglycan bind TGFβ through their core protein [24, 37] affecting its bioactivity. Furthermore, TGFβ had been shown to affect the production of both proteoglycans in different ways.

Biglycan is a member of the small leucine-rich proteoglycan family (SLRP) and its proposed function is dependent on its microenvironment [31] and hence a number of diverse functions have been attributed to it. In the blood vessels it had been shown to be involved in atherosclerotic plaques [38], in the skeleton it controls bone mass [19] and in the dentition biglycan affects amelogenesis [39]. Biglycan has also been shown to be expressed in cell surface and/or pericellulary [31, 40].The intense expression of BGN in the MEE of adhering palatal shelves further augments this pattern. Additionally, its expression on the apical surface of MEE greatly increases when contact between palatal shelves becomes imminent and at the point of contact (figure 6). The mRNA levels coincide with its protein pattern with an up regulation of its mRNA expression more than 6 fold when the palatal shelves are close to each other. The levels are then down regulated as the palatal shelves fuse and the MES is formed. This temporal expression of BGN in the apical surface of MEE of the fusing palatal shelves clearly demonstrates a

new function for BGN in palatal shelf adhesion. Biglycan expression can be modified by a number of growth factors including TGFβ. It had been shown that TGFβ enhances BGN mRNA and protein expression in numerous cell types including human skin, kidney, lung fibroblast [41], articular cartilage [42] and bone [43].The temporal co-expression of both BGN and TGFβ in the MEE cells as the palatal shelves adhere is suggestive that BGN expression is up regulated in the palatal epithelia as a result of TGFβ signaling. Further studies should be conducted to establish this relationship.

Decorin is the other most widely known SLRP family member. Although structurally very related, decorin and biglycan show considerable difference both in their functions and expression regulation. Decorin functions include regulating collagen fibrillogenesis, ECM production, cell-cycle progression, programmed cell death, neovascularization and maintaining corneal transparency [44, 45]. Our results show that decorin was also detected in a similar pattern as BGN and was also expressed in the apical surface of the MEE however, the immunolabeling was less intense. Furthermore its mRNA levels showed a significant down regulation of 2.5 and 5 fold in close and fused palatal selves respectively. The lack of correlation between DCN mRNA levels and the protein levels could be explained by either a more efficient translational rate or a slow turnover rate of DCN protein. The fact that decorin is mostly found bound to collagen in the ECM supports the second explanation. It has been reported that a reciprocal relationship exists between decorin and TGFβ [46, 47]. In contrast to biglycan, TGFβ had been reported to down regulate the expression of DCN [42, 47, 48]. In chondrocytes it had been demonstrated that the decreased DCN levels induced by

TGFβ was due to transcriptional down regulation [48]. This is consistent with the decline in DCN mRNA and it's coincident with the appearance of TGFβ expression in the MEE cells. Furthermore, previous studies have shown that decorin can interact with TGFβs through its core protein neutralizing its activity [26]. It was suggested that it forms complexes with TGFβ, sequesters the cytokine and prevents it from binding to its receptor [20, 24]. The improper sequestration of TGFβ in DCN/BGN double knockout is responsible for the osteoporotic bone phenotype [20]. Additionally, it was demonstrated that collagen-bound decorin is capable of sequestering TGFβ [49]. BGN, being located pericellularly, is less likely to trap the cytokine [35]. Moreover, as a result of this interaction decorin was studied as a potential therapeutic agent in treatment of TGFβ induced fibrosis (gene therapy) [50]. Hence, in palate adhesion, the transient expression of decorin and down regulation after fusion is required to allow for normal TGFβ expression and palatogensis process. Further studies will be required to conclusively establish the relationship between decorin and TGFβ in palatal adhesion.

MES disappearance is required to allow mesenchymal confluence and thus an essential process for palatogenesis. The MES disappears by means of cell death [51], epithelial to mesenchymal transformation [52] and migration orally or nasally [53]. Epithelial to mesenchymal transformation (EMT) is an important phenomenon of wound healing, tumor metastasis and embryonic development including palatogenesis [54, 55]. It encompasses a number of events including reorganization of cytoskeleton and mobility of epithelial cells [56]. Furthermore the process is regulated by a number of growth factors including TGFβ and extracellular matrix molecules [57]. Both decorin

and biglycan had been reported to affect cellular adhesion and increase cellular migration [58, 59]. Decorin influences cellular adhesion through interaction with a number of ECM proteins. For example, DCN decreases cellular attachment to fibronectin [60] and thrombospondin[61]. In addition it can bind integrin $\alpha_2\beta_1$ and act as a competitive inhibitor[62]. Decorin has been found to induce metalloproteinase synthesis including MMP-1,-2 and -14 [63]. MMPs are required for the ECM breakdown during epithelial to mesenchymal transformation process. Biglycan expression on the other hand, had been associated in recent studies with increased tumor invasiveness and association with EMT [64, 65]. Moreover, BGN up regulation parallels changes in morphology and gene expression associated with TGF-β-induced EMT. Our results clearly demonstrate that both DCN and BGN are expressed in the lateral cell membrane compartment in both MEE and MES. Their inductive cellular anti-adhesive and migratory properties and regulation by TGFβ is suggestive that they may be important in the morphological and cytoskeletal changes during EMT of the MEE cells.

On a final note, neither DCN nor BGN single or double knockout transgenic mice develop cleft palate. Recently more factors had been discovered to play a role in palatal shelf adhesion. Examples include the glycoprotein fibronectin and its receptor α5β1-integrin, the cellular adhesion molecule nectin-1 [6] , the epithelial adhesion molecule CEACAM-1 [66] and the Src family kinases (SFK) [67]. The mouse knockout phenotype of some of these molecules also do not develop cleft palates suggesting that opposing MEE adhesion results from interaction of different cell adhesion molecules.

These factors in addition to other undiscovered proteoglycans may compensate for the deficiency of DCN and BGN in these transgenic mice.

In conclusion, our results were the first to characterize an expression pattern of decorin and biglycan on the MEE and MES during palatal adhesion, which is highly indicative of an important role in both palatal shelves adhesion and involvement in the EMT process. Though the two small chondroitin/dermatan sulphate proteoglycans are very similar in structure, they differ in the distribution and function during palatogenesis. This opens up the doors for more studies focusing on the regulation of decorin and biglycan in the palatal epithelia during adhesion and their interaction with TGFβ.

The discovery of new genes involved in this process will help in the understanding of palatal fusion and thus aid in the development of approaches to enhance it and improve treatment for cleft palate.

REFERENCES

1. Parker, S.E., et al., *Updated National Birth Prevalence estimates for selected birth defects in the United States, 2004-2006.* Birth Defects Res A Clin Mol Teratol, 2010. **88**(12): p. 1008-16.
2. Ferguson, M.W., *Palate development.* Development, 1988. **103 Suppl**: p. 41-60.
3. Tudela, C., et al., *TGF-beta3 is required for the adhesion and intercalation of medial edge epithelial cells during palate fusion.* Int J Dev Biol, 2002. **46**(3): p. 333-6.
4. Martinez-Alvarez, C., et al., *Bulging medial edge epithelial cells and palatal fusion.* Int J Dev Biol, 2000. **44**(3): p. 331-5.
5. Hayward, A.F., *Ultrastructural changes in the epithelium during fusion of the palatal processes in rats.* Arch Oral Biol, 1969. **14**(6): p. 661-78.
6. Martinez-Sanz, E., et al., *Alteration of medial-edge epithelium cell adhesion in two Tgf-beta3 null mouse strains.* Differentiation, 2008. **76**(4): p. 417-30.
7. Gato, A., et al., *TGF-beta(3)-induced chondroitin sulphate proteoglycan mediates palatal shelf adhesion.* Dev Biol, 2002. **250**(2): p. 393-405.
8. Zhang, Y., et al., *Differential expression of decorin and biglycan genes during palatogenesis in normal and retinoic acid-treated mice.* Dev Dyn, 2003. **226**(4): p. 618-26.
9. Schonherr, E. and H.J. Hausser, *Extracellular matrix and cytokines: a functional unit.* Dev Immunol, 2000. **7**(2-4): p. 89-101.

10. Meng, L., et al., *Biological mechanisms in palatogenesis and cleft palate.* J Dent Res, 2009. **88**(1): p. 22-33.
11. Taya, Y., S. O'Kane, and M.W. Ferguson, *Pathogenesis of cleft palate in TGF-beta3 knockout mice.* Development, 1999. **126**(17): p. 3869-79.
12. Kinsella, M.G., S.L. Bressler, and T.N. Wight, *The regulated synthesis of versican, decorin, and biglycan: extracellular matrix proteoglycans that influence cellular phenotype.* Crit Rev Eukaryot Gene Expr, 2004. **14**(3): p. 203-34.
13. Wadhwa, S., et al., *Impaired posterior frontal sutural fusion in the biglycan/decorin double deficient mice.* Bone, 2007. **40**(4): p. 861-6.
14. Tenorio, D.M., M.F. Santos, and T.M. Zorn, *Distribution of biglycan and decorin in rat dental tissue.* Braz J Med Biol Res, 2003. **36**(8): p. 1061-5.
15. Matheson, S., H. Larjava, and L. Hakkinen, *Distinctive localization and function for lumican, fibromodulin and decorin to regulate collagen fibril organization in periodontal tissues.* J Periodontal Res, 2005. **40**(4): p. 312-24.
16. Wang, L., et al., *Fibromodulin and Biglycan Modulate Periodontium through TGFbeta/BMP Signaling.* J Dent Res, 2014. **93**(8): p. 780-787.
17. Kuwabara, M., et al., *Biochemical and immunohistochemical studies of the protein expression and localization of decorin and biglycan in the temporomandibular joint disc of growing rats.* Arch Oral Biol, 2002. **47**(6): p. 473-80.

18. Danielson, K.G., et al., *Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility.* J Cell Biol, 1997. **136**(3): p. 729-43.

19. Xu, T., et al., *Targeted disruption of the biglycan gene leads to an osteoporosis-like phenotype in mice.* Nat Genet, 1998. **20**(1): p. 78-82.

20. Bi, Y., et al., *Extracellular matrix proteoglycans control the fate of bone marrow stromal cells.* J Biol Chem, 2005. **280**(34): p. 30481-9.

21. Quentin, E., et al., *A genetic defect in the biosynthesis of dermatan sulfate proteoglycan: galactosyltransferase I deficiency in fibroblasts from a patient with a progeroid syndrome.* Proc Natl Acad Sci U S A, 1990. **87**(4): p. 1342-6.

22. Schonherr, E., et al., *Interaction of biglycan with type I collagen.* J Biol Chem, 1995. **270**(6): p. 2776-83.

23. Iozzo, R.V. and L. Schaefer, *Proteoglycans in health and disease: novel regulatory signaling mechanisms evoked by the small leucine-rich proteoglycans.* Febs j, 2010. **277**(19): p. 3864-75.

24. Hildebrand, A., et al., *Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor beta.* Biochem J, 1994. **302 (Pt 2)**: p. 527-34.

25. Wu, Z., et al., *Biglycan and decorin differentially regulate signaling in the fetal membranes.* Matrix Biol, 2014. **35**: p. 266-75.

26. Seelan, R.S., et al., *Epigenetic analysis of laser capture microdissected fetal epithelia.* Anal Biochem, 2013. **442**(1): p. 68-74.

27. Nawshad, A., et al., *Laser capture microdissection (LCM) for analysis of gene expression in specific tissues during embryonic epithelial-mesenchymal transformation.* Dev Dyn, 2004. **230**(3): p. 529-34.

28. Huang, S.H., T.W. Gong, and S.G. Gong, *Isolation of epithelial cells in the developing primary lip and palate.* J Craniofac Surg, 2011. **22**(5): p. 1847-51.

29. Fisher, L.W., J.T. Stubbs, 3rd, and M.F. Young, *Antisera and cDNA probes to human and certain animal model bone matrix noncollagenous proteins.* Acta Orthop Scand Suppl, 1995. **266**: p. 61-5.

30. Scholzen, T., et al., *The murine decorin. Complete cDNA cloning, genomic organization, chromosomal assignment, and expression during organogenesis and tissue differentiation.* J Biol Chem, 1994. **269**(45): p. 28270-81.

31. Wadhwa, S., et al., *Regulation, regulatory activities, and function of biglycan.* Crit Rev Eukaryot Gene Expr, 2004. **14**(4): p. 301-15.

32. Murillo, J., et al., *Interactions between TGF-beta1 and TGF-beta3 and their role in medial edge epithelium cell death and palatal fusion in vitro.* Differentiation, 2009. **77**(2): p. 209-20.

33. Proetzel, G., et al., *Transforming growth factor-beta 3 is required for secondary palate fusion.* Nat Genet, 1995. **11**(4): p. 409-14.

34. Yang, L.T. and V. Kaartinen, *Tgfb1 expressed in the Tgfb3 locus partially rescues the cleft palate phenotype of Tgfb3 null mutants.* Dev Biol, 2007. **312**(1): p. 384-95.

35. Kresse, H. and E. Schonherr, *Proteoglycans of the extracellular matrix and*

growth control. J Cell Physiol, 2001. **189**(3): p. 266-74.

36. Iozzo, R.V., *The biology of the small leucine-rich proteoglycans. Functional network of interactive proteins.* J Biol Chem, 1999. **274**(27): p. 18843-6.

37. Yamaguchi, Y., D.M. Mann, and E. Ruoslahti, *Negative regulation of transforming growth factor-beta by the proteoglycan decorin.* Nature, 1990. **346**(6281): p. 281-4.

38. Williams, K.J., *Arterial wall chondroitin sulfate proteoglycans: diverse molecules with distinct roles in lipoprotein retention and atherogenesis.* Curr Opin Lipidol, 2001. **12**(5): p. 477-87.

39. Goldberg, M., et al., *Biglycan is a repressor of amelogenin expression and enamel formation: an emerging hypothesis.* J Dent Res, 2002. **81**(8): p. 520-4.

40. Groth, S., et al., *Adhesion and Rac1-dependent regulation of biglycan gene expression by transforming growth factor-beta. Evidence for oxidative signaling through NADPH oxidase.* J Biol Chem, 2005. **280**(39): p. 33190-9.

41. Bassols, A. and J. Massague, *Transforming growth factor beta regulates the expression and structure of extracellular matrix chondroitin/dermatan sulfate proteoglycans.* J Biol Chem, 1988. **263**(6): p. 3039-45.

42. Roughley, P.J., L.I. Melching, and A.D. Recklies, *Changes in the expression of decorin and biglycan in human articular cartilage with age and regulation by TGF-beta.* Matrix Biol, 1994. **14**(1): p. 51-9.

43. Heegaard, A.M., et al., *Transforming growth factor beta stimulation of biglycan gene expression is potentially mediated by sp1 binding factors.* J Cell Biochem, 2004. **93**(3): p. 463-75.

44. Mohan, R.R., et al., *Decorin biology, expression, function and therapy in the cornea.* Curr Mol Med, 2011. **11**(2): p. 110-28.

45. Iozzo, R.V., *Matrix proteoglycans: from molecular design to cellular function.* Annu Rev Biochem, 1998. **67**: p. 609-52.

46. Bianco, P., et al., *Expression and localization of the two small proteoglycans biglycan and decorin in developing human skeletal and non-skeletal tissues.* J Histochem Cytochem, 1990. **38**(11): p. 1549-63.

47. Kahari, V.M., H. Larjava, and J. Uitto, *Differential regulation of extracellular matrix proteoglycan (PG) gene expression. Transforming growth factor-beta 1 up-regulates biglycan (PGI), and versican (large fibroblast PG) but down-regulates decorin (PGII) mRNA levels in human fibroblasts in culture.* J Biol Chem, 1991. **266**(16): p. 10608-15.

48. Demoor-Fossard, M., et al., *A composite element binding the vitamin D receptor and the retinoic X receptor alpha mediates the transforming growth factor-beta inhibition of decorin gene expression in articular chondrocytes.* J Biol Chem, 2001. **276**(40): p. 36983-92.

49. Markmann, A., et al., *Influence of decorin expression on transforming growth factor-beta-mediated collagen gel retraction and biglycan induction.* Matrix Biol, 2000. **19**(7): p. 631-6.

50. Isaka, Y., et al., *Gene therapy by skeletal muscle expression of decorin prevents fibrotic disease in rat kidney.* Nat Med, 1996. **2**(4): p. 418-23.

51. Martinez-Alvarez, C., et al., *Medial edge epithelial cell fate during palatal fusion.* Dev Biol, 2000. **220**(2): p. 343-57.

52. Fitchett, J.E. and E.D. Hay, *Medial edge epithelium transforms to mesenchyme after embryonic palatal shelves fuse.* Dev Biol, 1989. **131**(2): p. 455-74.

53. Carette, M.J. and M.W. Ferguson, *The fate of medial edge epithelial cells during palatal fusion in vitro: an analysis by DiI labelling and confocal microscopy.* Development, 1992. **114**(2): p. 379-88.

54. Kang, P. and K.K. Svoboda, *Epithelial-mesenchymal transformation during craniofacial development.* J Dent Res, 2005. **84**(8): p. 678-90.

55. Griffith, C.M. and E.D. Hay, *Epithelial-mesenchymal transformation during palatal fusion: carboxyfluorescein traces cells at light and electron microscopic levels.* Development, 1992. **116**(4): p. 1087-99.

56. Hay, E.D., *An overview of epithelio-mesenchymal transformation.* Acta Anat (Basel), 1995. **154**(1): p. 8-20.

57. Thiery, J.P. and J.P. Sleeman, *Complex networks orchestrate epithelial-mesenchymal transitions.* Nat Rev Mol Cell Biol, 2006. **7**(2): p. 131-42.

58. Tufvesson, E. and G. Westergren-Thorsson, *Biglycan and decorin induce morphological and cytoskeletal changes involving signalling by the small GTPases RhoA and Rac1 resulting in lung fibroblast migration.* J Cell Sci, 2003. **116**(Pt 23): p. 4857-64.

59. Kinsella, M.G., et al., *Selective expression and processing of biglycan during migration of bovine aortic endothelial cells. The role of endogenous basic fibroblast growth factor.* J Biol Chem, 1997. **272**(1): p. 318-25.
60. Winnemoller, M., G. Schmidt, and H. Kresse, *Influence of decorin on fibroblast adhesion to fibronectin.* Eur J Cell Biol, 1991. **54**(1): p. 10-7.
61. Winnemoller, M., et al., *Interactions between thrombospondin and the small proteoglycan decorin: interference with cell attachment.* Eur J Cell Biol, 1992. **59**(1): p. 47-55.
62. Guidetti, G., et al., *The small proteoglycan decorin supports adhesion and activation of human platelets.* Blood, 2002. **100**(5): p. 1707-14.
63. Schonherr, E., et al., *Matrix metalloproteinase expression by endothelial cells in collagen lattices changes during co-culture with fibroblasts and upon induction of decorin expression.* J Cell Physiol, 2001. **187**(1): p. 37-47.
64. Hu, L., et al., *Biglycan enhances gastric cancer invasion by activating FAK signaling pathway.* Oncotarget, 2014. **5**(7): p. 1885-96.
65. Kiemer, A.K., K. Takeuchi, and M.P. Quinlan, *Identification of genes involved in epithelial-mesenchymal transition and tumor progression.* Oncogene, 2001. **20**(46): p. 6679-88.
66. Mima, J., et al., *Regulation of the epithelial adhesion molecule CEACAM1 is important for palate formation.* PLoS One, 2013. **8**(4): p. e61653.
67. Kitase, Y. and C.F. Shuler, *Palatal adhesion is dependent on Src family kinases and p38MAPK.* Int J Dev Biol, 2014. **58**(5): p. 335-41.