CHARACTERIZATION OF GROWTH AND TISSUE REMODELLING DURING THE MOUSE CRANIOFACIAL AND CARDIAC DEVELOPMENT

by

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ABSTRACT

Craniofacial and cardiac development share many common basic biological processes. Remodelling of the extracellular matrix (ECM) is believed to play an important role during mammalian embryogenesis. But the role of tissue remodelling during morphogenesis of the craniofacial complex and heart remains unclear. Therefore, I hypothesized that changes of basement membrane components and growth factors were associated with remodelling and growth of the embryonic primary palate, the future premaxillary area, and the heart.

The present investigation encompassed four projects. First was the characterization of the distribution of major basement membrane components; laminin, type IV collagen and fibronectin; by indirect immunofluorescence in the primary palate as the epithelial seam is disrupted and the mesenchymal bridge forms and enlarges. The results showed that localized disruption of basement membrane components occurred simultaneously with mesenchymal bridge formation and enlargement during primary palate formation.

The purposes of the second part were to characterize the distribution patterns of epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-α) and their receptor, epidermal growth factor receptor (EGF-R), by immunohistochemistry and to analyze regional patterns of cell proliferation by 5-bromodeoxyuridine (BrdU) incorparation and proliferating cell nuclear antigen (PCNA) immunolocalization during primary palate morphogenesis. The results showed that EGF, TGF-α, and EGF-R were labelled more intensely in the tips and peripheral regions of the facial prominences where cell proliferation was most pronounced. This suggested that EGF and TGF-α stimulate cell proliferation during outgrowth of prominences during primary palate morphogenesis.

The purpose of the third part was to look for the presence of enzymes involved in degradation of the basement membrane of the epithelial seam during primary palate morphogenesis. Protein expression of a candidate matrix metalloproteinase (MMP), 72-kDa gelatinase (MMP-2) was studied by indirect immunofluorescence and zymography. The results revealed that MMP-2 was present in the area of fusion in the primary palate and also in the tips and peripheral regions of the facial prominences. This localization of MMP-2 suggested that regional differences in tissue remodelling are involved in directional enlargement of the facial prominences. Gelatin zymography confirmed the presence of active and latent MMP-2 in the developing craniofacial complex.

The purpose of the last part was to examine the distribution of MMP-2 and its substrates: type IV collagen, laminin and fibronectin during heart morphogenesis by indirect immunohistochemistry. The results showed that the distribution patterns of MMP-2 were highly correlated with that of the ECM components from embryonic day 9-13.

Collectively, these data indicate that the mechanisms of growth and tissue remodelling during craniofacial and heart development are complex and may involve multiple interactions between various molecular factors; including ECM components (type IV collagen, laminin, and fibronectin), growth factors (EGF and TGF-α) and their receptor (EGF-R), and matrix metalloproteinase (MMP-2).

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ABC	avidin-biotin-peroxidase complex
AVC	atrio-ventricular canal
BMP	bone morphogenetic protein
BrdU	5'-bromodeoxyuridine
CL/P	cleft lip with or without cleft palate
CRABP	cellular retinoic acid binding protein
CRBP	cellular retinol binding protein
CT-1	cardiotrophin-1
DAB	3,3'-diaminobenzidine hydrogen peroxide
DiI	1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
EC	endocardial cushion
ECGF	endothelial cell growth factor
ECM	extracellular matrix
EGF	epidermal growth factor
EGF-R	epidermal growth factor receptor
EHS	Engelbreth-Holm-Swarm
ES	embryonic stem cells
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FITC	fluorescein isothiocyanate
HBGF	heparin-binding growth factor
HGF/SF	hepatocyte growth factor/scatter factor
IGF	insulin-like growth factor
IL-1	interleukin-1

LIF	leukemia inhibitory factor
LN	lateral nasal prominence
LPS	lipopolysaccharide
MEE	medial edge epithelium
MMP	matrix metalloproteinase
MN	medial nasal prominence
MT-MMP	membrane-type matrix metalloproteinase
MX	maxillary prominence
NGF	nerve growth factor
PA	plasminogen activator
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
RAR	retinoic acid receptor
RT-PCR	reverse transcription polymerase chain reaction
SPARC	secreted protein acidic and rich in cysteine
TGF-α	transforming growth factor- alpha
TGF-β	transforming growth factor-beta
TIMP	tissue inhibitor of matrix metalloproteinase
TNF	tumor necrosis factor
TPA	12-O-tetradecanoyl phorbol 13-acetate
TRE	TPA-responsive element
TRITC	tetramethylrhodamine isothiocyanate
TS	tail somite
VCAM-1	vascular cell adhesion molecule-1

PREFACE

Some of the material included in this thesis has been previously published or has been submitted, as noted below:

Iamaroon, A., and Diewert V.M. (1996): Distribution of basement membrane components in the mouse primary palate. J. Craniofac. Genet. Dev. Biol. 16:48-51.

Iamaroon, A., Tait, B., and Diewert, V.M. (1996): Cell proliferation and expression of EGF, TGF-α, and EGF-R in the developing primary palate. J. Dent. Res. (in press).

Iamaroon, A., Wallon, U.M., Overall, C.M., and Diewert, V.M. (1996): Expression of 72-kDa gelatinase (matrix metalloproteinase 2) in the developing craniofacial complex of the mouse embryo. Arch. Oral Biol. (in press).

Iamaroon, A., Wallon, U.M., Overall, C.M., and Diewert, V.M. (1996): Immunolocalization of 72-kDa gelatinase and extracellular matrix components during mouse cardiac development. J. Anat. (in revision).

These publications as well as this thesis are the principal work of the candidate, Anak Iamaroon. However, the thesis supervisor, Dr. V.M. Diewert, offered editorial comments on the manuscripts and contributed advice and suggestions throughout the course of the experiments that comprise these publications and this thesis. Drs. C.M. Overall and U.M. Wallon also provided comments on the manuscripts and were responsible for preparation, affinity purification, and specificity evaluation of the anti-72-kDa gelatinase antibody. B. Tait was responsible for animal preparation for BrdU injection and tissue sectioning. The candidate and thesis supervisor agree that contributions of the respective parties are as stated above.

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namo tassa bhagavato arahato sammasambuddhassa

For my father and mother whose endless support is beyond words
and for all teachers whom I have fortunately known.

CHAPTER 1: GENERAL INTRODUCTION

A. Review of the Literature

1. Morphogenesis of the primary palate and mandible

The human primary palate starts forming at about 33 days after conception (stage 15) as the area which will form the nose becomes induced and thickened to form the nasal placode (O'Rahilly and Muller, 1987). The nasal placode is flanked by two elevated prominences, the medial and lateral nasal prominences. A succession of different inductors including the central nervous system is believed to involve formation of the nasal organ (Jacobson, 1963a, 1963b). Previous studies in the chick (Johnson, 1966; LeLievre and Le Douarin, 1975; Lumsden *et al.*, 1991) and mouse embryos (Nichols, 1986; Serbedzija *et al.*, 1992; Osumi-Yamashita *et al.*, 1994; Trainor and Tam, 1995) have found that the mesenchyme underlying the placode and adjacent prominences originates from neural crest cells and mesodermal cells. Migrating neural crest cells reach the facial regions at the stages 10-14 in chick (Lumsden *et al.*, 1991) and 9-to-10-day-old mouse embryos (Osumi-Yamashita *et al.*, 1994). The muscles of mastication and superficial muscles of the face, on the other hand, are derived from the somitomeres of the head (Noden, 1988). More recently, the spatial distribution of the mesoderm derived from somitomeres and neural crest cells during mouse craniofacial morphogenesis was studied by micromanipulative cell grafting and cell labelling (Trainor and Tam, 1995). The results revealed a distinct segregation of these two cell populations within the first three branchial arches in which neural crest cells are located in the periphery of the branchial arches and envelop the somitomere-derived core tissues on the rostral, lateral, and caudal sides of the arch. However, cells from these two sources mixed extensively in the peri-ocular, peri-otic, frontonasal, and cervical mesenchyme.

At about 37 days after conception in humans (stage 16), the medial nasal prominence starts contacting and fusing with the lateral nasal and maxillary prominences (O'Rahilly and Muller, 1987). The area of contact is called the epithelial plate or seam or nasal fin. This structure forms the continuity between the nasal cavity and the roof of the stomodeum. At about 41 days after conception in humans (stage 17) (Figs. 1,2), the epithelial seam becomes disrupted and invaded by the mesenchyme from the prominences, forming the mesenchymal bridge (Streeter, 1948; Warbrick, 1960; Vermeij-Keers, 1972; Diewert and Shiota, 1990). Diewert and Van der Meer (1991) analyzed the growth of the epithelial and mesenchymal components during normal primary palate formation in humans. It was found that during stage 17, the mesenchymal bridge formed through the epithelial seam and the size of the mesenchymal bridge increased rapidly to occupy up to 50% the total area. By stages 18 and 19, total primary palate area increased and the mesenchymal bridge enlarged to constitute 65 to 85% of the total area. At about 44 days after conception in humans (stage 18), the nasal fin posterior to the area of the mesenchymal bridge persists forming an oronasal membrane which separates the nasal cavity from the stomodeal cavity. Subsequently, this epithelial membrane ruptures to form a communication between the respiratory passage and the pharynx, the primitive choana (Streeter, 1948; Warbrick, 1960; Tamarin, 1982).

Successful primary palate formation involves a closely timed sequence of local cellular events with spatial changes associated with craniofacial growth. Disruption of primary palate formation, predisposed both to genetic and environmental factors, leads to cleft lip malformation (see review in Diewert and Wang, 1992). Previous studies have shown that teratogenic agents can induce cleft lip malformation in animals (Sulik *et al.*, 1979; Trasler and Leong, 1982). More recently, genetic studies revealed that two epistatic loci are involved

with cleft lip malformation in mice (Juriloff, 1995). A multifactorial threshold model was proposed to explain how genetic and environmental factors contribute to cleft lip malformation in mice (Diewert *et al.*, 1993b).

Mouse embryos have been used as a model for the study of the primary palate development since their morphogenesis is similar to that of the human (see review in Diewert and Wang, 1992) (Fig. 3). The mouse primary palate starts forming at about 10 days and 18 hours (Reed, 1933; Trasler, 1968; Wang *et al.*, 1995) with the appearance of the lateral nasal and medial nasal prominences. Subsequently, the medial nasal prominence makes contact with the lateral nasal and maxillary prominences at the inferior part of the nasal groove forming an epithelial seam or nasal fin from posterior to anterior direction. The scanning and transmission electron microscopic studies showed that fusion between the prominences occurs by a temporal sequence of events which include the loss of microvilli by the epithelial cells (Millicovsky and Johnston, 1981). After a brief period of quiescence, the epithelial cells begin to fill the nasal groove by producing a series of surface projections that increase in size and complexity as the process of fusion nears termination.

Recently, Wang *et al.* (1995) have studied growth of the internal morphological components during primary palate morphogenesis in mice with and without genetic cleft lip liability. It was found that in cleft lip strains (A/WySn and CL/Fr) enlargement of the epithelial seam and replacement of epithelia by a mesenchymal bridge are delayed compared with noncleft lip strains (C57BL/6J and BALB/cByJ). However, in both cleft and noncleft lip strains, the primitive choanae open at the same stages. Therefore, cleft lip strains have a narrower window of primary palate formation. These may contribute to high frequencies of cleft lip malformation in cleft lip-liable strains (Wang, 1992; Diewert and Wang, 1992; Wang *et al.*, 1995).

Various ECM molecules (type IV collagen, laminin, fibronectin), growth factors (fibroblast growth factor-8, bone morphogenetic proteins) and receptors (retinoic acid receptors), oncoprotein (*ras*), secreted protein (*Wnt-5a*), and transcriptional factors (*AP-2, Msx-1, Msx-2, goosecoid, Pax-3, Pax-6*), have been found in the developing facial prominences and suggested to be important for the facial development (Table I).

Table I: Distribution of ECM and other molecules during facial development.

Molecules	Localization	References
AP-2	MXP, FNM, LNM, MNP, MD (mouse, days 10.5-12.5) ectoderm, mesenchyme	Mitchell *et al.*, 1991
BMP-2A	MNP, MD (mouse, days 11.5, 12.5)	Lyons *et al.*, 1990
	FNM, MXP,MD, LNP (chick, stages 20, 24, 28) ectoderm, mesenchyme	Francis-West *et al.*, 1994
BMP-4	FNM, MXP, MD (mouse, day 9)	Jones *et al.*, 1991
	FNM, MXP,MD, LNP (chick, stages 20, 24, 28) ectoderm, mesenchyme	Francis-West *et al.*, 1994
c-*met*	MNP, LNP, MXP, MD (mouse, days 9-10) ectoderm	Andermarcher *et al.*, 1996

Collagen IV	MXP, roof of stomodeum	Xu *et al.*, 1990
	(chick, stages 22-31)	
	basement membrane	
CRABP-I	FNM, MXP, MD	Maden *et al.*, 1991
	(chick, stage 20)	
	FNM, MD	Dolle *et al.*, 1990
	(mouse, days 10.5, 12.5)	
	mesenchyme	
CRBP	FNM, MD	Dolle *et al.*, 1990
	(mouse, days 10.5, 12.5)	
	mesenchyme	
FGF-8	MXP, MD, nasal placode,	Crossley and Martin, 1995
	nasal pit epithelia	MacArthur *et al.*, 1995
	(mouse, days 9.5, 10.5)	Ohuchi *et al.*, 1994
	ectoderm	
Fibronectin	MXP, roof of stomodeum, MD	
	(chick, stages 22-31)	Xu *et al.*, 1990
	(mouse, days 12-18)	Richman and Diewert, 1987
	basement membrane, mesenchyme	
goosecoid	LNP, MNP, MD	Gaunt *et al.*, 1993
	(mouse, day 10.5)	
	mesenchyme	
HGF/SF	MNP, LNP, MXP, MD	Andermarcher *et al.*, 1996
	(mouse, days 9-10)	
	mesenchyme	

Laminin	MXP, roof of stomodeum	Xu *et al.*, 1990
	(chick, stages 22-31)	
	basement membrane	
***Msx-1* (*Hox-7*)**	MXP, FNM, MD	Nishikawa *et al.*, 1994
	(chick, stage 25)	Brown *et al.*, 1993
	LNP, MNP, MXP, MD	Robert *et al.*, 1989
	(mouse, day 9.5-11.5)	Brown *et al.*, 1993
	mesenchyme	MacKenzie *et al.*, 1992
***Msx*-2 (*Hox-8*)**	MXP, FNM, MD	Nishikawa *et al.*, 1994
	(chick, stage 25)	
	LNP, MNP, MXP, MD	MacKenzie *et al.*, 1992
	(mouse, day 10.5)	
	ectoderm, mesenchyme	
Pax-3	nasal prominence, MXP, MD	Goulding *et al.*, 1991
	(mouse, days 10-13)	
	mesenchyme	
Pax-6	nasal placode	Grindley *et al.*, 1995
	(mouse, days 9.5, 10.5)	
	ectoderm	
RARα	LNP, MNP, MXP, MD	Osumi-Yamashita *et al.*, 1990
	(mouse, days 10, 11)	
	FNM, MD	Dolle *et al.*, 1990
	(mouse, day 12.5)	
	mesenchyme	
RARβ	LNP, MD	Osumi-Yamashita *et al.*, 1990
	(mouse, days 10, 11)	

	FNM, MD	Dolle *et al.*, 1990
	(mouse, day 10.5)	
	FNM, MXP, LNP	Rowe *et al.*, 1991
	(chick, stage 20)	
	mesenchyme	
RARγ	LNP, MNP, MXP, MD	Osumi-Yamashita *et al.*, 1990
	(mouse, days 10, 11)	
	FNM, MD	Dolle *et al.*, 1990
	(mouse, days 10.5, 12.5)	
	ectoderm, mesenchyme	
***ras* onco-**	LNP, MNP, MXP	Wang *et al.*, 1995
proteins	(mouse, days 11, 12)	
	ectoderm, mesenchyme	
Wnt-5a	LNP, MNP, MD	Gavin *et al.*, 1990
	(mouse, day 10.5)	
	ectoderm, mesenchyme	

BMP, bone morphogenetic protein; CRABP,cellular retinoic acid binding protein; CRBP, cellular retinol binding protein; FGF, fibroblast growth factor; FNM, frontonasal mass; HGF/SF, hepatocyte growth factor/scatter factor; LNP, lateral nasal prominence; MD, mandibular prominence; MNP, medial nasal prominence; MXP, maxillary prominence; RAR, retinoic acid receptor

The primary palate and the secondary palate share many similar developmental events including fusion of the facial prominences, epithelial seam formation and elimination. In addition, the maxillary prominence contributes to not only the lateral part of the primary palate, but also the whole

secondary palate. The secondary palate starts to form with bilateral outgrowths from the maxillary prominences at embryonic day 12 in mice, day 6 in chickens, and day 45 in humans (see review in Ferguson, 1988). Initially, the maxillary prominences grow vertically and then elevate to a horizontal position above the tongue. Subsequently, the two palatal shelves make contact and fuse to form a midline epithelial seam, similar to the primary palate. Disruption of the epithelial seam in the secondary palate have been extensively studied and at least three mechanisms for elimination of the medial edge epithelium (MEE) have been proposed. Programmed cell death or apoptosis was initially suggested as a mechanism of the seam elimination based on ultrastructural evidence of autolytic epithelial cells (Hudson and Shapiro, 1973; Greene and Pratt, 1976). More recently by using *in situ* labelling for nuclear DNA fragmentation (TdT-mediated dUTP-biotin nick end labelling, TUNEL, method), apoptotic cells were observed along the epithelial seams of both primary (Pellier and Astic, 1994) and secondary (Mori *et al.*, 1994) palates. Recent *in vitro* and *in vivo* studies by using vital cell labelling techniques have suggested that MEE cells undergo epithelial-mesenchymal transformation (Griffith and Hay, 1992; Shuler *et al.*, 1992). Alternatively, migration of MEE cells to the adjacent oral and nasal epithelia is also indicated, based on using DiI labelling and confocal microscopy (Carette and Ferguson, 1992). The fate of the epithelial cells during seam regression in the primary palate needs to be investigated.

The first branchial arch gives rise not only to the maxillary prominence but also the mandibular prominence. The mandible starts to develop by elongation and outgrowth of the mandibular prominence. Subsequently, differentiation of tissues, including Meckel's cartilage, membranous bone, tooth germs and muscles, takes place (Frommer and Margolies, 1971). The mesenchymal cells in the mandibular primordia include both mesodermally-

derived and neural crest-derived mesenchymal cells. The neural crest-derived mesenchymal cells will give rise to chondrocytes and osteoblasts (Smith and Hall, 1990), whereas the mesodermally-derived mesenchymal cells will give rise to skeletal muscles (Noden, 1988).

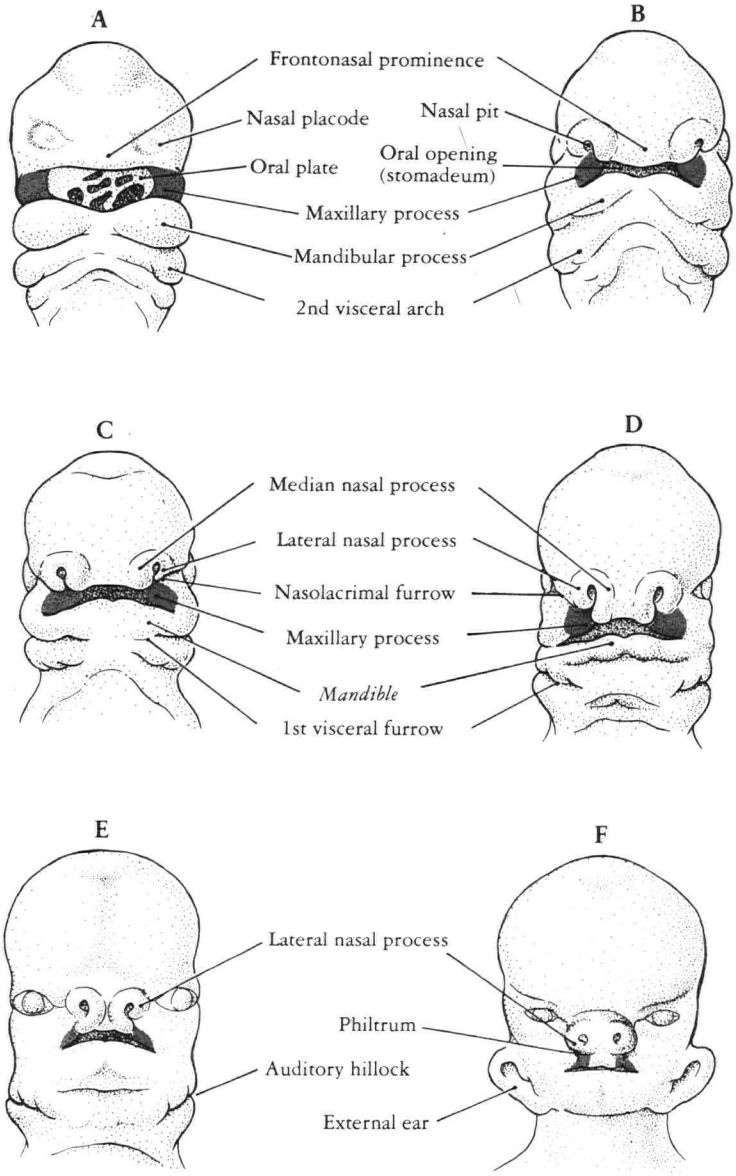
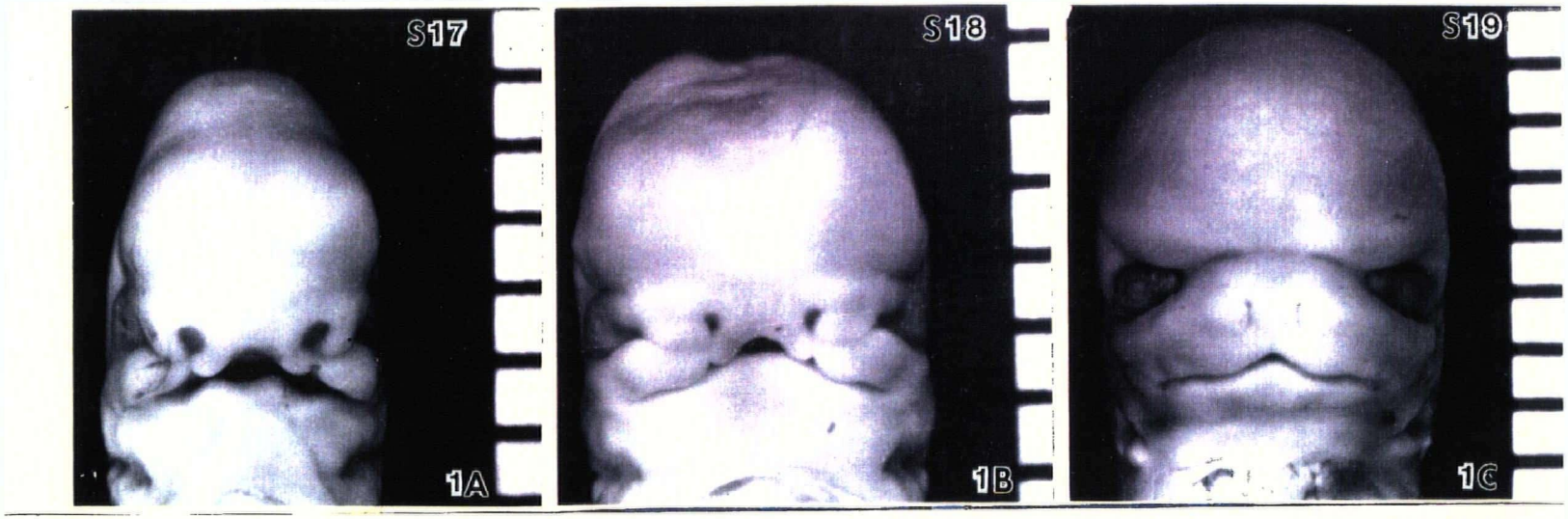


Diagram I: Frontal views of the human embryo showing development of the face. (From Foundations of Animal Development by Hopper A.F. and Hart N.H., 1980.)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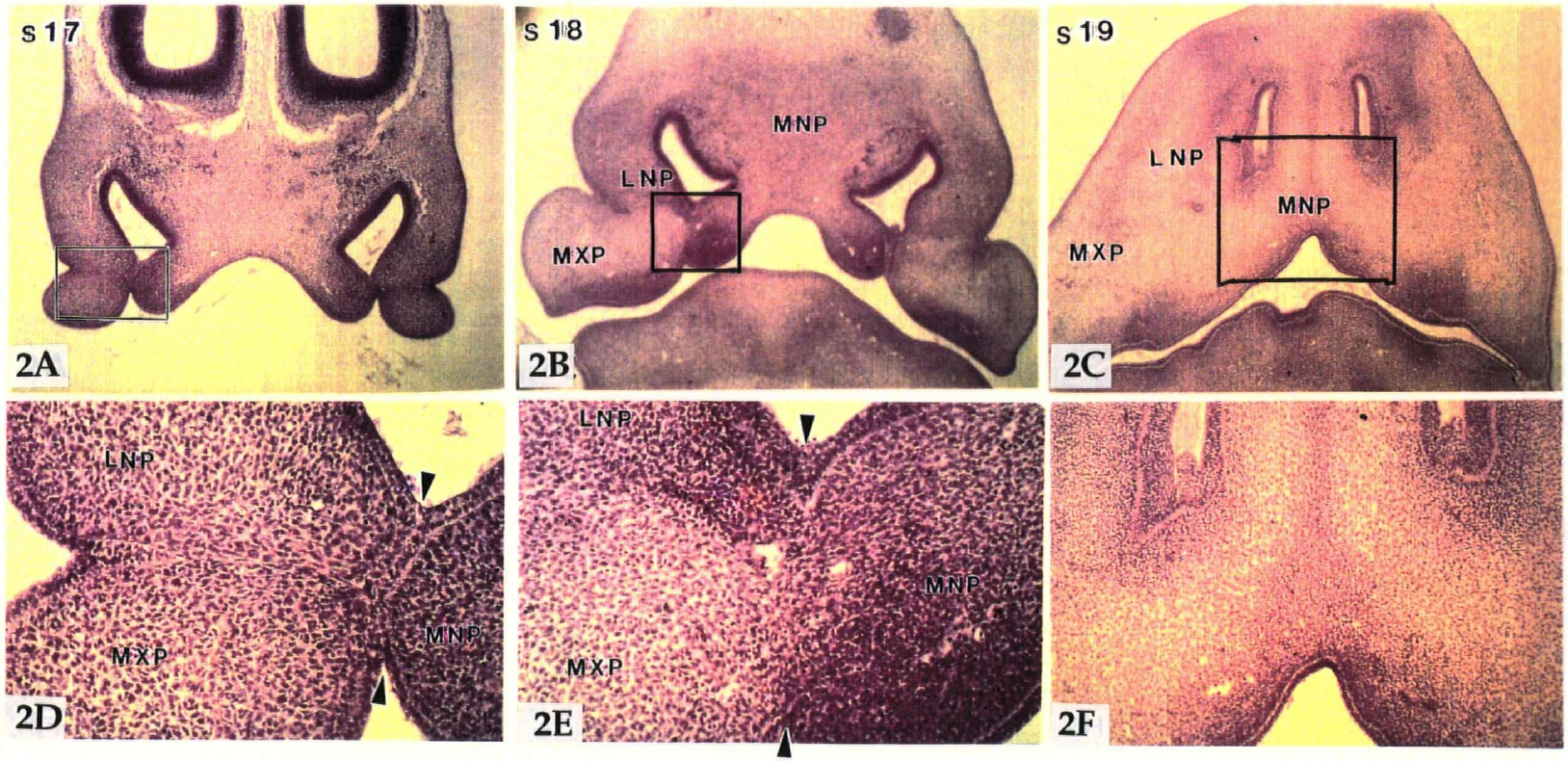
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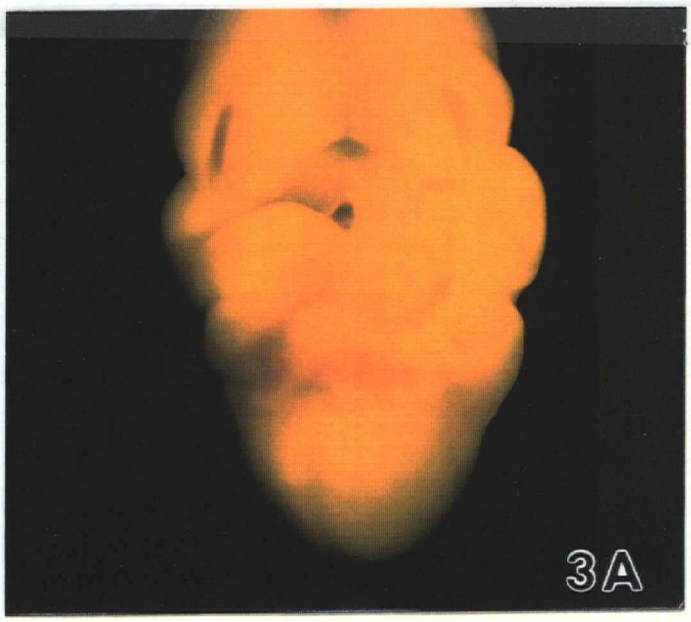
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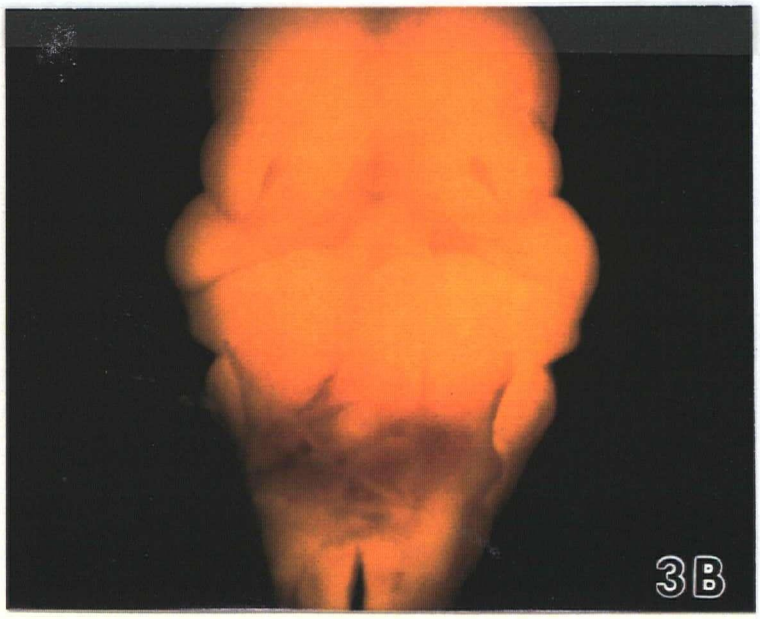
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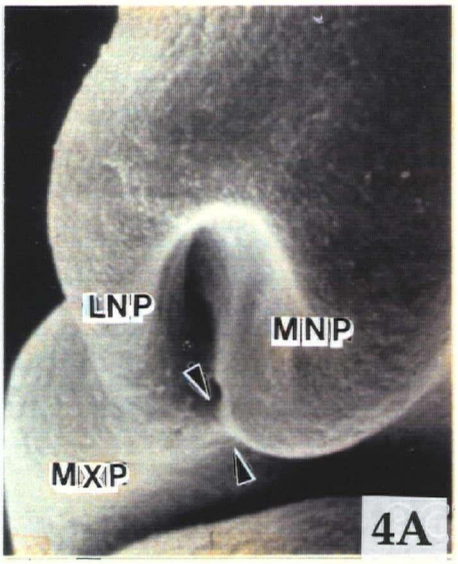
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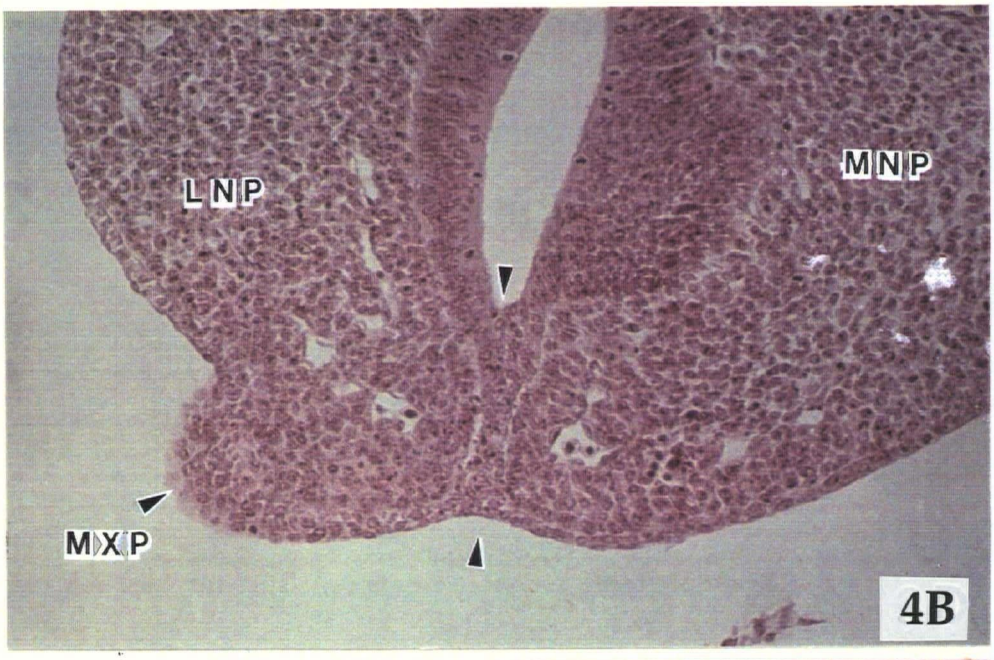
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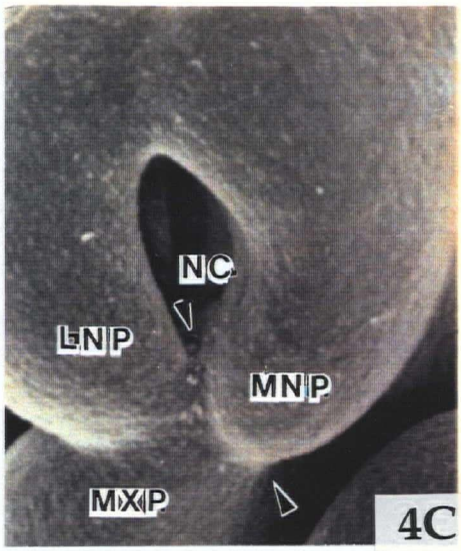
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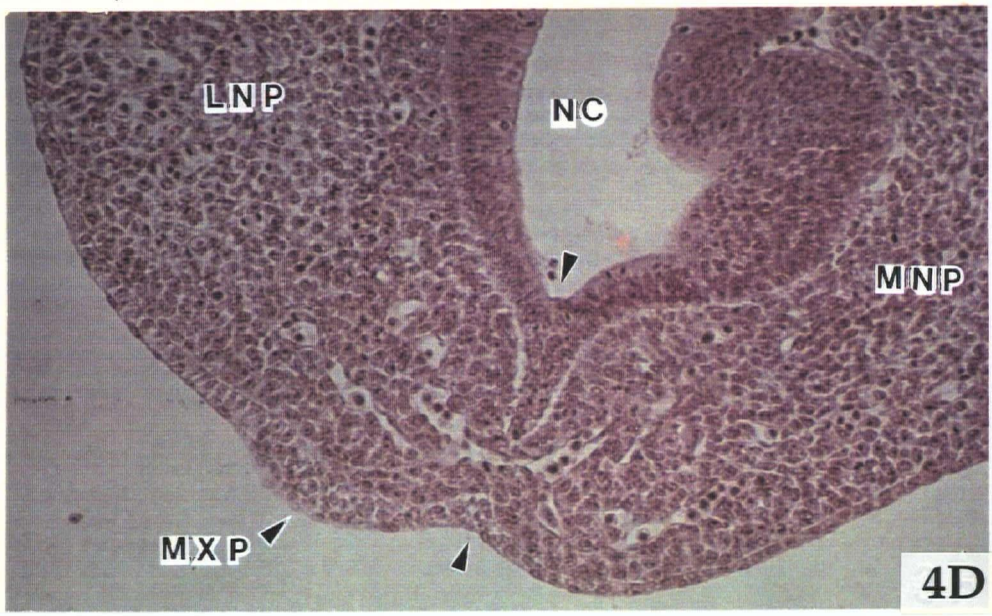
LNP
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MNP
MXP
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Figs. 1 A-C: Photographs of frontal views of the craniofacial morphology of the human embryos in the Kyoto Collection. At the stage (S)17 (A), the facial prominences have already made contact and formed the epithelial seam. At stage 18 (B), the facial prominences become more enlarged and completely form the primary palate at stage 19 (C). (modified from Diewert and Shiota, 1990)

Figs. 2 A-F: Frontal sections of human embryonic primary palate formation showing fusion between the medial nasal prominence (MNP) with the lateral nasal (LNP) and maxillary prominences (MXP) at early stage 17 (A). Higher magnification shows an epithelial seam (between arrows) (D). At stage 18, the epithelial seam becomes widely disrupted and replaced by a mesenchymal bridge (B). Higher magnification shows disruption of the epithelial seam with a replacement of a mesenchymal bridge (between arrows) (E). At stage 19, the mesenchymal bridge enlarges with the outgrowth of the primary palate (C). Grooves between prominences are markedly reduced. Higher magnification reveals a midline region of the primary palate (F). (modified from Diewert and Shiota, 1990)

Figs. 3 A,B: Photographs showing frontal views of the craniofacial morphology of mouse embryos during the stages of epithelial seam formation (A) and mesenchymal bridge formation (B).

Figs. 4 A-D: The scanning electron micrographs reveal the fusion between the medial nasal prominence with the lateral nasal and maxillary prominences (between arrows) during the stages of epithelial seam formation (A) and mesenchymal bridge formation (C). Frontal sections through the face showing an intact epithelial seam (between arrows) (B) and at the later stage, initial disruption of the epithelial seam and mesenchymal bridge formation (between arrows) (D). (modified from the Ph.D. thesis, Wang, 1992)

2. Morphogenesis of the heart

The heart, like the face, is formed from two types of mesenchyme: neural crest-derived ectomesenchymal and mesodermal. Cranial neural crest cells contribute not only to the mesenchyme of the branchial arches, but also to mesenchymal walls of the aortic arch arteries (Phillips *et al.*, 1987; Fukiishi and Morriss-Kay, 1992) and conduction tissue cells of the heart (Gorza *et al.*, 1988; Filogamo *et al.*, 1990). By using quail chick chimeras, neural crest cells presumptive for branchial arches 3, 4, and 6 were found distributed to the outflow tract region of the heart (Phillips *et al.*, 1987). Similarly, neural crest cells labelled with DiI between occipital somites 1 and 2 or 3 and 4 migrated within and dorsal to branchial arches 3 and 4 and into the outflow tract of the heart in rat embryos (Fukiishi and Morriss-Kay, 1992). Abnormal migration of cranial neural crest cells is believed to be the cause of DiGeorge anomaly which includes abnormalities of the craniofacial, thymus, parathyroid, thyroid, and cardiovascular systems (Lammer and Opitz, 1986).

Morphogenesis of the heart can be divided into two phases, the pretubular and tubular phases. The pretubular phase is initiated by anterior-lateral migration of the cardiogenic mesoderm cells from the primitive streak that leads to formation of the heart tube (Garcia-Martinez and Schoenwolf, 1993). The origin of the precursors of the cardiac myoblasts, however, remains controversial. In avian embryos, it was suggested that the precursors migrate into the prospective pericardial region of the intra-embryonic coelom from either side of the foregut diverticulum (Rosenquist and De Haan, 1966). In mouse embryos, it was found that the precursors develop *in situ* by the differentiation of coelomic mesothelial cells that line the wall of the prospective pericardial component of the intra-embryonic coelom (Kaufman and Navaratnam, 1981). More recently, by utilizing myosin immunostaining, it was proposed that the early rat

myocardium is a single epithelial unit lying in front of the prechordal plate and the heart tube is formed by cohesive movement of the myocardial epithelium (Suzuki *et al.*, 1995).

The heart primordium or cardiogenic plate is initially formed as a crescent-shaped zone of mesoderm cephalic to the embryonic disc at about embryonic day 15 in humans (Hopper and Hart, 1980) and embryonic day 7 in mice (Sissman, 1970). Subsequently, the heart primordium becomes canalized to form the endothelial tube on either side of the open gut. As the floor of the foregut is closed, the paired endothelial tubes are brought together and then fuse to form a single, endocardial tube lying in the midline. The endothelial tube is enveloped by the epimyocardial rudiments. Therefore, at this stage, the primitive heart tube is composed of two layers: an endocardium and a myocardium with ECM, the cardiac jelly, filled in between the two layers (Markwald *et al.*, 1984). As the heart further develops, the myocardium, particularly in the ventricles, loses its epithelial organization and forms trabeculations at about embryonic day 9 in mice shortly after the first myocardial contractions (Sissman, 1970). Subsequently, these trabeculations contribute to the interventricular septum and papillary muscles (Pexieder and Janecek, 1984; Hay *et al.*, 1984). In later development, the heart tube becomes regionalized, forming atria and ventricles. The endocardium in the regions of the atrioventricular canal and outflow tract undergoes epithelial-mesenchymal transformation (Manasek, 1976). As a result, endocardial cushion tissues are developed and give rise to septal and valvular structures (Van Mierop *et al.*, 1962). Formation of the endocardial cushion tissue is a biphasic process: initially, the endothelial cells become activated and subsequently, actively invade the underlying cardiac jelly (Runyan *et al.*, 1992). The endocardial cushion tissue will give rise to septal and valvular structures (Van Mierop *et al.*, 1962).

Extracellular matrix is believed to play an important role in heart development. The functions of the extracellular matrix include precardiac cell migration (Linask and Lash, 1988), epithelial-mesenchymal transformation (Icardo and Manasek, 1984; Mjaatvedt *et al.*, 1987; Litvin *et al.*, 1992), valve formation (Swiderski *et al.*, 1994), heart septation (Ahumada *et al.*, 1981), and epicardial formation (Giudice and Steinberg, 1981). Molecules known to be present in the cardiac jelly include hyaluronic acid (Manasek *et al.*, 1973), sulfated proteoglycan (Gessner *et al.*, 1965), fibronectin, laminin, types I-VI,VIII collagens (Kitten *et al.*, 1987; Little *et al.*, 1989; Kosher and Solursh, 1898; Drake *et al.*, 1990; Samuel *et al.*, 1994; Swiderski *et al.*, 1994), and elastin (Hurle *et al.*, 1994) (see Table II). Changes in the distribution of these ECM molecules occur throughout heart development. For example, during heart tube formation, fibronectin is found on the basal surfaces of the myocardium and endocardium. In later development, fibronectin increases in myocardium and endocardium at the onset of trabeculation and decreases as trabeculation is completed. These findings suggested that fibronectin is involved in cardiac trabeculation (Icardo and Manasek, 1983).

Table II: Distribution of ECM and other molecules during heart development.

Molecules	Localization	References
BMP-2A	atrial myocardium, AVC myocardium (mouse, days 9.5-14.5)	Lyons *et al.*, 1990

c-*met*	myocardium, endocardium, aorta (mouse, days 8, 9.5)	Andermarcher *et al.*, 1996
Collagen I	cardiac jelly (chick, stage 7)	Drake *et al.*, 1990
	subepicardium (chick, day 12)	Tidball 1992
Collagen II	between endocardium and epimyocardium (chick, stages 14-19)	Kosher and Solursh, 1989
	EC tissue, cardiac jelly, myocardium, valves (chick, stages 18-23)	Swiderski *et al.*, 1994
Collagen III	EC tissue, valves (chick, stages 18-23)	Swiderski *et al.*, 1994
	subepicardium (chick, day 8)	Tidball 1992
Collagen IV	cardiac jelly (chick, stages 7-9)	Drake *et al.*, 1990
	myocardium, endocardium (chick, stage 17)	Kitten *et al.*, 1987
Collagen V	EC tissue, valves (chick, stages 18-23)	Swiderski *et al.*, 1994
Collagen VI	EC tissue, epicardium, valves (chick, stages 28-40)	Hurle *et al.*, 1994

Collagen VIII	myocardium, valves	
	(mouse, days 11-17,	Sage and Iruela-Arispe, 1990
	chick stages 19-33)	Iruela-Arispe and Sage, 1991
CRABP-I	EC tissue	Dolle *et al.*, 1990
	(mouse, day 12.5)	
CRBP	EC tissue, epicardium	Dolle *et al.*, 1990
	(mouse, day 12.5)	
CT-1	myocardium	Sheng *et al.*, 1996
	(mouse, days 8.5-15.5)	
Elastin	OFT, EC tissue	Hurle *et al.*, 1994
	(chick, stages 22-29)	
	epicardium, valves	Hurle *et al.*, 1994
	(chick, stages 30-40)	
FGF-8	heart primordia	Crossley and Martin, 1995
	(mouse, days 7.75, 8.0)	
FGFR-1	heart primordia, myocardium	Sugi *et al.*, 1995
	(chick, stages 8-30)	
Fibronectin	myocardium, endocardium	Icardo and Manasek, 1983;
	(chick, stages 14-17)	Kitten *et al.*, 1987
	cardiac jelly, EC tissue	Icardo and Manasek, 1984
	(chick, stages 16-29)	
	pericardium, myocardium,	Tuckett and Morriss-Kay,
	endocardium	1986
	(rat, somite stages 4-16)	
HGF/SF	myocardium, endocardium	Andermarcher *et al.*, 1996
	(mouse, day 8)	

IGFs I & II	myocardium (chick, stage28)	Ralphs *et al.*, 1990
Interleukin-1α **(IL-1α)**	myocardium, endocardium, EC tissue (rat, days 10.5-14.5)	Nakagawa *et al.*, 1992
Laminin	cardiac jelly (chick, stages 9-15)	Drake *et al.*, 1990
	myocardium, endocardium (chick, stage 17)	Kitten *et al.*, 1987
	pericardium, myocardium, endocardium (rat, somite stages 8-16)	Tuckett and Morriss-Kay, 1986
MMP-2	myocardial cell lining (mouse, day 9)	Reponen *et al.*, 1992
Msx-1 (Hox-7)	EC tissue (mouse, day 10.5)	Robert *et al.*, 1989
RAR-α	myocardium, EC tissue (mouse, day 12.5)	Dolle *et al.*, 1990
RAR-β	no expression in heart	Dolle *et al.*, 1990
RAR-γ	EC tissue (mouse, day 12.5)	Dolle *et al.*, 1990
Rat interstitial **collagenase-3**	myocardium, endocardium, EC tissue (rat, days 10.5-14.5)	Nakagawa *et al.*, 1992

TGF-β1	EC tissue, endocardium (mouse, days 9.5, 10.5)	Akhurst *et al.*, 1990
TGF-β2	myocardium, AVC, OFT (mouse, days 9.5-12.5)	Dickson *et al.*, 1993
TGF-β3	OFT (mouse, day 8.5)	Dickson *et al.*, 1993
TIMP-3	myocardium, trabeculae (mouse, day 12.5)	Apte *et al.*, 1994
Urokinase PA	EC tissue, endocardium (quail, stage 17)	McGuire and Orkin, 1992
VCAM-1	epicardium, myocardium, intraventricular septum (mouse, days 8.75, 11.5)	Kwee *et al.*, 1995

AVC, atrio-ventricular canal; BMP, bone morphogenetic protein; *c-met*, HGF/SF receptor; CRABP, cellular retinoic acid binding protein; CRBP, cellular retinol binding protein; CT-1, cardiotrophin-1; EC, endocardial cushion; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; HGF/SF, hepatocyte growth factor/scatter factor; IGF, insulin-like growth factor; OFT, outflow tract; PA, plasminogen activator; RAR, retinoic acid receptor; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteases; VCAM-1, vascular cell adhesion molecule-1

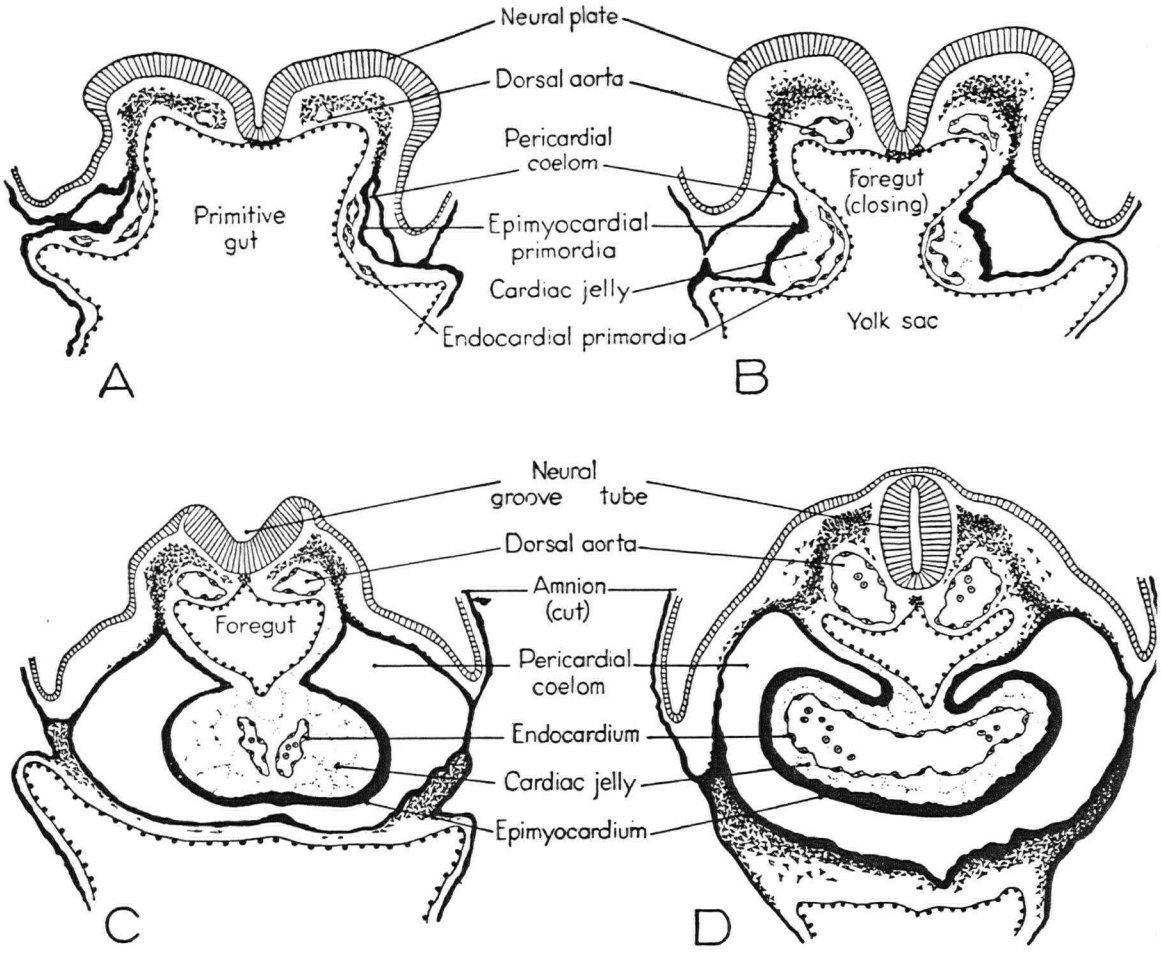


Diagram II: Transverse sections of human heart showing four stages in the fusion of the paired cardiac primordia. (From Patten's Human Embryology. Elements of Clinical Development. Corliss, 1976)

3. Morphogenesis of the eye

Eye morphogenesis is an important event during the craniofacial development. Often, the eyes are affected in craniofacial syndromes such as holoprosencephalies, fetal alcoholic syndrome, and cyclopia (see review in Johnston and Bronsky, 1995). The embryonic eye is formed as bilateral evaginations of the forebrain, the optic vesicles (human: 22d; mouse: 9d) (Mann, 1964; Hopper and Hart, 1980). The optic vesicles extend laterally until they make contact with the surface ectoderm of the head. Simultaneously, the surface ectoderm at the contact area becomes elongated and forms the lens placode. Subsequently, the lens placode and optic vesicle invaginate resulting in formation of the lens pit and double-layered optic cup. The lens pit continues to invaginate until it separates from the surface ectoderm and forms a hollow ball, the lens vesicle, embedded in the underlying mesenchyme (Fig. 4). Following formation of the lens vesicle, the epithelial cells at the posterior aspect, the posterior lens epithelium, are elongated and fill up the space of the lens vesicle, forming the lens fibers and producing lens-specific proteins, crystallins. As the optic cup forms, the inner cell layer differentiates into the neural retina, while the outer cell layer differentiates into the retinal pigment epithelium, producing melanin. The optic stalk provides a pathway for the future optic nerve (for reviews see McAvoy, 1980; Piatigorsky, 1981; Kaufman, 1992).

Invagination of the lens ectoderm is believed to involve a multistep process of tissue interactions and inductions. Initially, lens induction was speculated to depend on specific interactions between the presumptive lens ectoderm and optic vesicle (Spemann, 1901; Lewis, 1907). However, later experiments have shown that other tissues can also induce lens formation from the presumptive lens ectoderm (Karkinen-Jaaskelainen, 1978; Mizuno, 1972). These findings suggested that the presumptive lens ectoderm is predetermined

and capable of differentiation into lens under the appropriate conditions (Piatigorsky, 1981). Many mechanisms of invagination of the lens ectoderm have been proposed. These mechanisms include intracellular forces mainly from actin microfilaments (Wrenn and Wessells, 1969), forces generated by cell population pressure (Zwaan and Hendrix, 1973), localized cell death (Silver and Hughes, 1973), asymmetric migration of cells into the placode (Bancroft and Bellairs, 1977), and a close proximity of optic-vesicle ECM constituents which provide a stimulus for the basal growth of presumptive lens ectoderm (Parmigiani and McAvoy, 1984; Peterson *et al.*, 1995).

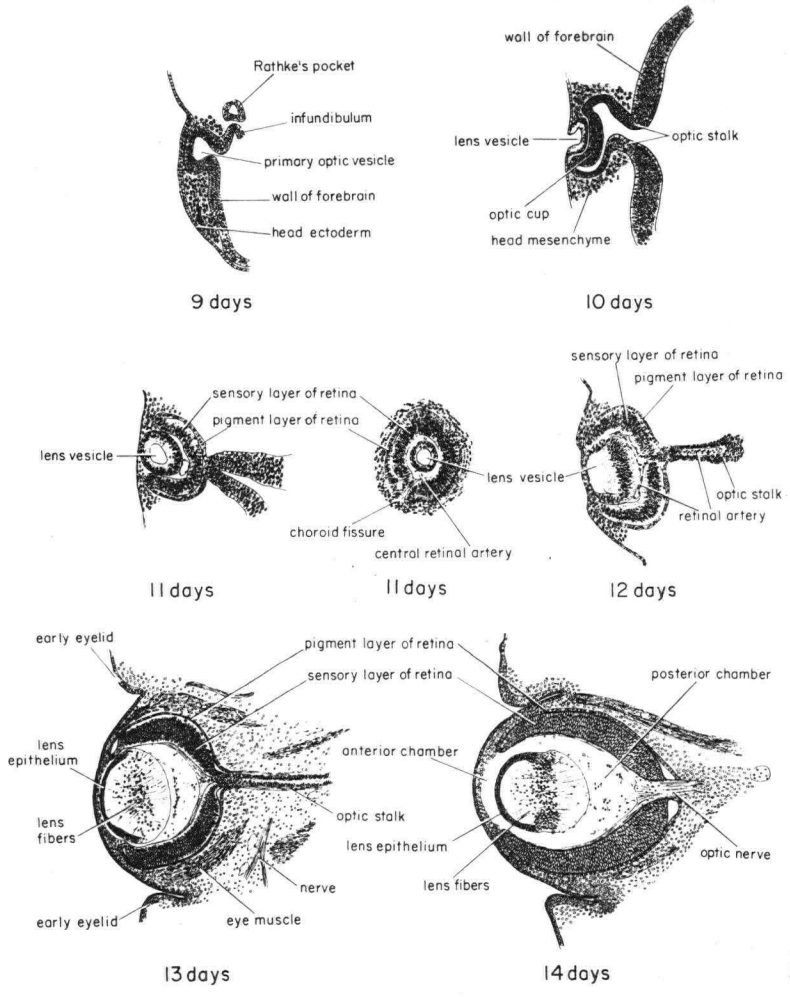


Diagram III: Sections of the developing eye of 9-to14-day-old mouse embryo. (from The mouse. Its Reproduction and Development. Rugh, 1968)

4. Extracellular matrix and its degradation in craniofacial and heart development

Tissue remodelling is believed to be important for embryogenesis particularly during cell migration, proliferation and differentiation and tissue outgrowth (Matrisian, 1992; Werb *et al.*, 1992). Many organ systems show changes of ECM molecules during their development. The mechanism of remodelling of ECM is thought to be involved with matrix metalloproteinases (MMPs) because MMPs are found to be physiologically and pathologically capable of degrading a wide variety of ECM molecules (reviewed by Birkedal-Hansen *et al.*, 1993; Overall, 1994). MMPs have been found to be expressed in a number of developmental events, for example blastocyst outgrowth (Behrendtsen *et al.*, 1992), tooth morphogenesis (Overall and Limeback, 1988; Sahlberg *et al.*, 1992; Heikinheimo and Salo, 1995), and branching morphogenesis of the salivary gland (Nakanishi *et al.*, 1986; Hayakawa *et al.*, 1992). The expression of 72-kDa gelatinase, in particular, was studied by *in situ* hybridization and found in the mesenchyme of the developing branchial arches, cornea, limb, kidney, lung, and bone (Reponen *et al.*, 1992). These data suggest that MMPs may play a significant role in tissue remodelling in the embryo.

The basement membrane acts as an interface between histologically dissimilar tissues that arise from different primary germ layers. Ultrastructurally, the basement membrane can be divided into three major zones; (1) the lamina lucida, an electron lucent zone 25-50 nm beneath the plasma membrane, (2) the lamina densa, an amorphous or finely fibrillar electron-dense layer lying beneath the lamina lucida and extending for a thickness of 20-50 nm, and (3) the basement membrane reticulum, a fibrillar area below the lamina densa containing bundles of microfibril-like structures, anchoring fibrils (see review in Burgeson, 1993). The major molecular components of basement membranes are type IV collagen, laminin, entactin, and heparan sulfate proteoglycan.

Immunoelectron microscopic studies revealed a preferential localization of type IV collagen, laminin, proteoglycan, and entactin in the lamina densa with occasional projections into the lamina lucida (see review in Timpl and Dziadek, 1986). These components are present in virtually all basement membranes, although their proportions may vary among basement membranes (see review in Furthmayr, 1993). Other basement membrane components including fibronectin, SPARC, amyloid P, some complement components, and molecular isoforms of laminin and type IV collagen, are more restricted in their distribution (see reviews in Timpl, 1989; Fitch and Linsenmayer, 1994).

In development, some the major components of the basement membrane are present in mesenchyme. Type IV collagen is present not only in basement membranes, but also in the mesenchyme of the limb bud (Solursh and Jensen, 1988), tooth mesenchyme (Heikinheimo and Salo, 1995), and cardiac jelly of the heart (Little *et al.*, 1989). Similarly, laminin was also detected in the mesenchyme of the mandibular arch and the guidance pathway of the trigeminal axon in chicken embryos (Riggott and Moody, 1987), the cranial mesenchyme (Tuckett and Morriss-Kay, 1986), and early ganglia and nerve roots (Rogers *et al.*, 1986).

Tissue interactions are defined as events in which dissimilar cell populations act on one another to alter cell behavior in developmentally significant ways. It is believed that every organ in the adult body of many species arises as a result of tissue interactions (Wessells, 1977). The basement membrane has been speculated to play a crucial role in epithelial-mesenchymal interactions during embryogenesis (Grobstein, 1954; Bernfield *et al.*, 1984). Alterations of the basement membrane components are important for branching morphogenesis of the salivary gland (Bernfield *et al.*, 1984) and the lung (Grant *et al.*, 1983; Schuger *et al.*, 1990). Degradation of the basement membrane is believed to allow the transmission of inductive signals from one tissue to another, for example in

formation of kidney tubules (Wartiovaara *et al.*, 1974; Ekblom, 1981) and tooth morphogenesis (Thesleff, 1981; Sahlberg *et al.*, 1992). Epithelial-mesenchymal interactions have been found to be important for facial morphogenesis in chick embryos (Wedden, 1987; Richman and Tickle, 1989,1992). The facial ectoderm, particularly, is required for the outgrowth and accompanying differentiation of cartilage to form rod-like structures within the frontonasal mass and mandible in chick embryos (Wedden, 1987; Richman and Tickle, 1989). Recombinations between epithelium and mesenchyme from different prominences showed that the epithelia are interchangeable and appear to be equivalent (Richman and Tickle, 1989). It was also found that removal of the epithelium from the maxillary prominence explant disturbed the continued growth of the explant (Saber *et al.*, 1989). Recombination experiments from maxillary epithelium-maxillary mesenchyme, limb apical ectodermal ridge-maxillary mesenchyme, and stage 28 epithelium-stage 22 mesenchyme indicated that direct epithelial-mesenchymal contact and normal architecture of the interface between the two tissues are important for the viability of the tissue explant. Therefore, it was suggested that the basement membrane within the tissue interface may influence epithelial-mesenchymal interactions. Each of the major extracellular molecules will be discussed in the next section.

4.1 Laminin

Laminin is the most abundant glycoprotein in the basement membrane composed of three genetically distinct polypeptide chains referred to as α (A) (400 kDa), β (B1) (220 kDa), and γ (B2) (200 kDa) chains. These three chains are connected to each other by disulfide bonds (Timpl, 1989; Burgeson *et al.*, 1994). Laminin is synthesized by different types of cells including epithelial, and

endothelial cells, muscle cells and fibroblasts (Paulsson, 1992). Molecules known to bind laminin include laminin, type IV collagen, heparan sulfate proteoglycan, entactin, heparin, cell-surface glycoproteins, sulfatides, and gangliosides (Kleinman *et al.*, 1987). Laminin interacts with cells via the transmembrane glycoprotein, integrin. This interaction is believed to mediate the signal to the cytoskeleton and influence cell proliferation and differentiation (Paulsson, 1992). So far, eight different laminin chains; including α1, α2 (M), α3 (K), β1, β2 (S), β3, γ1, and γ2 (B2t); and seven different heterotrimeric assembly forms; including laminins-1, -2, -3, -4, -5, -6, and -7; have been characterized (see reviews in Burgeson *et al.*, 1994; Timpl and Brown, 1994). These findings suggest that the functional diversity of basement membranes arises in part from the particular laminin isoforms they contain (Miner and Sanes, 1994). For example, laminin α chain polypeptide was mainly detected in basement membranes of epithelial cells, suggesting that this chain is important for morphogenesis of the epithelial sheets (Klein *et al.*, 1990). Renal glomerular basement membrane contains α, β2 and γ chains, while basement membrane of the extrasynaptic muscle contains α2, β and γ chains (Sanes *et al.*, 1990). Antibodies are available to various isoforms of laminin (Miner and Sanes, 1994; Durham and Snyder, 1995).

Laminin appears as the first ECM protein during embryogenesis as early as the two-cell stage (Dziadek and Timpl, 1985). Only the β and γ polypeptides are synthesized until the 8-cell stage. Then an α chain starts to appear from the 16-cell stage (Cooper and MacQueen, 1983). Laminin plays a diverse role in numerous biological activities including neurite outgrowth, cell adhesion, proliferation, migration, epithelial cell differentiation, phagocytosis, angiogenesis, and tumor metastases (see reviews in Ekblom, 1993; Kleinman *et*

al., 1993; Yurchenco, 1994). Laminin is suggested to be important for embryonic lung morphogenesis since anti-laminin-treated explants showed a marked inhibition of branching morphogenesis and a distortion of the bronchial tree (Schuger *et al.*, 1990, 1991). Different temporal patterns of laminin α, β and γ chains during lung development suggested that each chain is independently regulated and may play important, but different roles in fetal lung development (Durham and Snyder, 1995). In kidney development, when the mesenchyme transforms into the epithelium and forms tubular structures, the mesenchyme expresses primarily the laminin β and γ chains, based on immunofluorescence, Western and Northern blot analyses (see review in Ekblom, 1993). Later, as the epithelial cells develop, the mRNA expression of laminin α1 chain increases. These findings were confirmed by *in situ* hybridization results in which the expression of the α1 chain was mainly localized to areas of developing epithelial cells (Ekblom *et al.*, 1990). These data suggest that local cell-cell interactions enhance production of laminin chains and epithelial cell polarization may be associated with the presence of laminin containing the α chain (Ekblom, 1993). Recently, mutations in human γ2 chain gene was found to be correlated with a skin disease, epidermolysis bullosa (Pulkkinen *et al.*, 1994).

4.2 Type IV collagen

Type IV collagen is the principal collagenous component found as a molecular network in the basement membrane. One of the major functions of type IV collagen is to provide mechanical support for the tissues (Timpl, 1989). Type IV collagen also possesses binding activities for ECM such as laminin, proteoglycan, entactin (nidogen), and cells (Timpl, 1989). Type IV collagen

comprises three polypeptide chains [α1(IV)2 α2(IV)]. Recently, variant type IV collagen α3, α4, α5 and α6 chains have been identified and found in a more restricted tissue distribution (Hudson *et al.*, 1993). For example, the α3 and α4(IV) collagen chains are present in the synaptic basal lamina at the neuromuscular junction, while the α1 and α2(IV) collagen chains are found extrasynaptically (Sanes *et al.*, 1990). In rat kidney development, α1 and α2(IV) collagen chains and the laminin B1 appear to be fetal components of the glomerular basement membrane. Subsequently, there is a developmental switch to α3-α5(IV) chains and S-laminin in mature glomerular basement membrane (Miner and Sanes, 1994).

Type IV collagen is believed to be the first collagen to appear in the embryo. It was found in the inner cell mass of the 3- to 4-day-old blastocysts (Leivo *et al.*, 1980) and subsequently in the basement membranes of the ectoderm and endoderm, notochord and neural tube (Hay, 1991). Immunohistochemical studies revealed that the pathways for neural crest migration not only contain fibronectin, type I collagen, laminin, heparan sulfate proteoglycan and entactin but also type IV collagen. Studies of neural crest cell migration on various substrates *in vitro* demonstrated that type IV collagen, laminin, fibronectin and type I collagen permit migration whereas type II collagen does not (Fitch and Linsenmayer, 1994). Type IV collagen and laminin were implicated as important for endothelial cell attachment and polarization during embryonic angiogenesis (Grant *et al.*, 1990). Mutation of type IV collagen gene, that causes impairment of triple helix stability, leads to embryonic lethality in *C. elegans* (Guo *et al.*, 1991).

4.3 Fibronectin

Fibronectin is a glycoprotein characteristically present in adult connective-tissue stroma and embryonic mesenchyme. The protein comprises a dimer of two similar 250-kDa subunits held together by a pair of disulfide bonds at their carboxyl termini. Fibronectin is encoded by a single gene. However, several isoforms occur as a result of an alternative splicing of its precursor mRNA (Yamada, 1991). Fibronectin is not only found in the ECM of the mesenchyme but also in basement membranes of the embryo (Stenman and Vaheri, 1978; Timpl and Martin, 1982; Yamada, 1991). However, the origin of basement membrane fibronectin remains controversial. Since fibronectin is present in plasma, therefore fibronectin can be deposited in the basement membrane by simple infiltration. Alternatively, fibronectin can be synthesized by cells resting on the basement membrane (Liotta *et al.*, 1986).

During mouse embryogenesis, fibronectin is first detected in the inner cell mass during primitive endoderm formation and at the the interface between the trophectoderm and the parietal endoderm (Zetter and Martin, 1978: Leivo *et al.*, 1980). Subsequently, fibronectin is present in between the mesodermal and ectodermal layers (Wartiovaara *et al.*, 1979). Later in development, fibronectin has been found to become associated with different tissues. Thus, it is speculated that fibronectin plays different roles in different regions of the embryo at specific periods of time (Thiery *et al.*, 1989). These include cell adhesion, cell proliferation, migration of mesenchymal and neural crest cells, cell differentiation, spreading and growth of endothelial cells and structural organization of embryonic matrix (Bronner-Fraser, 1986; Yamada, 1991; Lesot *et al.*, 1990; Thiery *et al.*, 1989; Hay, 1991). Recently, fibronectin null homozygous mouse embryos were generated and found to implant and initiate gastrulation normally (George *et al.*, 1993). However, in later development various detected

abnormalities were found including deformed neural tubes, absence of notochord and somites, deformed vessels and heart, and severe defects in mesodermally-derived tissues all of which led to early embryonic lethality. These findings suggested that these abnormalities arise from fundamental deficits in mesodermal migration, adhesion, proliferation or differentiation as a result of the absence of fibronectin.

4.4 Remodelling of the ECM

During embryonic development, ECM molecules, which are the major components of the tissue stroma, have to be locally and co-ordinately degraded and synthesized in order to facilitate morphogenesis.

The basement membrane is a good example for remodelling of the ECM since it is found to be rapidly remodelled during embryonic development (Bernfield, 1981; Timpl and Dziadek, 1986). There are a number of situations in which the basement membrane is completely degraded as the organs or structures undergo regression, differentiation or invasion. For example, type IV collagen, laminin, heparan sulfate proteoglycan, and fibronectin were found to become irregular and discontinuous, as the male Mullerian duct regresses (Ikawa *et al.*, 1984). These findings indicate that degradation of the ECM around the male Mullerian duct is a prior event to regression of the structure. During early tooth development, the basement membrane between the enamel organ and the dental papilla is thought to play a role in mediating epithelio-mesenchymal interactions, resulting in odontoblast differentiation. In the late bell stage of tooth development, when the odontoblasts secrete predentine, the dental basement membrane degrades. Subsequently, the preameloblast undergoes differentiation (Hurmerinta and Thesleff, 1981; Thesleff and Hurmerinta, 1981; Sahlberg *et al.*, 1992). During the involution of the mammary gland, the

basement membrane around the secretory alveoli becomes discontinuous and degraded as the secretory alveolar structures degenerate (Warburton *et al.*, 1982). In early secondary palate formation, the midline epithelial seam is formed by fusion of the medial edge epithelia of the palatal shelves (Ferguson, 1988). Subsequently, the epithelial seam as well as the basement membrane becomes disrupted with ingrowth of the mesenchymal tissue. Breakdown of the seam basement membrane was demonstrated by immunolocalization of type IV collagen (Ferguson, 1988) and laminin (Shuler *et al.*, 1992). Similar events, in which the basement membrane is degraded, have also found in ductal growth of the submandibular gland (Bernfield *et al.*, 1984), angiogenesis (Form *et al.*, 1986), and tumor invasion and metastasis (Barsky *et al.*, 1983). However, the molecular events of ECM remodelling during embryonic development are addressed in the present work.

5. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs)

In general, degradation of the extracellular matrix involves at least four distinct pathways including plasminogen-dependent, matrix metalloproteinase, phagocytic, and osteoclastic pathways (see review in Birkedal-Hansen *et al.*, 1993). This review focuses on the matrix metalloproteinase pathway because it is thought to play a major role in tissue remodelling during the embryonic development.

5.1 MMPs and TIMPs

Matrix metalloproteinases (MMPs) are believed to play a major role in physiological degradation of ECM because they are found to be present in various types of both adult and embryonic cells and tissues and able to degrade a wide variety of ECM molecules including type I-V, VII-X collagens, gelatin, fibronectin, laminin, proteoglycans, and elastin (see Table III). MMPs are classified into the same family on the basis of extracellular substrate specificity, zinc dependence, inhibition by tissue inhibitors of metalloproteinases (TIMPs), secretion as a zymogen, and DNA sequence relatedness (Matrisian, 1990; Alexander and Werb, 1991). MMP family is composed of four members; (1) the collagenases, (2) gelatinases/type IV collagenases, (3) stromelysins, and (4) membrane-type MMPs (MT-MMPs) (Table III). The collagenases specifically degrade native type I-III collagens to produce thermally unstable degradation fragments. Upon collagen denaturation, gelatin and the collagenase cleavage products can be further degraded by other MMPs including gelatinases and stromelysins (reviewed by Overall, 1994). The 72-kDa (MMP-2) and 92-kDa type IV gelatinases (MMP-9) also degrade native types IV,V (Fessler *et al.*, 1984), VII (Seltzer *et al.*, 1989), X (Welgus *et al.*, 1990) collagens and elastin (Senior *et al.*,

1991). Although 72-kDa gelatinase can degrade fibronectin and laminin, 92-kDa gelatinase does not (Okada *et al.*, 1990; Nagase *et al.*, 1991). Stromelysins have a broader substrate specificity including proteoglycans, laminin, fibronectin, gelatin, and the globular portion of type IV collagen (Galloway *et al.*, 1988; Nicholson *et al.*, 1989).

Recently, nucleotide and amino acid sequences of human and mouse 72-kDa gelatinases were characterized. It was found that the overall amino acid sequence similarity between the mouse and human enzymes proper (active enzyme form) is 96.6%. The zinc-binding domain is 100% identical. The sequence of the mouse carboxyl terminus contains two residues more than the human enzyme (Reponen *et al.*, 1992). Human 92-kDa gelatinase exhibits 49% homology to human 72-kDa gelatinase (Wilhelm *et al.*, 1989).

Table III: MMP family members.

MMPs	Substrates
I. Collagenases	
-Interstitial collagenase	collagens I,II,III,VII,
(MMP-1, fibroblast collagenase)	VIII,X, gelatin
-PMN collagenase	collagens I,II,III,VII,
(MMP-5)	VIII,X, gelatin
II. Gelatinases	
-Gelatinase A	gelatin, collagens IV,V,
(MMP-2, 72-kDa gelatinase,	VII,X,XI, elastin,
72-kDa type IV collagenase)	fibronectin, laminin

-Gelatinase B	gelatin, collagens IV,V,
(MMP-9, 92-kDa gelatinase,	elastin, fibronectin
92-kDa type IV collagenase)	
III. Stromelysins	
-Stromelysin 1	proteoglygans, laminin,
(MMP-3, transin)	fibronectin, gelatin,
	collagens IV,V,IX,X
-Stromelysin 2	proteoglycans, laminin,
(MMP-10, transin-2)	fibronectin, gelatin,
	collagens III,IV,V,IX
-Stromelysin 3	?
(MMP-11)	
-Matrilysin	proteoglycans, gelatin,
(MMP-7, PUMP-1)	fibronectin, elastin,
	laminin, collagen IV
IV. Membrane-type MMPs (MT-MMPs)	
-MT-MMP-1	progelatinase A
-MT-MMP-2	progelatinase A

(Modified from Matrisian,1992; Birkedal-Hansen *et al.*,1993; and Birkedal-Hansen, 1995)

Regulation of MMP activity occurs at different levels of expression. At the transcriptional level, many growth factors, cytokines, oncogene products, tumor promoters, and hormones have been found to either stimulate or suppress transcription of MMPs (see reviews in Woessner, 1991; Birkedal-Hansen *et al.*, 1993). For example, 12-O-tetradecanoyl phorbol 13-acetate (TPA) can affect

transcription of MMPs via the AP-1/TRE binding site (Angel *et al.*, 1987). MMPs are secreted as a latent, inactive form and can be *in vitro* activated by many mechanisms including treatment with organic mercurides, chaotropic agents, or proteases. For example, trypsin was found to be able to activate almost all MMPs (Liotta *et al.*, 1979; Salo *et al.*, 1983; Saari *et al.*, 1990), while chymotrypsin can activate stromelysin-1 (Nagase *et al.*, 1990) and neutrophil collagenase (Saari *et al.*, 1990). Activation of the precursors of interstitial collagenase and stromelysin can occur via a plasminogen-plasmin-MMP cascade (Chapman *et al.*, 1988; Thomson *et al.*, 1989). Activated stromelysin, in turn, can degrade collagenase into highly active collagenase (Nagase *et al.*, 1991). Therefore, it was proposed that, *in vivo*, the precursors of interstitial collagenase and stromelysin are activated by a protease cascade mechanism (Diagram III) (Matrisian, 1992).

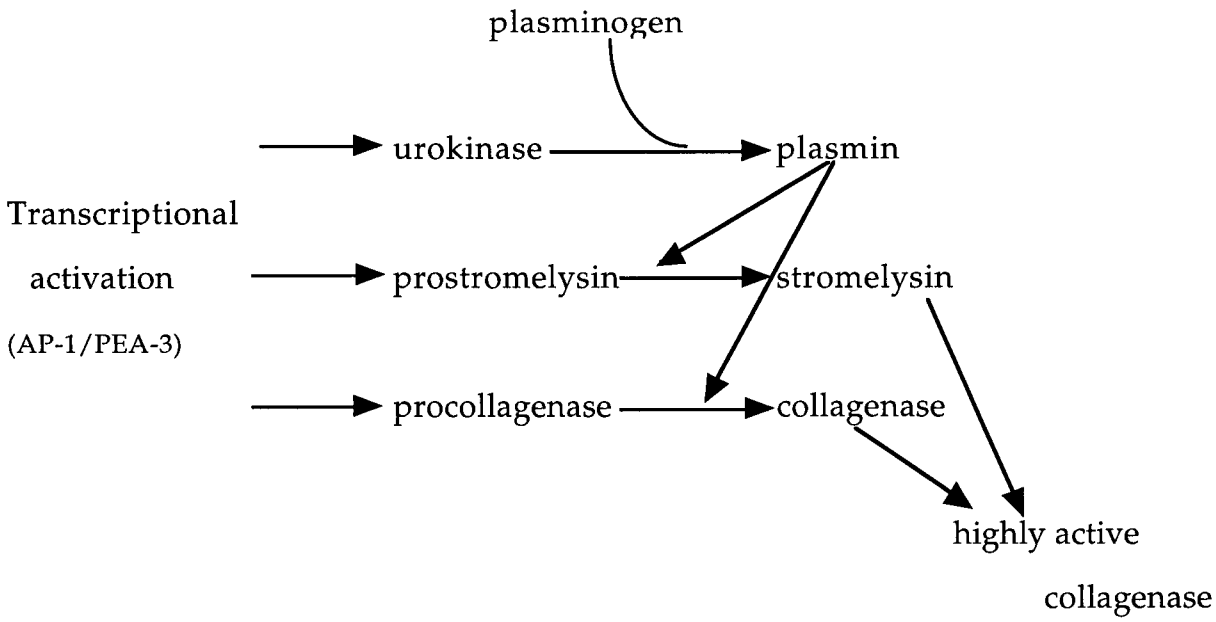


Diagram III: A protease cascade of activation of MMPs *in vivo* (Matrisian, 1992).

The *in vivo* mechanism of activation of progelatinase A is believed to be different from the other types of MMPs as shown above. Indeed, activation of progelatinase A has been found to be plasma membrane-dependent (Ward *et al.*, 1994) and associated with a new member of MMPs, membrane-type MMP (MT-MMP-1) (Sato *et al.*, 1994; Strongin *et al.*, 1993, 1995). MT-MMP-1 is an integral plasma membrane protein with the molecular weight of 66 kDa. It was found that expression of MT-MMP-1 on the cell surface induces specific activation of progelatinase A (Sato *et al.*, 1994). Mt-MMP-1 was also found to be expressed in the human lung carcinoma (Sato *et al.*, 1994). Strongin *et al.* (1993,1995) have demonstrated that MT-MMP-1 acts as a cell surface receptor for TIMP-2. By binding its carboxyl-end domain to MT-MMP•TIMP-2 complex at the cell surface, the proenzyme of 72-kDa gelatinase becomes activated. More recently, MT-MMP-2 cDNA was isolated from a human placenta cDNA library (Takino *et al.*, 1995). Like MT-MMP-1, MT-MMP-2 can induce processing of progelatinase A into the activated forms.

TIMPs are molecules found to be able to form non-covalent bimolecular complexes with the active forms of MMPs and, in some instances, with latent MMP precursors. Thus, it was implicated that the local activity of MMPs is regulated by TIMPs (see review in Matrisian, 1992). TIMP-1 (Cawston, 1986) inhibits all MMPs as does TIMP-2 (Stetler-Stevenson *et al.*, 1989). TIMP-2, however, is 2 to 10 fold more effective than TIMP-1 at inhibiting 72-kDa and 92-kDa gelatinases; whereas interstitial collagenase is inhibited by TIMP-1 more than twofold more effectively than it is by TIMP-2 (Howard *et al.*, 1991). Recently, the primary structure of mouse TIMP-2 was characterized and found to have extraordinarily high homology (97%) to human TIMP-2, suggesting a fundamental biological role of the protein (Shimizu *et al.*, 1992). Besides inhibition of MMP activities, both TIMP-1 and TIMP-2 have a potent growth-

promoting activity for a wide range of cells. This activity was suggested to be a direct cellular effect mediated by a cell surface receptor (Hayakawa, 1994). TIMP-3 (ChIMP-3, chicken inhibitor of metalloproteinases) was recently characterized (Apte *et al.*, 1994) and appeared to have similar fuctions as TIMP-1 and TIMP-2 (Hayakawa, 1994). Very recently, a novel TIMP-4 was cloned from a human heart cDNA library (E. Shi, personal communication). Its function remains to be determined.

MMPs and TIMPs have been detected in a wide variety of embryonic tissues and suggested to play an important role in tissue invasion, cell migration, proliferation, and differentiation during embryogenesis. There are numerous examples of expression of ECM-degrading proteinases and/or TIMPs in developmental processes including ovulation (Curry *et al.*, 1990), embryonic pre- and peri-implantations (Brenner *et al.*, 1989; Behrendtsen *et al.*, 1992; Lefebvre *et al.*, 1995; Harvey *et al.*, 1995; Reponen *et al.*, 1995; Canete-Soler *et al.*, 1995), endoderm differentiation (Adler *et al.*, 1990; Brenner *et al.*, 1989), branching morphogenesis of salivary gland (Nakanishi *et al.*, 1986; Hayakawa *et al.*, 1992) and lung (Ganser *et al.*, 1991), involution of the tadpole tail (Gross and Bruschi, 1971), bone and cartilage formations (Dean *et al.*, 1985; Edwards *et al.*, 1992; Flenniken and Williams, 1990; Apte *et al.*, 1994), neurite extension (Pittman, 1985; Muir, 1994), tooth morphogenesis (Overall and Limeback, 1988; Nomura *et al.*, 1989; Sahlberg *et al.*, 1992), heart development (Nakagawa *et al.*, 1992; McGuire and Orkin, 1992), retina development (Sheffield, 1992; Sheffield *et al.*, 1994), myogenesis (Guerin and Holland, 1995), secondary palate development (Brinkley *et al.*, 1995; Du *et al.*, 1996; Morris-Wiman *et al.*, 1996), mammary gland development (Talhouk *et al.*, 1991, 1992; Dickson and Warburton, 1992; Sympson *et al.*, 1994) and mandibular condyle development (Breckon *et al.*, 1994).

Collectively, MMPs and TIMPs are suggested to play a significant role in embryogenesis. However, the *in vivo* mechanism of tissue remodelling remains speculative and circumstantial. Involvement of MMPs and TIMPs during pre- and peri-implantation and blastocyst outgrowth of the mammalian embryo has been extensively studied. At about 4.5 days p.c. in mice, the trophectodermal cells of the blastocyst penetrate the uterine epithelium and its basement membrane and then invade the uterine decidual stroma. Studies of embryonic implantation in culture have been studied by placing the blastocyst on a complex ECM (Glass *et al.*, 1983). Following attachment of the blastocyst to the ECM, trophoblast cells migrate out and invade the ECM by degrading it. Several MMPs, including collagenase, stromelysin, 72-kDa and 92-kDa gelatinases, uPA and TIMP-1,-2,-3 are secreted by trophoblast cells (Brenner *et al.*, 1989; Behrendtsen *et al.*, 1992; Harvey *et al.*, 1995). Exogenous TIMP-1 was found to stimulate the migration of parietal endoderm-like cells from the blastocyst outgrowths (Werb *et al.*, 1992). These data suggest that the ability of the embryo to invade the uterine epithelium and stroma is, at least in part, regulated by MMPs and TIMPs (Matrisian, 1990). By *in situ* hybridization, Reponen *et al.* (1995) and Canete-Soler *et al.* (1995) found that 92-kDa gelatinase but not 72-kDa gelatinase was strongly expressed in invading trophoblasts. TIMP-3 but not TIMP-1 or TIMP-2 was intensely expressed in maternal cells in the area surrounding the invading embryonic tissue (Reponen *et al.*, 1995). These results suggested a cooperative role between 92-kDa gelatinase and TIMP-3 in the ECM proteolysis associated with implantation of the early embryo. During pre-implantation development, it was found that gelatinases are markedly induced by TGF-α *in vitro* (Dardik *et al.*, 1993). These data suggest that upon stimulation by TGF-α, gelatinases, secreted from the blastocyst, may be involved in blastocoel ECM remodelling and

migration of the parietal endoderm cells. More recently, growth factors and cytokines, particularly EGF and leukemia inhibitory factor (LIF), were found to stimulate uPA and MMP-9 activities in the blastocyst cultures during the early, highly invasive phase of implantation (Harvey *et al.*, 1995). In the later stages, LIF was found to decrease production of both proteinases, while EGF had no effect. These results suggested that LIF may also down-regulate invasiveness of the blastocyst in later development.

MMPs and TIMPs are also believed to participate in branching morphogenesis of the salivary gland and lung (Nakanishi *et al.*,1986; Fukuda *et al.*, 1988; Hayakawa *et al.*, 1992; Ganser *et al.*, 1991), a process that requires both ECM stabilization and degradation, as well as cell migration and proliferation (Werb *et al.*, 1992). Upon stimulation by EGF and TGF-α, the gelatinases, particularly 72-kDa gelatinase, were secreted by the lung explants (Ganser *et al.*, 1991). Exogenous collagenase can inhibit branching of the salivary gland (Nakanishi *et al.*,1986; Fukuda *et al.*, 1988) and lung (Ganser *et al.*, 1991) in culture. TIMP, on the other hand, can stimulate supernumerary budding of the salivary gland. These findings suggested that collagenase and TIMP regulate branching morphogenesis through remodelling of the ECM molecules (Hayakawa *et al.*, 1992).

Tissue remodelling during secondary palate formation was also shown by immunolocalization of several ECM components (Morriss-Wiman and Brinkley, 1992). It was found that type III collagen, fibronectin, and hyaluronate are the major components of the ECM infrastructure of the developing secondary palate. As development proceeds, hyaluronate was found to be expanded, while type III collagen and fibronectin become more circumscribed. Recently, the expression of both mRNA and protein of MMPs and TIMPs have been investigated in the developing mouse secondary palate (Brinkley *et al.*,

1995; Du *et al.*, 1996; Morris-Wiman *et al.*, 1996). By utilizing zymography and reverse transcriptase polymerase chain reaction (RT-PCR) techniques, urokinase plasminogen activator (uPA), MMP-1, -2, and -9 were identified *in vivo* (Brinkley *et al.*, 1995) and uPA MMP-1, -2, -3, -7, and -9 were detected in secondary palate explants (Morris-Wiman *et al.*, 1996). TIMP-1 and TIMP-2 were also found to be expressed during *in vivo* and *in vitro* palatogenesis (Du *et al.*, 1996). These data suggest that MMPs, serine protease, and TIMPs are involved in ECM remodelling during secondary palate morphogenesis.

5.2 MMPs, TIMPs and growth factors

In most cell types, genes of MMPs are not constitutively expressed but rather are regulated at the transcriptional level by treatment with a variety of factors including growth factors/cytokines, okadaic acid, bacterial lipopolysaccharide (LPS), hormones, oncogene products and tumor promotors (see review in Birkedal-Hansen *et al.*, 1993). For example, expression of some MMPs is induced by interleukin-1β, tumor necrosis factor-α (MacNaul *et al.*, 1990), platelet-derived growth factor (PDGF), EGF (Kerr *et al.*, 1988), bFGF (Edwards *et al.*, 1987) and nerve growth factor (NGF) (Machida *et al.*, 1991). TGF-β can repress the transcription of collagenase and stromelysin-1 (Edwards *et al.*, 1987; Kerr *et al.*, 1990) but upregulates 72-kDa and 92-kDa gelatinases (Overall *et al.*, 1991; Salo *et al.*, 1991).

TIMP-1 and -2 genes are differently regulated. TIMP-1 expression is stimulated by EGF, TNF-α (Mawatari *et al.*, 1989), TGF-β (Edwards *et al.*, 1987), retinoids and glucocorticoids (Clark *et al.*, 1987); whereas, TIMP-2 expression is down regulated by TGF-β (Stetler-Stevenson *et al.*, 1990). TIMP-1, in particular, has been localized to the same embryonic tissues where TGF-β expression has

been detected (Heine *et al.*, 1987; Flenniken and Williams, 1990). These findings suggested that there is a functional interaction between TIMP-1 and TGF-β *in vivo* (Flenniken and Williams, 1990).

Based on substrate specificity, 72-kDa gelatinase appears to be a major enzyme for basement membrane degradation since it can degrade type IV collagen, laminin, and fibronectin (see Table III). In addition, 72-kDa gelatinase has been found to be associated with disruption of the basement membrane of the developing mammary gland (Dickson and Warburton, 1992) and the developing tooth (Sahlberg *et al.*, 1992; Heikinheimo and Salo, 1995). Accordingly, in the present study, 72-kDa gelatinase was hypothesized to be involved in epithelial seam disruption during primary palate morphogenesis and remodelling of the basement membrane during heart morphogenesis.

6. Growth factors

Growth factors are small polypeptides functioning mainly in cell proliferation and differentiation. They have been found in a variety of tissues particularly embryonic and wound tissues. In addition, they have also been identified in some neoplasia and certain diseases. Growth factors can be classified into at least five families based on nucleotide and amino acid sequence homology and similar receptor-binding activity. These growth factor families include (1) EGF, (2) insulin-like growth factor (IGF), (3) fibroblast growth factor (FGF), (4) platelet-derived growth factor (PDGF) and (5) transforming growth factor-beta (TGF-β) families (Table III). Growth factors can act in autocrine, paracrine or endocrine regulation pathways (for reviews see Mercola and Stiles, 1988; Pusztai *et al.*, 1993).

Table IV: Growth Factor Families

Family	Members
Epidermal growth factor	EGF, TGF-α, amphiregulin (AP), heparin-binding EGF-like growth factor (HB-EGF), Vaccinia virus growth factor (VGF), myxomavirus growth factor (MGF), Shope fibroma growth factor (SFGF), Schwannoma-derived growth factor (SDGF), Cripto, gp30
Fibroblast growth factor (Heparin-binding growth factor)	FGF-1 (acidic FGF, aFGF, endothelial cell growth factor, ECGF)

	FGF-2 (basic FGF, bFGF)
	FGF-3 (*int*-2), FGF-4 (k-FGF)
	FGF-5, FGF-6
	FGF-7 (keratinocyte growth factor, KGF)
	FGF-8, FGF-9
Insulin-like growth factor	IGF-I, IGF-II (Somatomedin-C)
	Relaxin
Platelet-derived growth factor	PDGFαα, PDGFββ, PDGFαβ
Transforming growth factor-β	TGF-β1, TGF-β2, TGF-β3,
	TGF-β4, TGF-β5, Inhibin α,
	Inhibin/activinβA, Inhibin/activinβB,
	Mullerian inhibitory substance (MIS),
	DPP-C (decapentaplegic gene),
	60A,
	Vg1 (vegetal pole gene1),
	Vgr-1 (vegetal-pole-gene-related-1), Vgr-2,
	DVR (decapentaplegic-Vg-related)/BMP-2-4,
	DVR/BMP-5-7,
	Osteogenin,
	OP-1 (osteogenic protein-1), OP-2,
	GDF-1 (growth and differentiation factor-1),
	GDF-3, GDF-9,
	Nodal

(modified from Derynck, 1992; Heikinheimo, 1993; Pusztai *et al.*, 1993; Miller and Rizzino, 1994)

EGF Family

Growth factors play an important role in wound healing and embryogenesis by stimulating cell proliferation and enhancing cell differentiation (see reviews in Mercola and Stiles, 1988; Milos, 1992). The EGF family including EGF and TGF-α is one of the major growth factor families detected in embryonic tissues. Previous studies of the expression of EGF, TGF-α and their common receptor, EGF-R suggest that these growth factors participate in growth and development of various embryonic tissues for example the blastocyst (Adamson, 1993; Chia *et al.*, 1995), tooth (Partanen and Thesleff, 1987; Partanen, 1990; Shum *et al.*, 1993), mandible (Kronmiller *et al.*, 1993; Shum *et al.*, 1993), and secondary palate (Dixon *et al.*, 1991). However, the expression of EGF, TGF-α, and EGF-R and their relationship with cell proliferation during primary palate morphogenesis were unknown. Therefore, in the present study, the expression of these molecules in the developing primary palate was investigated.

EGF and TGF-α are the primary EGF family members expressed in mammalian tissues. EGF and TGF-α are similar in many aspects, for example they exert their effects via the same integral membrane glycoprotein receptor, EGF-receptor (Cohen *et al.*, 1980; Todaro *et al.*, 1980; Massague, 1983), they are mitogenic peptides that regulate cell proliferation and differentiation (de Larco and Todaro, 1978; Derynck *et al.*, 1984), and the molecular weight of each mature peptide are approximately 6 kDa (Carpenter and Wahl, 1991; Derynck *et al.*, 1984). TGF-α shares about 33-44% homology with EGF (Heath, 1993). However, the transmembrane and cytoplasmic domains of TGF-α and EGF demonstrate no similarity (Marquardt *et al.*, 1984). The lack of similarity in these regions suggests a difference in the function of these two growth factors (Kumar *et al.*, 1995). TGF-

α does not cross-react with anti-EGF antibodies suggesting structural differences between EGF and TGF-α (Schlessinger *et al.*, 1983). EGF and TGF-α and their receptor (EGF-R) have been detected in various embryonic and adult tissues and in both normal and pathological conditions.

6.1 EGF

EGF mRNA encodes a transmembrane precursor glycoprotein of 1207 amino acids. In mouse submandibular gland, the precursor molecule is rapidly processed to the 53-amino acid form of EGF. Both the precursor and mature forms can bind to EGF-R and activate the target cells (Carpenter and Wahl, 1991). EGF was first isolated from mouse submandibular salivary glands (Cohen, 1962). Later by amino acid analysis, mouse EGF was shown to be identical with human β-urogastrone (Gregory, 1975).

EGF transcripts and protein were initially detected in the human preimplantation embryo (Chia *et al.*, 1995). In later development, EGF expression has been detected in the developing mandible (Kronmiller *et al.*, 1991a), tooth, lung (Snead *et al.*, 1989), pancreas, skin, kidney, and submandibular salivary gland (Miettinen, 1993). EGF protein has been identified in various fetal tissues including the kidney, gastrointestinal tract, submandibular gland, lung, liver, brain, placenta, and amnion (Kasselberg *et al.*, 1985; Poulsen *et al.*, 1986; Stahlman *et al.*, 1989; Shigeta *et al.*, 1993). EGF and EGF-R were also found to be coexpressed in the mouse embryonic mandible from E10 through E15 by using RT-PCR technique (Shum *et al.*, 1993). EGF transcripts were localized to ectomesenchymal cells associated with precartilage, cartilage, bone, and tooth-forming cells (Shum *et al.*, 1993). More recently, EGF immunoreactivity was studied in human fetuses and found to be present in the placenta, skin, distal

tubules of the kidney, surface epithelium of the stomach, tips of the small intestinal villi, and glandular structures of the stomach, duodenum, pancreas, and trachea (Poulsen *et al.*, 1996).

Physiological roles of EGF in development have been studied *in vivo* and *in vitro*. Upon injection into newborn mice, EGF stimulated precocious eye opening and incisor eruption (Cohen, 1962). EGF also enhanced growth and keratinization of epithelial cells (Cohen, 1962; Cohen and Elliot, 1963). It has been proposed that EGF is necessary for the initiation of odontogenesis (Kronmiller *et al.*, 1991b; Shum *et al.*, 1993). However, exogenous EGF altered the pattern of the dental lamina, producing supernumeraries in the diastema region in organ cultures (Kronmiller, 1995), inhibited early mouse tooth morphogenesis and cytodifferentiation *in vitro* (Partanen *et al.*, 1985) and reduced tooth size *in vivo* (Rhodes *et al.*, 1987). Taken together, these results suggest that EGF plays a role in tooth morphogenesis. The location of EGF transcripts in the mesenchyme adjacent to the mandibular epithelium suggested a paracrine mechanism in the stimulation of epithelial proliferation in dental lamina formation (Kronmiller, 1995). Addition of exogenous EGF to lungs in culture resulted in significant stimulation of branching morphogenesis (Warburton *et al.*, 1992). By using an antisense oligodeoxynucleotide directed against EGF precursor mRNA in embryonic mouse lung in culture, the expression of EGF and branching morphogenesis were inhibited (Seth *et al.*, 1993). These data suggest that EGF is important for pulmonary organogenesis.

6.2 TGF-alpha

TGF-α mRNA encodes a transmembrane precursor glycoprotein of 160 amino acids which is processed into a 50 amino acid mature protein (Derynck, 1984). TGF-α is highly conserved between man and mouse and shares about 33-44% homology with EGF (Heath, 1993). TGF-α was first isolated from culture medium of mouse sarcoma virus transformed cells (de Larco and Todaro, 1978) and later found to be highly conserved between human and mouse (Heath, 1993). Like EGF, both precursor and mature forms of TGF-α can bind to EGF-R and activate the target cells (Wong *et al.*, 1989). Binding of TGF-α precursor with EGF-R, so-called "juxtacrine" stimulation, is proposed to mediate cell-cell adhesion and trigger a cascade of events leading to cell duplication (Massague, 1990). More recently, a role for membrane-bound TGF-α-mediated cell-cell interactions during the peri-implantation of mammalian development was suggested (Paria *et al.*, 1994).

By using RT-PCR technique, TGF-α transcripts are able to be detected as early as in the unfertilized egg and during preimplantation stage. TGF-α protein was immunolocalized in all cells of the blastocyst (Rappolee *et al.*, 1988). In later stages, TGF-α has been localized in embryonic tissues particularly ectodermal origin for example in the branchial arches, oral and nasopharyngeal epithelia, otic vesicle, mesonephric tubules of the kidney (Wilcox and Derynck, 1988), pancreas and gastrointestinal tract (Hormi and Lehy, 1994). TGF-α transcripts were also identified by PCR in the mouse mandible at days 9 and 10 (Kronmiller *et al.*, 1993). Initially, TGF-α was considered as a fetal form of EGF. However, it has also been identified in various types of normal adult tissues including skin

(Coffey *et al.*, 1987), gastrointestinal tract (Cartlidge and Elder, 1989), and ovarian tissues (Chegini and Williams, 1992) and pathological conditions including psoriatic skin (Kondo *et al.*, 1992) and neoplasia (Derynck *et al.*, 1987; Barton *et al.*, 1991).

TGF-α is frequently a superagonist of EGF (Derynck, 1992). Similar to EGF, injection of exogenous TGF-α into newborn mice also activated precocious eye opening and incisor eruption (Smith *et al.*, 1985; Tam, 1985). It was proposed that the major role of TGF-α in development is to drive the proliferation of various cell types, especially epithelial cells. The coexpression of TGF-α and EGF-R genes by many epithelial cells suggests that TGF-α may act in an autocrine manner (Derynck, 1992). TGF-α was found to promote the proliferation of the medial edge epithelial cells until fusion of the palatal shelves occurs in secondary palate cultures (Lee and Hahn, 1991). The *in vitro* study showed that TGF-α can stimulate ECM formation in the developing mouse secondary palate (Dixon and Ferguson, 1992). Overexpression of TGF-α induced epithelial hyperplasia in the liver, pancreas, mammary gland and skin of transgenic mice, indicating that TGF-α functions in epithelial proliferation (Matsui *et al.*, 1990; Sandgren *et al.*, 1990; Vassar and Fuchs, 1991). More recently, mice homozygous for a disrupted TGF-α (TGF-α -/-) gene appeared to be viable and fertile, suggesting that TGF-α may not be absolutely essential for embryonic development (Mann *et al.*, 1993; Luetteke *et al.*, 1993). These findings, however, do not rule out a role for TGF-α under natural conditions since other growth factors such as EGF might be able to compensate the effects of TGF-α in the knockout mice (Wiley *et al.*, 1995).

6.3 EGF-Receptors

EGF-R is a transmembrane glycoprotein of 1186 amino acids with a molecular weight of 170 kDa (Cohen *et al.*, 1980). EGF-R is also known as c-*ErbB*1 since it is the proto-oncogene precursor of *v-erb*B found in chicken erythroblastosis virus (Adamson, 1990). Other related members of this family are *ErbB*2, *ErbB*3, and *ErbB*4 (see reviews in Pusztai *et al.*, 1993; Wiley *et al.*, 1995). EGF-R referred in the present work is the prototype of EGF-R, *c-ErbB*1. Upon binding to an extracellular ligand, the monomeric EGF-R protein becomes dimerize and autophosphorylated. Subsequently, signal transduction events take place that lead to changes in cell proliferation, cell differentiation, cell adhesion, and/or cell migration (see reviews in Hernandez-Sotomayor and Carpenter, 1992; Wiley *et al.*, 1995). EGF-R has been detected in various embryonic and adult tissues, and found to be overexpressed in some neoplasia (Gullick *et al.*, 1986; Carpenter and Wahl, 1991). During preimplantation period, EGF-R has been detected in mouse (Adamson, 1990) and human (Adamson, 1993; Chia *et al.*, 1995) blastocysts. Therefore, it was suggested that EGF-R takes part in growth regulation of the early embryo, particularly in the process of implantation (Adamson, 1990). EGF binding studies have been used to characterize EGF-R function in many developing organ systems: heart, kidney (Shigeta *et al.*, 1993), tooth (Partanen and Thesleff, 1987), kidney, liver, lung (Nexo and Kryger-Baggesen, 1989; Shigeta *et al.*, 1993) and secondary palate (Sharpe *et al.*, 1992). EGF-R mRNA and/or protein have been found in the developing tooth (Abbott and Pratt, 1988), pancreas (Miettinen and Heikinheimo, 1992), gastrointestinal tract (Hormi and Lehy, 1994), mandible (Shum *et al.*, 1993) and secondary palate (Dixon *et al.*, 1991). More recently, the knockout mice lacking EGF-R were generated and showed multiple abnormalities dependent on genetic background (Threadgill *et al.*, 1995; Sibilia and Wagner, 1995). For example, mutant fetuses

died at mid-gestation due to placental defects on a 129/Sv background. On a CD-1 background, the mutants lived up to three weeks and showed abnormalities in skin, kidney, brain, liver and gastrointestinal tract (Threadgill *et al.*, 1995). These results suggested that EGF-R participates in a wide range of cellular activities particularly epithelial proliferation and differentiation.

Although TGF-α can bind EGF-R with an affinity comparable to that of EGF, there are distinguishable differences in their biological activities (see reviews in Derynck, 1992; Hernandez-Sotomayor and Carpenter, 1992). For example, both TGF-α and EGF can induce neovascularization *in vivo*. But TGF-α is found to be more potent than EGF (Schreiber *et al.*, 1986). A monoclonal antibody to EGF-R (13A9) can block the binding of TGF-α to human EGF-R but has no affect on the affinity of EGF for binding to EGF-R (Winkler *et al.*, 1898). These results indicated either that the antibody stabilizes a conformation of EGF-R which is not favorable for TGF-α binding or that it blocks a part of the surface of the receptor which is necessary for TGF-α binding but not EGF binding.

7. Cell proliferation in the developing primary palate.

During primary palate formation, morphogenetic changes occur in the craniofacial complex. By using morphometric analysis and three-dimensional computer reconstructions in different stages of human embryos (Diewert and Wang, 1992; Diewert and Lozanoff, 1993), it has been found that the frontonasal prominence elongates vertically (height increases by seven times) and narrows to approximately half the width. The brain and the face become vertically separated. The area of the maxillary region increases extensively, particularly in the distal region, as the maxillary prominences grow forward to meet the lateral nasal and medial nasal prominences. The growth of the lateral nasal region increases primarily in width. Whereas, the growth of the medial nasal prominences appears to increase only slightly. These normal growth patterns are believed to be important for facilitating contact between facial prominences and primary palate morphogenesis (Diewert and Wang, 1992). Regional differences in cell proliferation and ECM composition in the facial prominences are believed to contribute to these changes during primary palate morphogenesis (Diewert *et al.*, 1993a).

During embryonic development, there is ongoing division of cells, resulting in tissue proliferation and morphogenesis. As this occurs, the parent cell accumulates certain proteins and doubles its DNA content to provide for the progeny. Cell proliferation and DNA synthetic activity can be studied by many approaches. 5-Bromodeoxyuridine (BrdU), an analogue of thymidine, has been utilized for this purpose since it can be incorporated into DNA during the S-phase of the cell cycle. Its localization in the nuclei can be detected by using the anti-BrdU antibody (Gratzner, 1982). Proliferating cell nuclear antigen (PCNA), an auxiliary protein to DNA polymerase delta, has also been proposed as a marker of replicating cells since its cell concentration is directly correlated with

the proliferative state of the cell. PCNA was shown to increase through G1, peak at the G1/S-phase interface, decrease through G2 and reach low levels in M-phase and interphase (Celis and Celis, 1985; Kurki *et al.*, 1986; Casasco *et al.*, 1993). By using immunohistochemistry with the anti-PCNA antibody, the proliferation index of certain tissues, including embryonic (Leibovici *et al.*, 1992; Sanders *et al.*, 1993) and neoplastic tissues (Hall *et al.*, 1990; Pendleton *et al.*, 1993), can be characterized. The advantages of using PCNA to measure growth fractions and labelling indices include ease of staining and elimination of the need for injections of BrdU for the labelling of S-phase fractions. However, PCNA is less suitable for determination of kinetic parameters such as phase durations and cell cycle time and generally cannot be used to trace lineages and migrations of cells during differentiation (Dolbeare, 1995). A study of cell proliferation in the gastrulating chick embryo showed that immunolocalization of PCNA gave generally similar distribution patterns to those of BrdU incorporation, although there were always more PCNA-positive nuclei than BrdU-positive nuclei (Sanders *et al.*, 1993). Similarly, a comparative study of rat pancreatic growth between PCNA labelling and [3]H-thymidine autoradiography showed that PCNA index is higher than that of [3]H-thymidine. These two studies suggest that PCNA-positive cells are found not only in S-phase cells, but also in cells that have recently completed the cell cycle (Elsasser *et al.*, 1994). Labelling of BrdU and and [3]H-thymidine were also compared and showed similar results in murine tumors (Wilson *et al.*, 1987). In addition, BrdU labelling demonstrated greater sensitivity with little background interference in transformed Syrian hamster cells (Cawood and Savage, 1983).

Cell proliferation has been studied in the embryonic face. Analysis of labelling indices by labelling chick embryos with [3]H- thymidine indicated that rates of cell proliferation varied within each of the facial prominences of the

chick embryo. Regions where rates of proliferation were maintained at elevated levels were the boundary areas of the facial prominences particularly the anterior tip of the maxillary and the lateral nasal prominences and the zones of attachment between the maxillary and the lateral nasal prominences (Minkoff, 1980a). The percentage of labelled cells in all areas declines with advancing developmental age (Minkoff and Kuntz, 1978; Minkoff, 1980a). A pattern of declining rates of cell proliferation with advancing developmental age has also been observed in morphogenesis of the fronto-nasal prominence (Minkoff and Kuntz, 1977), limb (Ede *et al.*, 1975), and secondary palate (Nanda and Romeo, 1975). Recently, cell densities and proliferation were studied in rhesus monkey embryos labelled with [3]H-thymidine during primary palate formation (Diewert *et al.*, 1993a). The results showed that labelling indices were consistently higher in facial prominences than in midline tissues ventral to the brain (Diewert *et al.*, 1993a). Similarly, cell proliferation of the facial prominences was studied by BrdU incorporation in mouse embryos (Gui *et al.*, 1993). Cell proliferation rates of the mesenchyme in the facial prominences were found to remain unchanged during the early stages of development and to decrease in the later stages. These data suggested that a differential rate may be operative as a morphogenetic mechanism during enlargement and union of the facial prominences. It was also found that epithelial cells in the prospective fusion area showed decreased DNA synthesis in comparison with those in the nonfusing areas, indicating that epithelial cell proliferation converts to a differentiation-type pattern (Gui *et al.*, 1993). Although the study of cell proliferation by Gui *et al.* (1993) was done during embryonic mouse day 10, regional differences in cell proliferation in later stages (embryonic day 11): fusion of the facial prominences and mesenchymal bridge enlargement was not studied. Therefore, in the present work, regional cell proliferation at different stages during primary palate formation was undertaken.

B. Hypotheses

Normal tissue remodelling involves well-balanced and coordinated synthesis and degradation of the ECM molecules. Remodelling of the ECM is believed to play an important role during embryonic development particularly in cell migration, proliferation, differentiation, cell-cell interactions, and tissue outgrowth and invasion (Matrisian, 1992; Werb *et al.*, 1992). Morphogenesis of the tissues in the craniofacial complex; especially the primary palate, mandible and eye; and heart share many common basic biological processes for example cell migration, proliferation, differentiation, and tissue interactions. However, the mechanisms of tissue remodelling and outgrowth during craniofacial morphogenesis and tissue remodelling during heart morphogenesis remain unclear.

I, therefore, tested the hypotheses that temporo-spatial changes of certain molecular factors take place during craniofacial and heart morphogenesis and contribute to the regulation of tissue remodelling and outgrowth of the craniofacial complex and heart such as;

1. basement membrane components of the epithelial seam; including laminin, type IV collagen, and fibronectin; are gradually disrupted in a sequence during primary palate morphogenesis,

2. elevated levels of expression of growth factors and their receptors, particularly EGF, TGF-α, and EGF-R, are present in rapidly growing regions of the developing primary palate and are correlated with the sites of increased cell proliferation,

3. 72-kDa gelatinase is expressed at high levels in the area of the epithelial seam as the basement membrane becomes disrupted during primary palate morphogenesis,

4. 72-kDa gelatinase co-localized with the enzyme's substrates; type IV collagen, laminin, and fibronectin; during heart morphogenesis.

General Aim

The aim was to increase our understanding of the molecular mechanisms of the development of the craniofacial and heart morphogenesis in the mouse.

Specific Aims

1. To characterize changes of the major components of the basement membrane; laminin, type IV collagen, and fibronectin; that accompany the regression of the epithelial seam during morphogenesis of the mouse primary palate,

2. To characterize the distribution of the growth factors and their receptors; particularly EGF, TGF-α, and EGF-R; during morphogenesis of the mouse primary palate,

3. To analyze regional proliferation of the facial prominences by BrdU incorporation and PCNA immunulocalization during morphogenesis of the mouse primary palate,

4. To characterize the distribution of 72-kDa gelatinase in association with tissue outgrowth and morphogenesis of the mouse craniofacial complex particularly the primary palate, mandible and eye,

5. To characterize the co-localization of EGF, TGF-α and 72-kDa gelatinase in association with morphogenesis of the mouse primary palate,

6. To characterize the distribution of 72-kDa gelatinase in correlation with changes of the distribution of the enzyme substrates; type IV collagen, laminin, and fibronectin; during morphogenesis of the mouse heart.

CHAPTER 2: Distribution of Basement Membrane Components in the Developing Mouse Primary Palate

Introduction

The basement membrane is an ubiquitous extracellular matrix mainly found in epithelial, nerve, and fat tissues (Timpl, 1989). Various molecules have been identified in the basement membrane such as laminin, type IV collagen, heparan sulfate proteoglycan, fibronectin, nidogen (entactin), SPARC, amyloid P, and some complement components (Timpl, 1989).

The primary palate is formed by fusion of the medial nasal prominence with the lateral nasal and maxillary prominences. Initially, the epithelial coverings of the prominences adhere and establish an epithelial seam which becomes disrupted and replaced by a mesenchymal bridge which enlarges and unites the prominences together as a single organ, the primary palate (Streeter, 1948; Diewert and Shiota, 1990; Diewert and Wang, 1992; Diewert and Lazanoff, 1993; Diewert *et al.*, 1993b; Wang *et al.*, 1995).

I predicted that during primary palate formation, laminin, type IV collagen, and fibronectin in the basement membrane of the epithelial seam are disrupted and rapidly disappear in association with mesenchymal bridge formation. The temporo-spatial distributions of laminin, type IV collagen, and fibronectin were determined by means of immunohistochemistry (Iamaroon and Diewert, 1996).

Materials and Methods

Animal Maintenance and Breeding

All mice were housed in facilities approved by the Canadian Council of Animal Care and experimental protocols used were approved by the Animal Care Committee of the University of British Columbia. Mice were maintained on a diet of Purina mouse chow and filtered water, in the animal unit in the Faculty of Dentistry at the University of British Columbia, and in windowless rooms on a 12 hour light (7:00 am to 7:00 pm), 12 hour dark cycle. The temperature was controlled at about 22° C. Three or four adult females were caged overnight with a male and were examined in the morning for the presence of a vaginal plug. Ovulation was assumed to occur at midnight, therefore 9 am of the day the plug found was designated as day 0 hour 9 of gestation (Snell *et al.*, 1940).

Mouse Stocks

The strains of mice used in the present study were CD1 (Charles River, Wilmington, MA), BALB/cBy (Jackson Laboratory, Bar Harbor, Maine), CL/Fr (developed in the laboratory of Dr. Fraser in McGill University and kindly provided to our laboratories by Dr. Trasler in 1985), A/WySn (Jackson, Bar Harbor, Maine), and A/J (Jackson, Bar Harbor, Maine).

Tissue Collection and Preparation

Pregnant mice were killed at various times from day 10 through 11 in a carbon dioxide chamber. Gravid uteri were removed and placed in normal saline solution. Individual embryos were dissected from the uterine decidua and staged by counting the number of tail somites (TS) from the caudal edge of the hide

limb to the end of the tail (Wang, 1992). Embryonic heads were removed from the body with a No. 15 surgical blade, fixed in 4% (w/v) paraformaldehyde for 12-24 hours, and processed for paraffin embedding. The specimens were serially sectioned coronally or horizontally at a thickness of 7 μm and placed on 3-aminopropyl-triethoxy-silane-coated slides (Sigma, St. Louis, MO), then dried overnight.

Deparaffinized sections were incubated with 0.4% (w/v) pepsin (Sigma, St. Louis, MO) in 0.02M HCl for 5 min. The purpose of proteolytic digestion with pepsin was to remove the cross-linking in paraformaldehyde-fixed tissues and render tissue antigen reactive. Sections without pepsin treatment were also included and found weak staining. Sections were incubated with 25% (v/v) normal goat serum for 30 min, washed with phosphate-buffered saline (PBS), and incubated with anti-laminin (Sigma, St. Louis, MO, rabbit anti-EHS mouse sarcoma, L-9393), anti-type IV collagen (Chemicon, Temecula, CA, rabbit anti-human, AB748), or anti-fibronectin (Dako, Glostrup, Denmark, rabbit anti-human, A 245) antibody with concentrations of 1:100, 1:25, and 1:100, respectively overnight at 4° C. Subsequently, the sections were incubated in fluorescein isothiocyanate (FITC)-conjugated (sheep anti-rabbit, Sigma, St. Louis, MO, anti-rabbit IgG, F-7512) or tetramethylrhodamine isothiocyanate (TRITC)-conjugated (goat anti-rabbit, Sigma, St. Louis, MO, anti-rabbit IgG, T-6778) secondary antibody with the concentration of 1:100 for 1 hour at 4° C and washed. Within each group of the sections, a negative control slide was incubated with normal goat serum as a replacement of the primary antibody. The dilution tests were performed on the antibodies against laminin, type IV collagen, and fibronectin. The results showed that the staining intensity decreased gradually as the dilution of the antibodies increased, as predicted. Positive control tissues included embryonic basement

membranes of the surface ectoderm for the antibodies against laminin, type IV collagen, and fibronectin (Timpl, 1989). Positive immunoreactivity was found in basement membranes of the surface ectoderm as predicted (data not shown).

Results

At least 33 CD1 mouse embryos were used in these studies (see Appendix 4). Consecutive sections were not employed in these studies but selected comparable sections were used for each antibody staining. Morphogenesis of the primary palate can be categorized into three stages; (1) epithelial seam formation (7-11 TS), (2) epithelial seam disruption (12-17 TS), and (3) mesenchymal bridge enlargement (18-20 TS).

Epithelial seam formation (7-11 TS)

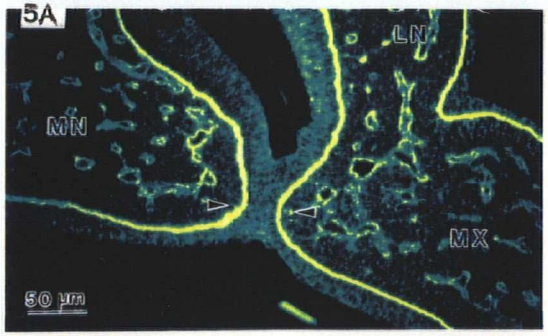
Sections of CD1 embryos at the 7-11 tail somite stage showed intense localization of laminin, type IV collagen, and fibronectin in the basement membrane of the epithelial seam during the primary palate formation (Figs. 5A,B,C). The staining was present from the anterior to posterior of the epithelial seam and was continuous with the basement membranes of the stomodeal and nasal epithelia. The staining patterns of laminin, type IV collagen, and fibronectin were uniform throughout all basement membranes. The staining of the basement membrane components was also present around the blood vessels showing extensive vascularization of the primary palate. Fibronectin was also intensely labelled in the mesenchyme of the facial prominences (Fig. 5C). The staining of laminin, type IV collagen, and fibronectin appeared to be more diffuse in the tip of the maxilla than in the other regions of the basement membrane (Figs 8A,B,C). These findings were also observed in later stages of the primary palate morphogenesis.

Epithelial seam disruption (12-17 TS)

The staining of laminin, type IV collagen, and fibronectin in the basement membrane of the epithelial seam became rapidly discontinuous and fragmented in the later stages of primary palate formation (Figs. 6A,B,C). However, basement membranes of the stomodeal and nasal epithelia remained intact. A close association between the areas of basement membrane breakdown and mesenchymal ingrowth was evident. Disruption of the basement membrane components appeared to be a prior event to disappearance of the epithelial seam. The basement membrane components were also disrupted at the nasal and oral margins where merging of facial prominences continued as the primary palate enlarged. Disruption of the basement membrane appeared to progress from central to anterior of the seam.

Mesenchymal bridge enlargement (18-20 TS)

At the 18-20 TS stages, the epithelial seam and its basement membrane largely disappeared simultaneously with mesenchymal bridge enlargement in the primary palate (Figs. 7A,B,C). Basement membrane components were intact around the margins of the primary palate. Grooves between the prominences were markedly reduced. Fibronectin was also intensely localized to the mesenchyme of the peripheral regions of the facial prominences; whereas it was less pronounced in the midline region. In the region posterior to the mesenchymal bridge, basement membrane components of the the nasal fin remained intact where formation of the primitive choana occurred (Fig. 7D).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MN
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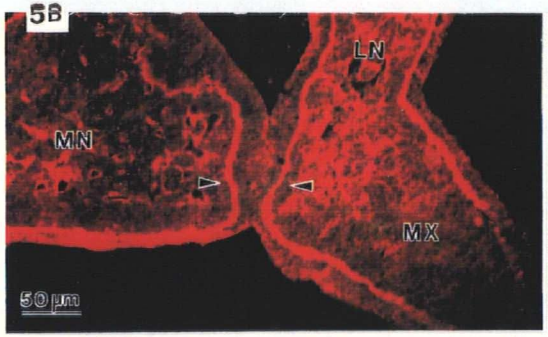
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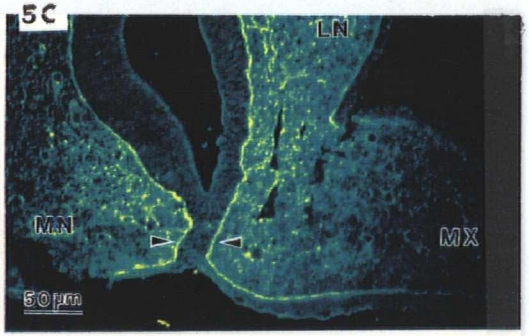
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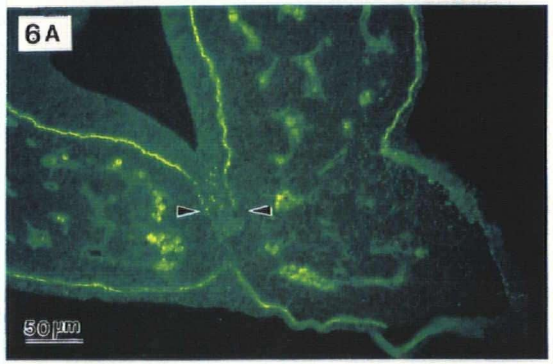
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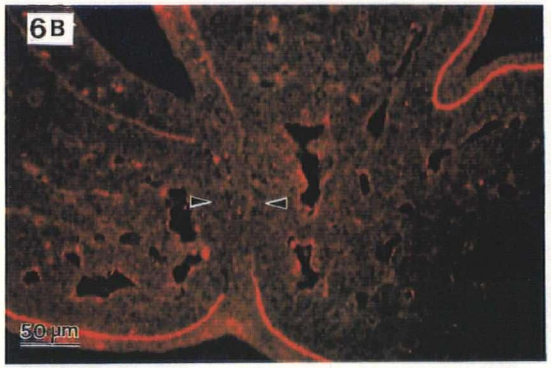
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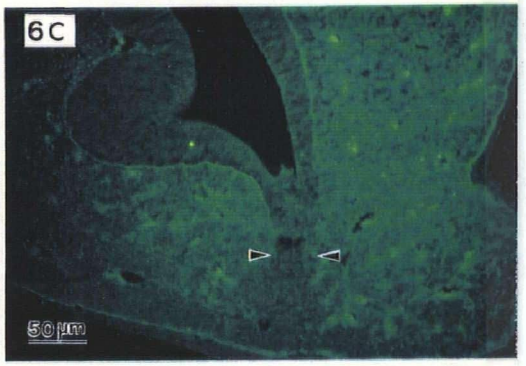
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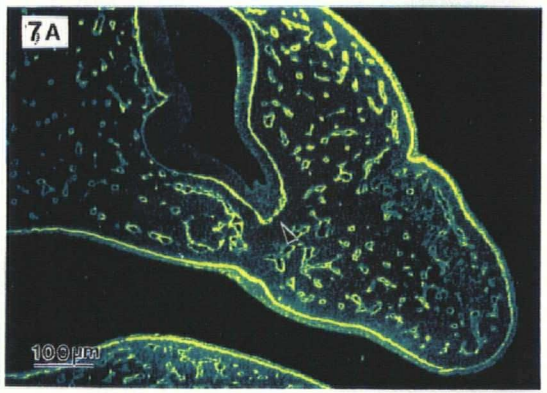
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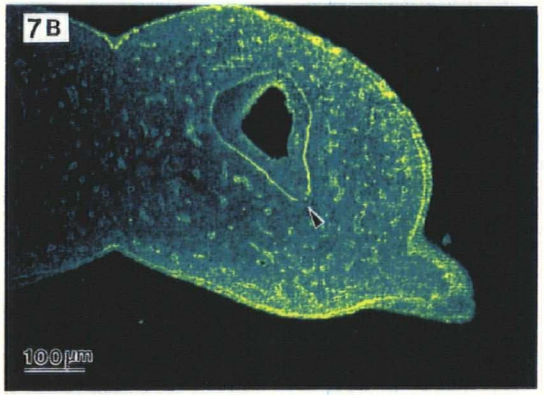
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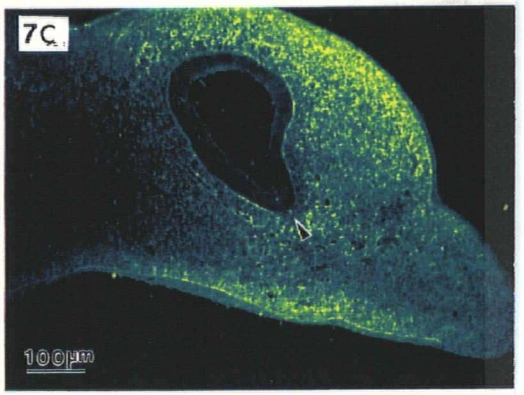
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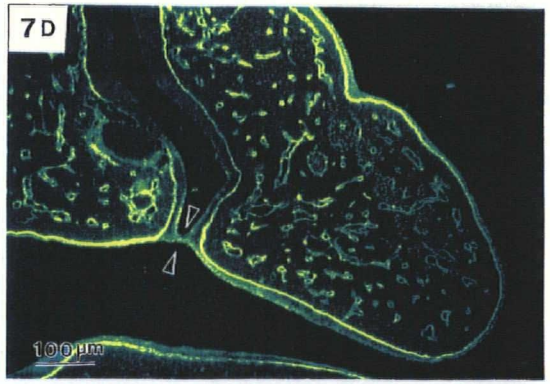
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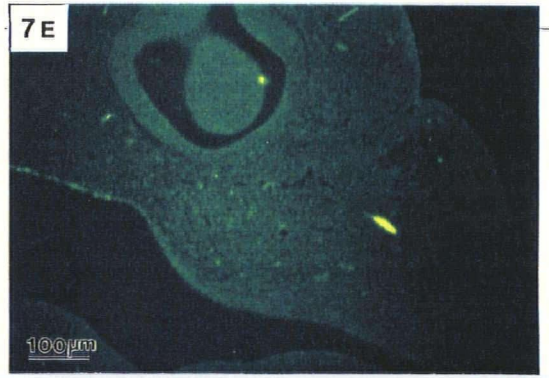
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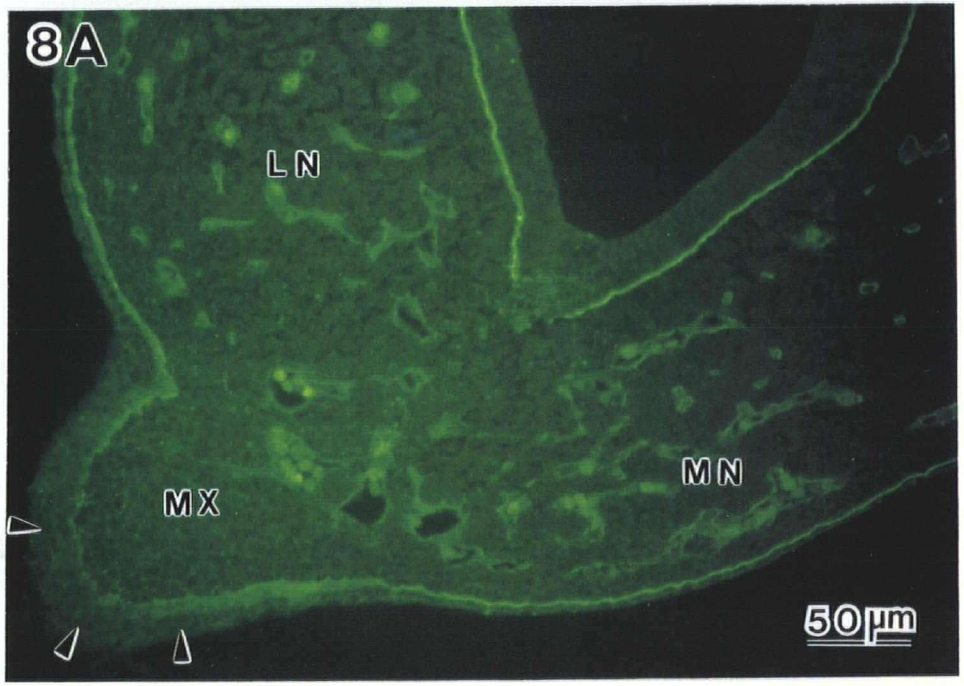
8A
LN
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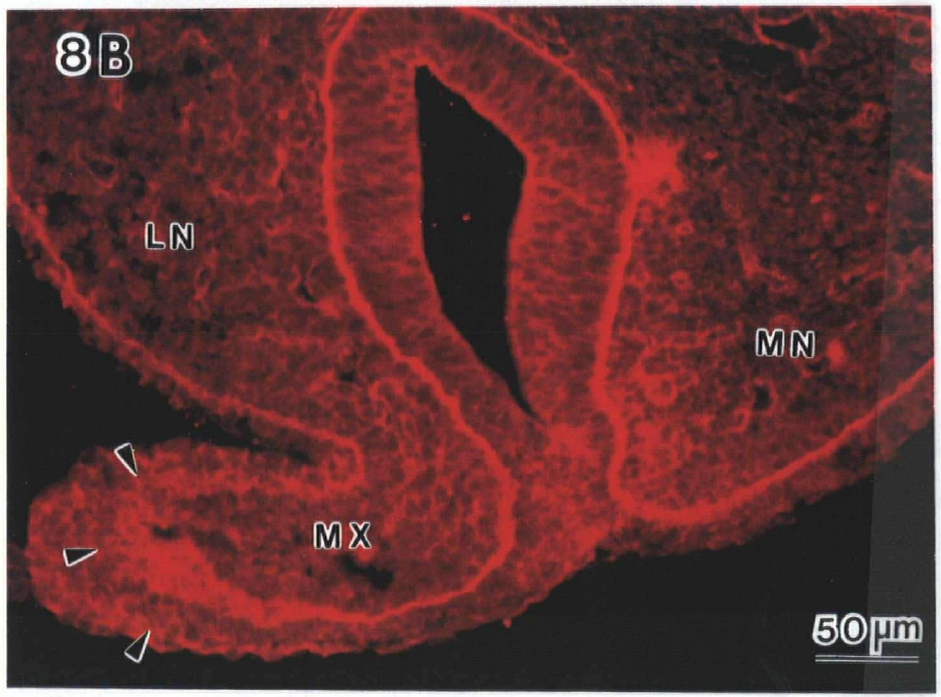
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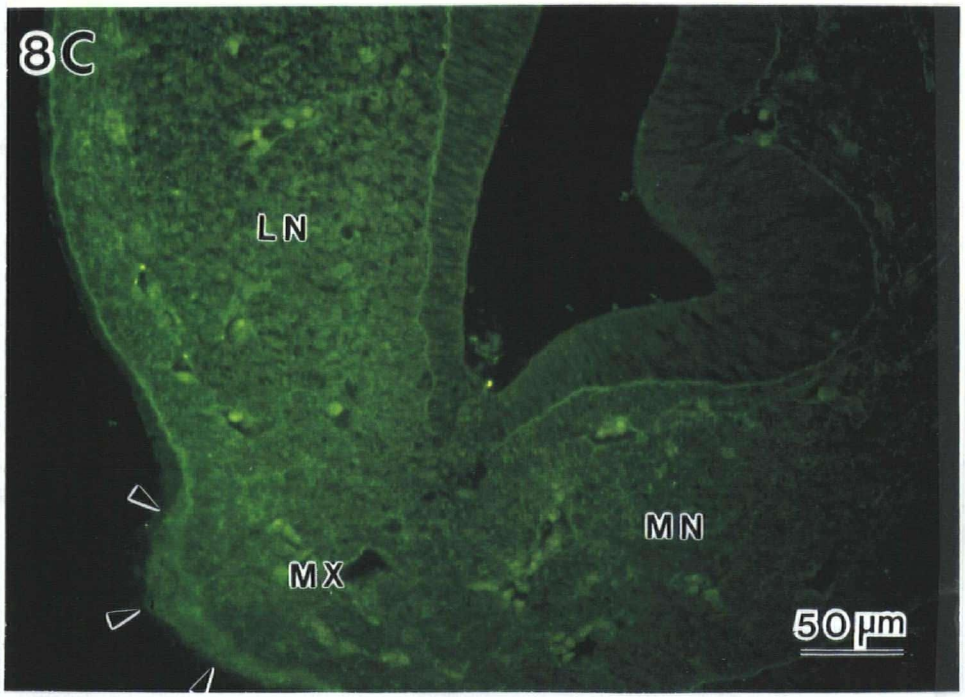
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Figs. 5A-C: Epithelial seam formation: Sections show intense staining of laminin (A), type IV collagen (B), and fibronectin (C) in basement membranes of the epithelial seam (between arrows), and nasal and stomodeal epithelia. The staining of fibronectin was also present in the mesenchyme of the facial prominences. LN, lateral nasal; MN, medial nasal; MX, maxillary prominences.

Figs. 6A-C: Epithelial seam breakdown: Sections show disruption of the stainings of laminin (A), type IV collagen (B), and fibronectin (C) in the basement membrane of the epithelial seam (between arrows). However, the staining in basement membranes of the nasal and stomodeal epithelia remained intact.

Figs. 7A-C: Mesenchymal bridge enlargement: Sections show disappearance of the epithelial seam and its basement membrane. There was a reorganization of the stainings of laminin (A), type IV collagen (B), and fibronectin (C) around the margins of the primary palate (arrows). The staining of fibronectin was intensely localized to the mesenchyme particularly in the peripheral regions of the facial prominences.

Figs. 7D-E: In the region posterior to the mesenchymal bridge, the staining of laminin remained intact in the basement membrane of the forming primitive choana (between arrows) (D). The negative control section, incubated with normal goat serum as a replacement of the primary antibody, showed absent staining in the tissue (E).

Figs. 8A-C: The staining of laminin (A), type IV collagen (B), and fibronectin (C) appeared to be more diffuse in the basement membrane at the tips of the maxilla.

Discussion

In this study, we characterized the presence of the major components of the basement membrane in the primary palate by means of immunohistochemistry. During epithelial seam formation, basement membrane components were intact. Subsequently, the basement membrane components of the epithelial seam became patchy and then entirely disrupted as the mesenchymal bridge formed and enlarged. This coincides with rupture of the oronasal membrane or nasal fin resulting in formation of the primitive choana and completion of the primary palate (Tamarin, 1982).

Previous studies have implicated the basement membrane in mediation of epithelio-mesenchymal interactions in several developing organ systems, such as the tooth (Thesleff *et al.*, 1991), salivary glands (Bernfield *et al.*, 1984), lung (Jaskoll and Slavkin, 1984), kidney (Ekblom, 1981), and genitourinary system (Ikawa *et al.*, 1984). Indeed, epithelial-mesenchymal interactions in the facial prominences were suggested to be mediated by the epithelial-mesenchymal interface (Saber *et al.*, 1989). During primary palate formation, elevated rates of cell proliferation beneath the ectoderm of the facial prominences are believed to be maintained by these epithelial-mesenchymal interactions (Minkoff, 1980a,b; Diewert *et al.*, 1993a; Gui *et al.*, 1993).

Disruption of the major basement membrane components of the epithelial seam was clearly demonstrated in this study. This phenomenon occurs rapidly after fusion of the facial prominences and formation of the epithelial seam. Similar situations, with breakdown of the basement membrane and regression of the epithelial components, have also been found in some other organ systems, such as the Mullerian ducts of male embryos (Trelstad *et al.*, 1982; Ikawa *et al.*, 1984), involuted mammary glands (Warburton *et al.*, 1982; Talhouk

et al., 1992), and developing secondary palate (Ferguson, 1988). The mechanism of the disruption of the basement membrane and the epithelial seam has drawn considerable interest from investigators since persistence of the epithelial seam may be the abnormality leading to cleft formation (Stark, 1954; Warbrick, 1960; Diewert and Wang, 1992). A mesenchymally-produced soluble factor, hepatocyte growth factor/scatter factor (HGF/SF), was isolated (Stoker *et al.*, 1987) and proposed to be associated with the disruption of the midline epithelial seam in the developing secondary palate (Ferguson, 1988). More recently, the mRNA expression of HGF/SF and its receptor, c-*met* were studied and found in the mesoderm and endoderm along the rostro-intermediate part of the primitive streak (Andermarcher *et al.*, 1996). In later development, HGF/SF and c-*met* were expressed in the cardiovascular system, migrating neural crest cells, branchial arches, early limb buds, and some visceral organs. However, their expression in the secondary palate is unknown.

Matrix metalloproteinases (MMPs), especially type IV collagenases/gelatinases and stromelysins, may be good candidates for this degradative process since they have been found to be capable of degrading type IV collagen, laminin, and fibronectin (Fessler *et al.*, 1984; Galloway *et al.*, 1988; Okada *et al.*, 1990; Nagase *et al.*, 1991). Indeed, the proteins expression of 72-kDa gelatinase (MMP-2) and stromelysin (MMP-3) were found in the rat mammary gland and implicated to be responsible for basement membrane degradation during mammary gland involution (Dickson and Warburton, 1992). 72-kDa gelatinase was also found to be associated with breakdown of type IV collagen in the dental basement membrane during mouse (Sahlberg *et al.*, 1992) and human (Heikinheimo and Salo, 1995) tooth morphogenesis.

Disruption of the basement membrane appears to be a common process during embryogenesis since it has been found to be a key event prior to

migration of neural crest cells (Duband and Thiery, 1982a; Duband and Thiery, 1987), somitic mesenchyme formation (Solursh *et al.*, 1979), mesoderm formation from the primitive streak (Duband and Thiery, 1982b), and ameloblast differentiation during tooth formation (see review in Thesleff and Hurmerinta, 1981). Disruption of the basement membrane of the epithelial seam of the secondary palate appears to occur prior to epithelial-mesenchymal transformation (Griffith and Hay, 1992) and/or migration of the medial edge epithelial cells to the stomodeal and nasal epithelia (Carette and Ferguson, 1992), the two mechanisms proposed for seam elimination in the developing secondary palate. It was suggested that degradation of the basement membrane allows the transmission of inductive signals from one tissue to another in kidney tubule formation (Wartiovaara *et al.*, 1974; Ekblom, 1981) and tooth morphogenesis (Sahlberg *et al.*, 1992). This inductive transmission may be also important for the fate of the medial edge epithelial cells of the secondary palate. In the primary palate, the smaller number of cells present in the epithelial seam makes study of their fate even more difficult than that of the secondary palate (Diewert and Wang, 1992; Diewert *et al.*, 1993b).

In this study, the presence of fibronectin in the extracellular matrix is particularly interesting since its differential distribution was observed in the 18-20 TS facial primordia. Fibronectin was intensely labelled in the peripheral regions of the facial prominences; whereas the staining was less pronounced in the midline regions. The pattern of distribution of fibronectin was consistent with previous studies of cell density during primary palate formation in which the cell densities were high in the facial prominences and low in midline tissues (Diewert *et al.*, 1993a; Minkoff, 1980a,b). Changes in cell dispersion and extracellular matrix content have been studied and found to be involved in growth of embryonic primordia (Burk, 1983). The differential distribution of

fibronectin was also observed in the developing secondary palate in which staining was found mainly in the mesenchyme and basement membrane but appeared to be fibronectin-free in the mesenchymal core of the palatal shelves (Morris-Wiman and Brinkley, 1992). Recently, the knock-out mice with mutant fibronectin gene were generated and found to have various severe abnormalities that lead to early embryonic lethality (George *et al.*, 1993). These abnormalities in mouse embryos were suggested to arise from fundamental deficiencies in mesodermal migration, adhesion, proliferation or differentiation as a result of the absence of fibronectin. Collectively, the differential distribution of fibronectin in the developing primary palate suggests that the mesenchymal cells in the peripheral regions of the facial prominences may utilize fibronectin as a substrate for cell adhesion, proliferation, and differentiation. As a result, successful outgrowth of the primary palate takes place.

Distribution of type IV collagen, laminin, and fibronectin was also studied during maxillary process formation in the chick embryo between stages 22 and 31 (Xu *et al.*, 1990). It was found that the staining of type IV collagen was more intense in the basement membranes lining the roof of the stomodeum than in the maxillary process at all staged examined, whereas laminin appeared to be uniformly stained throughout the basement membrane of all regions examined. It was proposed that maxillary process outgrowth may be related directly to changes in the distribution of type IV collagen. Laminin, on the other hand, may serve mainly as a structural support (Xu *et al.*, 1990). In the present study, the staining of type IV collagen, laminin, and fibronectin was also found to be more diffuse particularly in the basement membrane of the tip of the maxilla. These findings suggest that the basement membrane becomes less organized in the region that needs to expand and outgrow.

In summary, the basement membrane components; laminin, type IV collagen, and fibronectin; were found to become rapidly disrupted in the epithelial seam during the primary palate formation. These results indicate that disruption of the basement membrane is important for epithelial seam regression that leads to successful mesenchymal bridge formation and fusion of the facial prominences.

CHAPTER 3: Cell Proliferation and Distribution of EGF, TGF-α, and EGF-Receptor in the Developing Mouse Primary palate

Introduction

Both EGF and TGF-α are members of the EGF family found in various embryonic and adult tissues. They exert their actions via the same receptor, EGF-R (Massague, 1983; Hernandez-Sotomayor and Carpenter, 1992). The expression of these three molecules has been found in many organ development systems including the preimplantation embryo (Adamson, 1993; Chia *et al.*, 1995), tooth (Partanen and Thesleff, 1987; Partanen, 1990; Shum *et al.*, 1993), mandible (Kronmiller *et al.*, 1993; Shum *et al.*, 1993), secondary palate (Dixon *et al.*, 1991), and gastrointestinal tract (Miettinen et al., 1989; Miettinen, 1993; Hormi and Lehy, 1994).

Nonsyndromic cleft lip with or without cleft palate (Cl/P) is one of the most common human birth defects, with frequencies of 1/1000 in Caucasians (Thompson *et al.*, 1991), 1.7/1000 in Asians (Kobayashi, 1958; Neel, 1958), and 2.75/1000 in North American Indians in British Columbia (Lowry and Renwick, 1969). The eitiology of Cl/P is complex and associated with heterogenous factors including anatomical variations, racial differences, genetic and environmental factors (see review in Johnston and Bronsky, 1995). Recent studies have suggested that genetic factors play an important role in Cl/P malformation and may involve several susceptibility loci in humans (Farrall and Holder, 1992; Mitchell and Risch, 1992) and mice (Juriloff, 1995). In human studies, TGF-α locus has been indicated to be one of the potential Cl/P susceptibility loci based on population association studies (Ardinger *et al.*, 1989; Chenevix-Trench *et al.*, 1991, 1992; Holder *et al.*, 1992; Sassni *et al.*, 1993; Feng *et al.*, 1994). Therefore, it

was suggested that allelic variants of TGF-α may have differential effects on primary palatal epithelial cells (Feng *et al.*, 1994). A variant molecule, in combination with other factors, may cause the epithelial cells either to proliferate excessively or to differentiate prematurely in some way that preclude successful fusion of the facial prominences. However, involvement of TGF-α during primary palate morphogenesis remains unknown.

In the present study, I, therefore, undertook a comprehensive study on the temporo-spatial distribution of TGF-α along with its receptor, EGF-R, and a related growth factor, EGF during primary palate morphogenesis by means of indirect immunohistochemistry with conventional and/or confocal laser scan microscopes. In addition, the regional cell proliferation within the developing primary palate was studied by using 5-bromodeoxyuridine (BrdU) incorporation and proliferating cell nuclear antigen (PCNA) immunolocalization (Iamaroon *et al.*, 1996a).

Materials and Methods

Tissue collection and preparation

CD1 mice were mated overnight and embryos were collected on days 10 and 11 (plug = day 0). A small number of CL/Fr mice which showed normal development of the primary palate (20-30% of embryos have spontaneous cleft lip) were also used in this study. The TS number from the caudal edge of the hind limb was determined (Wang, 1992). The heads were fixed in 4% (w/v) paraformaldehyde in PBS for labelling of EGF, TGF-α, EGF-R and PCNA, and in 70% ethanol for labelling of BrdU for 24 hours. The paraffinized specimens were

serially sectioned coronally at a thickness of 7 μm. Information of animal breeding and maintenance and mouse stocks is shown in Chapter two.

For BrdU labelling, on days 10 and 11, the pregnant mice were injected intraperitoneally with 0.2 ml of 10 mg/ml BrdU (Sigma, St. Louis, MO) and killed 2 hours following injection. The embryos were obtained and processed as mentioned above.

Immunohistochemistry

For the staining of EGF and TGF-α, an indirect immunofluorescence technique was used. Briefly, deparaffinized sections were incubated with 0.4% (w/v) pepsin (Sigma, St. Louis, MO) in 0.02M HCl for 5 min. The purpose of proteolytic digestion with pepsin was to remove the cross-linking in paraformaldehyde-fixed tissues and render tissue antigen reactive. Sections without pepsin treatment were also included and found weak staining. Subsequently, the sections were incubated with normal goat serum for 20 min at room temperature and a polyclonal anti-EGF (rabbit anti-mouse, Sigma, St. Louis, MO, E-2635) or polyclonal anti-TGF-α (sheep anti-human recombinant, Chemicon, Temecula, CA, AB1412) antibody both at the concentration of 1:50 overnight at 4° C. After washing with PBS, the sections were incubated with the TRITC (goat anti-rabbit, Sigma, St. Louis, MO, T-6778) or FITC (donkey anti-sheep, Sigma, St. Louis, MO, F-7634) conjugated secondary antibody for 1 hour at 4° C both at the concentration of 1:200.

For labelling of EGF-R, the avidin-biotin-immunoperoxidase (ABC) technique was employed as previously described (Hsu *et al.*, 1981). Briefly, deparaffinized sections were incubated with 3% (v/v) hydrogen peroxide for 20 min to eliminate endogenous peroxidases, washed with PBS, and then digested

with 0.4% (w/v) pepsin (Sigma, St. Louis, MO) in 0.02M HCl for 5 min. After washing with PBS, the slides were incubated with normal goat serum for 20 min at room temperature and then with a monoclonal anti-EGF-R (mouse anti-human, Clone F4, Sigma, St. Louis, MO, E-3138) at the concentration of 1:20 overnight at 4° C. After washing with PBS, the slides were incubated with a biotin-conjugated secondary antibody (goat anti-mouse, Vector, Burlingame, CA, PK-4002) and then with StreptABComplex (Dakopatts, Santa Barbara, CA, K377) both at the concentration of 1:100 for 30 min at room temperature. Dark brown staining was developed by using 3,3'-diaminobenzidine hydrogen peroxide (DAB) as a substrate. The section were counterstained with methyl green. For PCNA (monoclonal mouse anti-human PC10 antibody, Dimension, Mississauga, ONT, Canada) labelling, sections were treated as those of EGF-R except the absence of incubation with 0.4% (w/v) pepsin in 0.02M HCl. The slides were observed and photographed with the Zeiss photomicroscope and/or the Zeiss confocal laser scan microscope.

For BrdU labelling, deparaffinized sections were treated with 2N HCl for 10 min and neutralized by using sodium tetraborate (pH 9) for 10 min. Subsequently, the sections were incubated with normal goat serum for 20 min and a monoclonal anti-BrdU antibody (mouse anti-human, Clone BU-1, Amersham, Oakville, ONT, Canada, RPN202) at 4° C overnight, followed by incubation with FITC conjugated secondary antibody (goat anti-mouse, Sigma, St. Louis, MO, F-9006) at the concentration of 1:200 overnight at 4° C.

The specificity of antibodies against EGF-R (Gullick *et al*, 1986), TGF-α (Ju *et al*, 1991), EGF (Beerstecher *et al.*, 1988), BrdU (Gonchoroff *et al.*,1986), and PCNA (Waseem and Lane, 1990) have been partially characterized by others. The monoclonal antibody against EGF-R specifically recognizes the cytoplasmic

tyrosine kinase catalytic domain of EGF-R and cross-reacts with mouse tissue (Fowler *et al.*, 1995). This antibody recognizes *c-ErbB1* form of EGF-R. The specificity of anti-human TGF-α antibody was also characterized and found not to cross-react with EGF or TGF-β (Chemicon, Temecula, CA). Positive control tissues used for anti-EGF antibody was embryonic mouse lung (Warburton *et al.*, 1992); for anti-TGF-α antibody, human oral squamous cell carcinoma (Christensen *et al.*, 1993, kindly provided by Dr. L. Zhang), for anti-PCNA antibody, human tonsil (kindly provided by Dr. L. Zhang) and mouse intestine (Hall *et al.*, 1990; Dolbeare, 1995); and for anti-BrdU antibody, mouse intestine (Dolbeare, 1995). All positive control tissues showed positive immunoreactivity as predicted. Within each group of the sections, one negative control slide was incubated with normal serum as a replacement of the primary antibodies. The results showed that there was no staining on the negative control slides. The dilution tests were performed on the antibodies against EGF, TGF-α, EGF-R, and BrdU. The results showed that the staining intensity decreased gradually as the dilution of the antibodies increased, as predicted. A series of dilution test for anti-PCNA antibody (PC10) was characterized (Hall *et al.*, 1990) and the optimal dilution for the immunostaining was used as recommended (Pendleton *et al.*, 1993). An irrelevant monoclonal antibody was also used as a control (mouse anti-human epithelial keratin, AE1, ICN, Costa Mesa, CA, 69-140). The anti-AE1 antibody recognizes most of the acidic (type I) keratins and cross-reacts with mouse keratins. The sections of the mouse primary palate were treated similar to those of BrdU and EGF-R. The result showed intense staining of keratin particularly in the stomodeal epithelial cells (Fig. 18D).

Confocal microscopy

Tissues from the primary palate stained with antibodies against EGF and TGF-α were examined with both epifluorescence and confocal microscopy using a 20X objective on the Zeiss Confocal Laser Scan Microscope (LCM10). An argon laser (λ_{max}=488 nm) and a helium-neon laser (λ_{max}=543 nm) were used for FITC- and rhodamine-labelled tissues, respectively. Tissues were sectioned at 1 or 2 μm intervals and images were printed using a video printer (UP-5000, Sony Canada) and the Polaroid 6000 film recorder system.

Results

EGF, TGF-α, and EGF-Receptor

At least 33 embryos were used in these study (see Appendix 4). Consecutive sections were not employed in these studies but selected comparable sections were used for each antibody staining. The stainings of EGF, TGF-α, and EGF-R showed to have similar distribution patterns at all stages examined during morphogenesis of the primary palate. A small number of CL/Fr strain of mice which showed normal development of the primary palate were also used in these studies and showed similar results to those of CD1 strain of mice. CL/Fr mouse embryos with cleft fomation were excluded.

Epithelial seam formation

In the anterior region of the face, the staining of EGF, TGF-α, and EGF-R (Figs. 9A-C) was found in epithelial and mesenchymal tissues, particularly at the tips and peripheral regions of the lateral and medial nasal prominences. Upon

fusion of the medial nasal prominence with the lataral nasal and maxillary prominences, labelling of EGF, TGF-α, and EGF-R (Figs. 10A-C) was intensely present in the fusion and peripheral areas of the facial prominences. The expression of the three molecules decreased in the deeper regions of the facial prominences (Figs. 11A-B). However, the staining remained pronounced in the peripheral regions of the prominences. The brain and nasal epithelium were minimally stained at all stages examined.

Epithelial seam disruption and mesenchymal bridge enlargement

As the epithelial seam was disrupted with an enlargement of the mesenchymal bridge, the staining of EGF, TGF-α, and EGF-R (Figs. 12A-C) was absent in the disrupted epithelial seam and the midline tissues. However, the staining remained intense in the epithelial and mesenchymal cells in the peripheral regions of the maxillary and nasal prominences.

BrdU and PCNA

At least twenty-two embryos were used in these studies (see Appendix 4). BrdU and PCNA were found to have similar distribution patterns at all stages examined during morphogenesis of the primary palate.

Epithelial seam formation

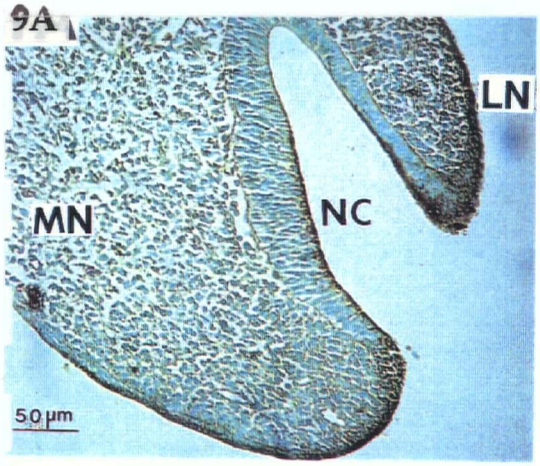
In the anterior region of the face, the staining of BrdU and PCNA (Figs. 13A,B) was intensely localized in the nuclei of the epithelial and mesenchymal cells of the lateral and medial nasal prominences. The central aspect of the medial nasal prominence appeared to have fewer labelled cells than other areas. Upon fusion of the medial nasal prominence with the lateral nasal and

maxillary prominences, BrdU and PCNA (Figs. 14A,B) appeared to be intensely labelled at the peripheral regions of the facial prominences. The fusion areas showed decreased labelling. In the deeper regions, the labellings of BrdU and PCNA (Figs. 15A,B; 17A,B) became dramatically reduced in the midline regions. The peripheral regions of the maxillary and nasal prominences were stained more intensely. The brain showed positive stained cells particularly at its superior and inferior aspects. Labelling indices were not calculated in these studies.

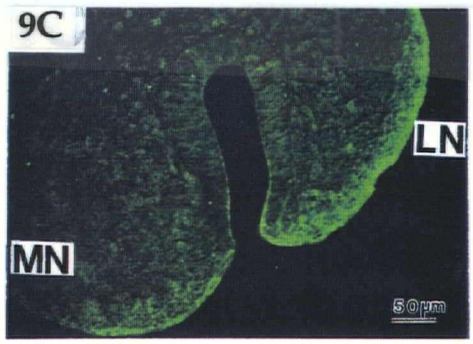
Epithelial seam disruption and mesenchymal bridge enlargement

As the epithelial seam regressed and was replaced by the mesenchymal bridge, the staining of BrdU and PCNA (Figs. 16A,B) appeared to be most pronounced in the epithelial and mesenchymal cells at the peripheral regions of the maxillary and nasal prominences. The disrupted epithelial seam and the midline areas showed decreased labellings of BrdU and PCNA.

The negative controls sections, incubated with 20-25% (v/v) normal goat or donkey serum as a replacement of the primary antibodies, showed absent staining (Figs. 18A-C). A monoclonal antibody against epithelial keratin (anti-AE1 antibody) was also used as a control and found to be stained appropriately on the surface ectoderm as predicted (Fig. 18D). Mouse lung buds were used as a positive control for anti-EGF antibody (Fig. 18E) and found positive as predicted while anti-TGF-α antibody gave a negative result (Fig. 18F).


9A
LN
NC
MN
50 µm


9B
LN
MN
50µm


9C
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MN
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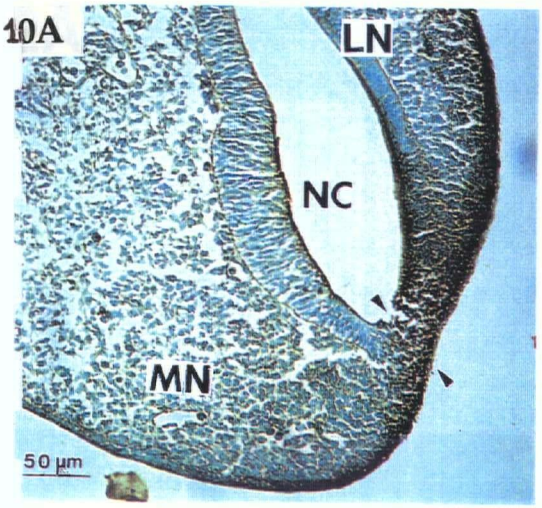
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MN
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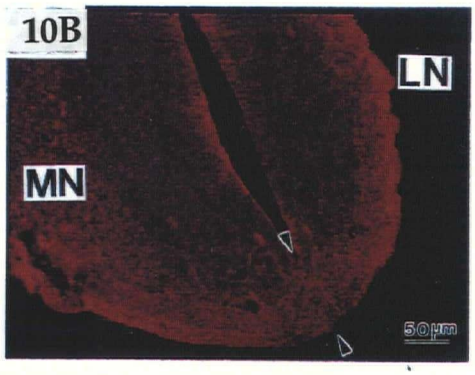
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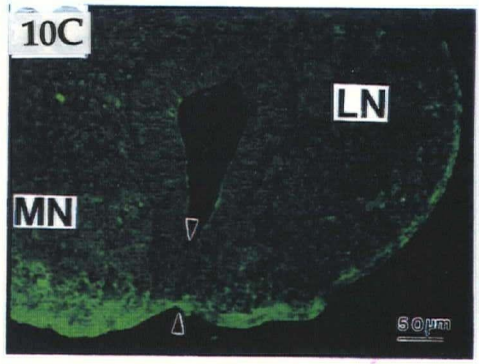
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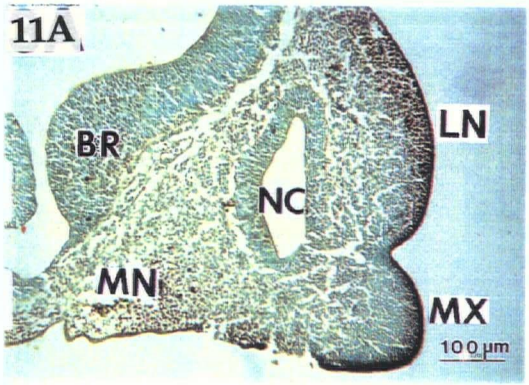
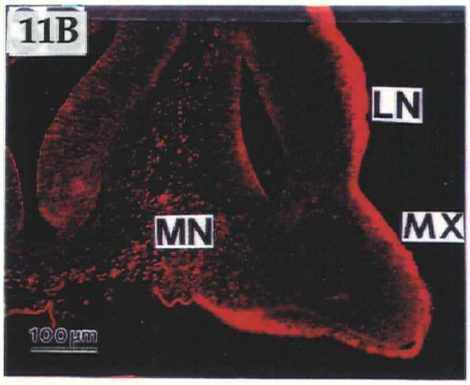
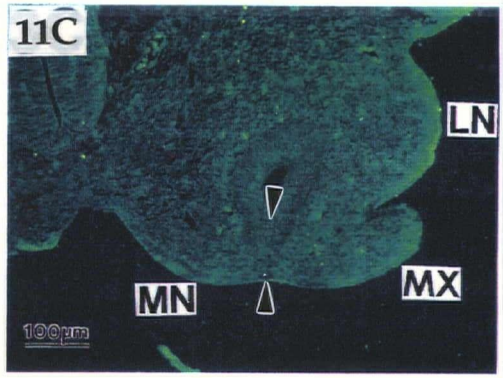
LN
MN
50µm


11A
BR
LN
NC
MN
MX
100 µm


11B
LN
MN
MX
100µm


11C
LN
MN
MX
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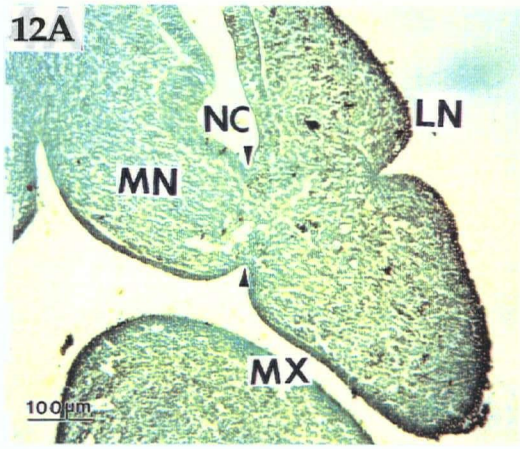
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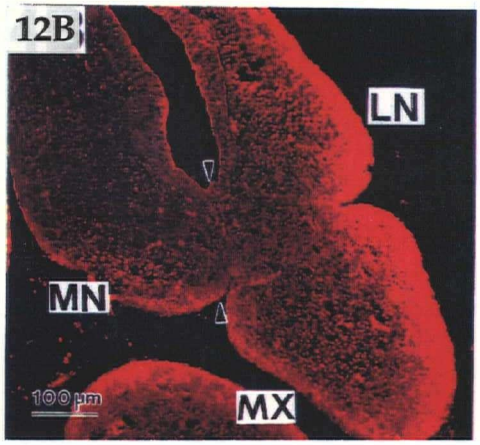
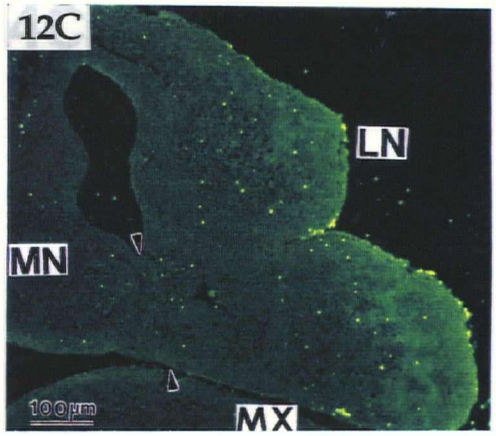

12B
LN
MN
100µm
MX


12C
LN
MN
100µm
MX

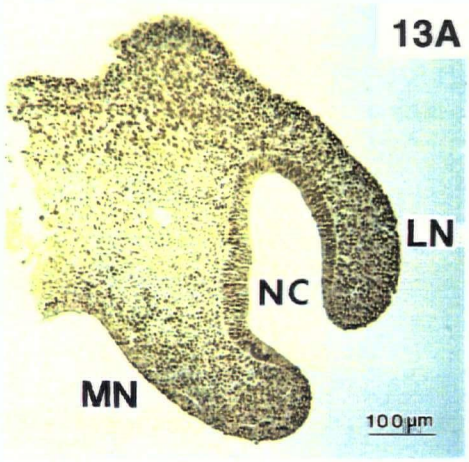
Figs. 9-12: Frontal sections through the developing mouse nasal region during formation of the primary palate. The sites of fusion between the prominences are shown by the arrows.
BR, brain; LN, lateral nasal prominence; MN, medial nasal prominence; MX, maxillary prominence; NC, nasal cavity

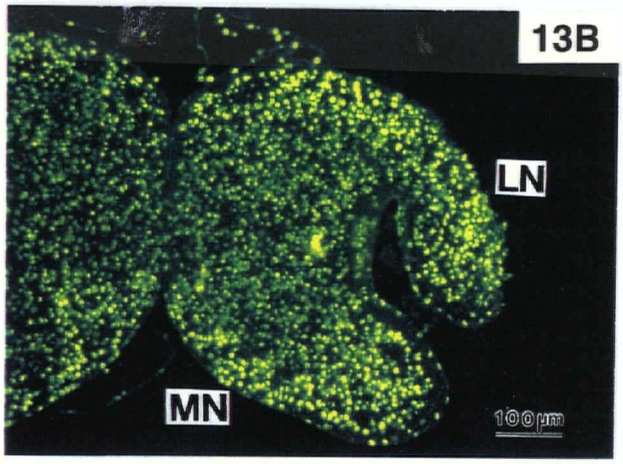
Fig. 9: During epithelial seam formation, anteriorly, EGF-R was intensely localized to the tips and peripheral regions of the LN and MN (A). The confocal micrographs show similar patterns of labelling of EGF (B) and TGF-α (C).

Fig. 10: Upon fusion of the facial prominences, the staining of EGF-R was intensely present at the fusion (between arrows) and peripheral areas of the LN and MN (A). The confocal micrographs show similar patterns of labelling of EGF (B) and TGF-α (C).

Fig. 11: In the deeper regions, the staining of EGF-R remained present at the peripheral regions of the MX, LN, and MN (A). The confocal micrographs show similar patterns of labelling of EGF (B) and TGF-α (C). The midline and nasal fin (between arrows) areas were negative for staining.

Fig. 12: During epithelial seam disruption and mesenchymal bridge enlargement, the stainings of EGF-R (A), EGF (B), and TGF-α (C) were absent in the disrupted epithelial seam (between arrows) but remained intense in the peripheral regions of the facial prominences.


13A
LN
NC
MN
100μm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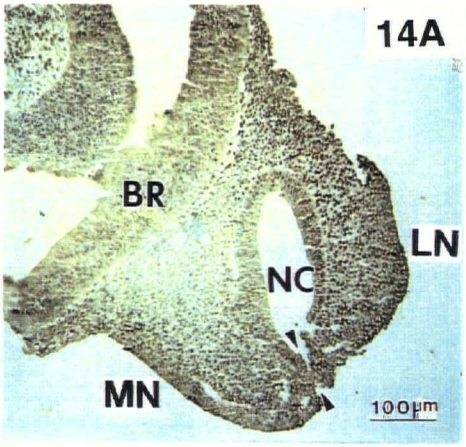
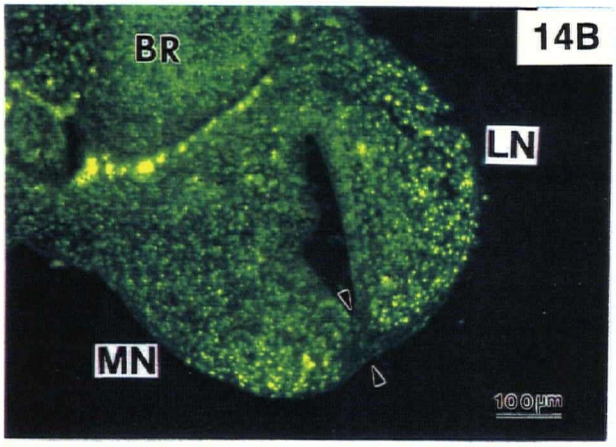
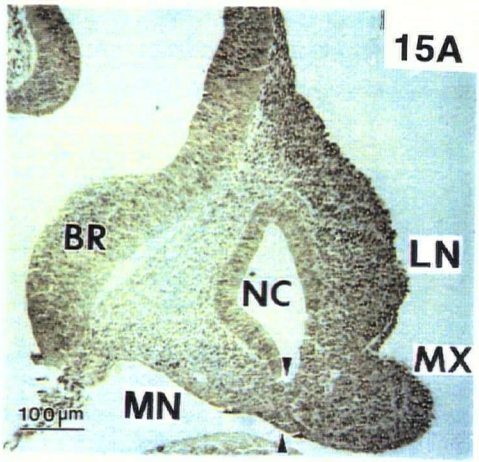
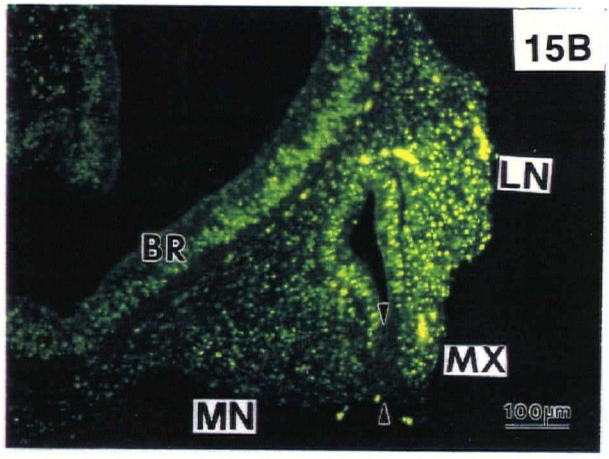
MN
100μm


14A
BR
LN
NC
MN
100μm


14B
BR
LN
MN
100μm


15A
BR
LN
NC
MX
MN
100μm


15B
LN
BR
MX
MN
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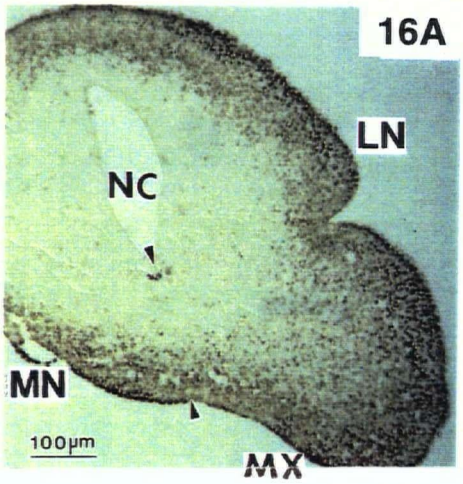
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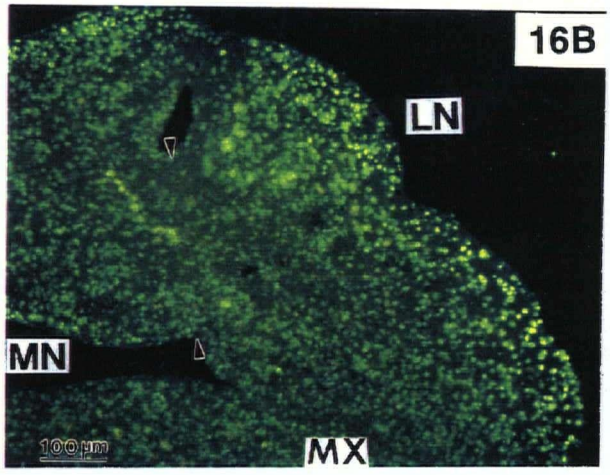
NC
MN
100μm
MX


16B
LN
MN
100μm
MX

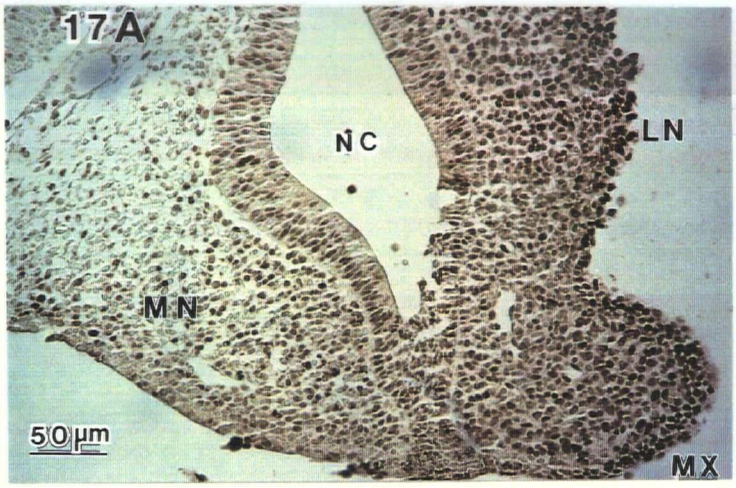
Figs. 13-16: Frontal sections through the developing mouse nasal region during formation of the primary palate. The sites of fusion between the prominences are shown by the arrows.
BR, brain; LN, lateral nasal prominence; MN, medial nasal prominence; MX, maxillary prominence; NC, nasal cavity

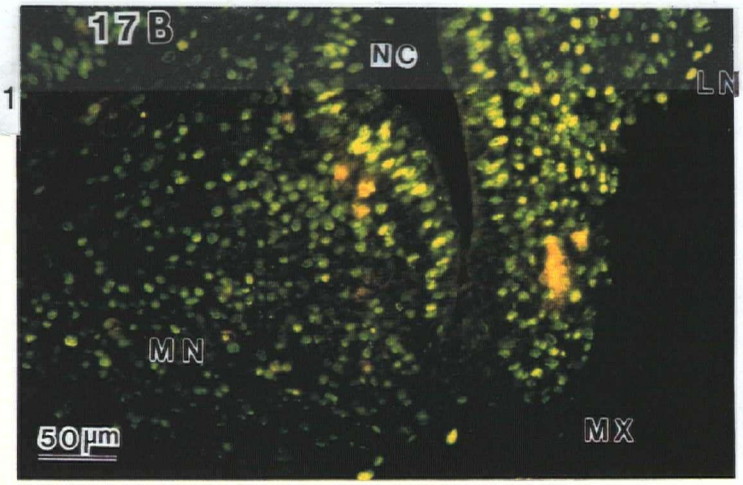
Fig. 13: During epithelial seam formation, anteriorly, PCNA (A) and BrdU (B) labelled cells were present in the epithelium and mesenchyme particularly of the LN and the tip of the MN.

Fig. 14: Upon fusion of the facial prominences, the stainings of PCNA (A) and BrdU (B) were intensely present at the LN and the tip of the MN. The epithelial seam (between arrows) showed reduced staining.

Fig. 15: Posteriorly, the stainings of PCNA (A) and BrdU (B) were mainly present at the peripheral regions of the facial prominences. The nasal fin (between arrows) area showed decreased staining.

Fig. 16: During epithelial seam disruption and mesenchymal bridge enlargement, the stainings of PCNA (A) and BrdU (B) were generally reduced but remained pronounced in the peripheral regions of the facial prominences. The disrupted epithelial seam (between arrows) showed markedly reduced staining.


17A
NC
LN
MN
50μm
MX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MN
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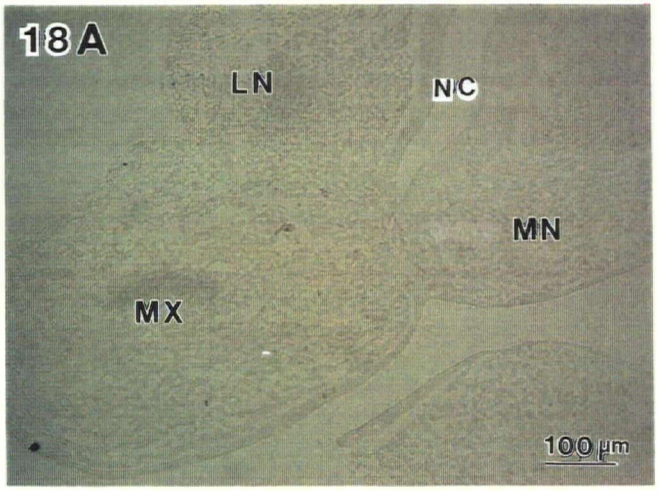
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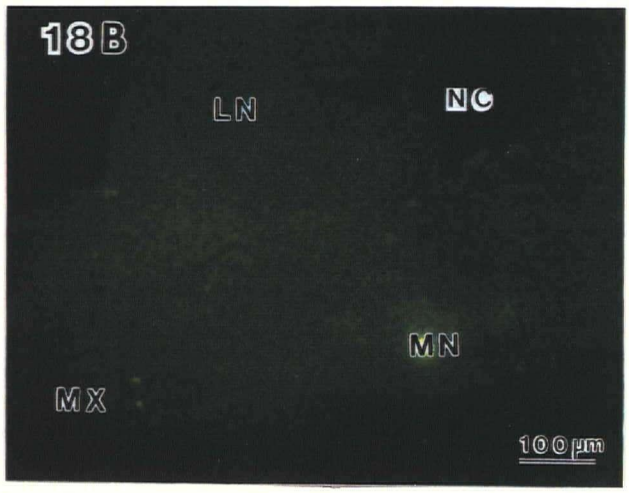
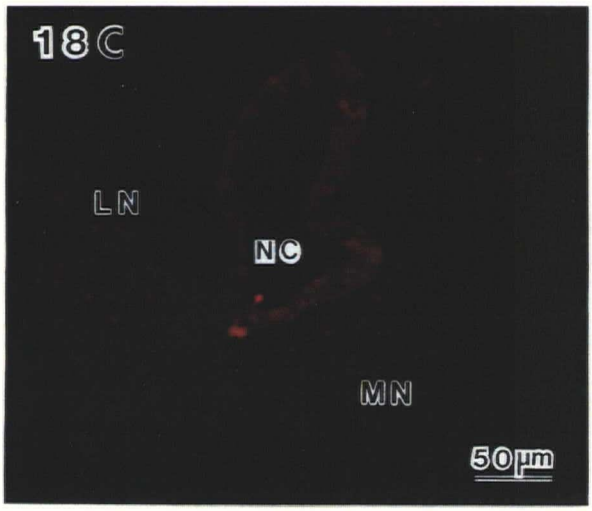
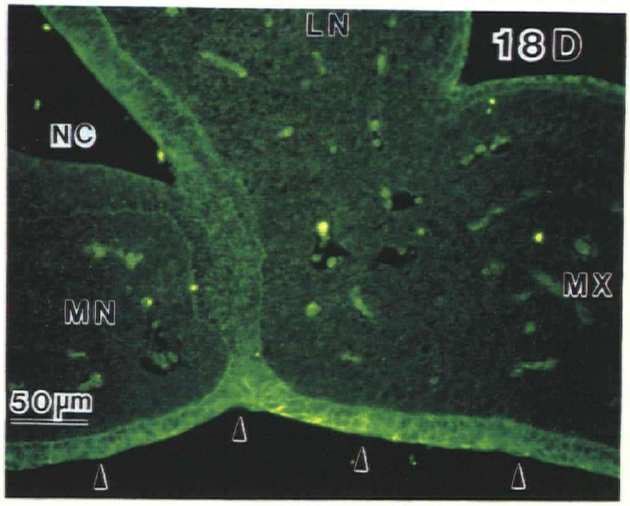
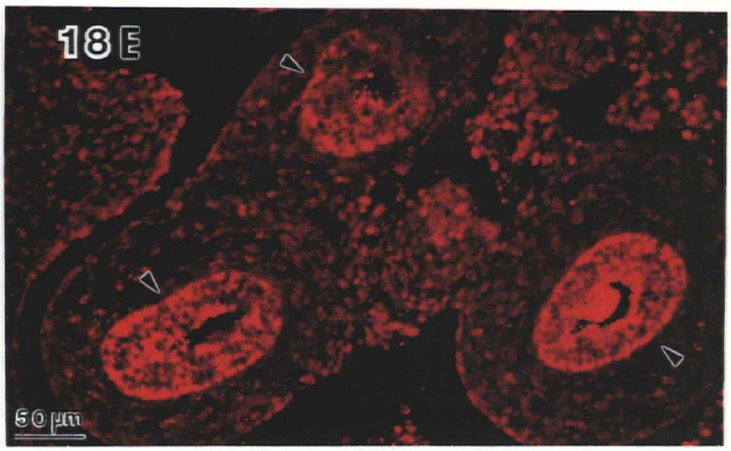
LN
NC
MN
MX
100μm


18B
LN
NC
MN
MX
100μm


18C
LN
NC
MN
50μm


LN
18D
NC
MX
MN
50μm


18E
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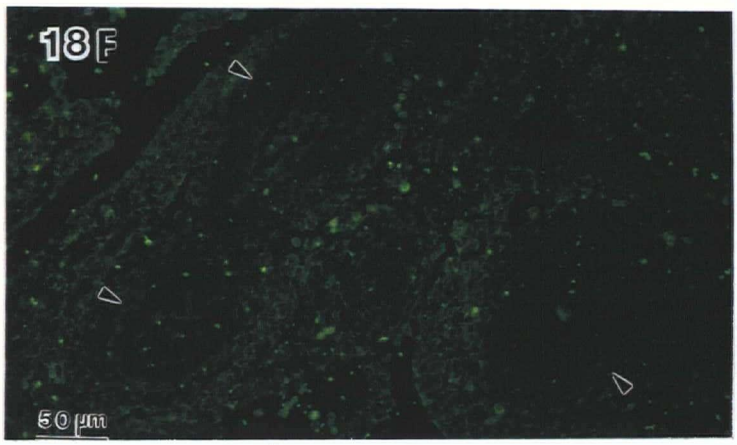

18F
50μm

Figs. 17A,B: High magnifications of the developing primary palate during the stage of epithelial seam formation demonstrate intense labelling of PCNA (A) and BrdU incorporation (B) particularly in the lateral nasal and maxillary prominences.
LN, lateral nasal prominence; MN, medial nasal prominence; MX, maxillary prominence; NC, nasal cavity

Figs. 18A-C: The negative control sections, incubated with normal serum as a replacement of the primary palate and biotin- (A), FITC- (B), or rhodamine-conjugated secondary antibody, showed absent staining in the developing primary palate.

Fig. 18D: A monoclonal antibody, mouse anti-human epithelial keratin antibody (AE-1), was used as a control revealing intense staining particularly in the stomodeal epithelial cells (arrowheads). The nasal and surface epithelia showed less intense labelling.

Fig. 18E,F: The epithelial linings of the developing mouse lung buds (arrowheads) demonstrated pronounced labelling for EGF (E) but absent staining for TGF-α (F).

Discussion

In the present study, the expression of EGF, TGF-α, and their receptor, EGF-R (*c-erbB1*) was characterized by means of indirect immunohistochemistry. Interestingly, the distribution patterns of the three molecules showed striking similarity at all stages examined during primary palate morphogenesis. These observations suggest an autocrine mode of growth regulation for these growth factors during outgrowth and fusion of the facial primordia. Previous studies have also shown the coexpression of these growth factors and their receptor during mandibular morphogenesis (Shum *et al.*, 1993), embryonic preimplantation (Johnson *et al.*, 1994; Chia *et al.*, 1995) and malignant transformation (Derynck *et al.*, 1987; Christensen *et al.*, 1993; Barton *et al.*, 1991). Alternatively, a paracrine mode of regulation may also be operative since the strongest signals of EGF and TGF-α appeared to be at the surface ectoderm. The growth factors may diffuse into the underlying mesenchyme, bind to EGF-R at the surfaces of the mesenchymal cells and stimulate cell proliferation. As a result, a differential proliferation of the mesenchymal cells occurred as shown in the studies of immunolocalization of PCNA and BrdU incorporation.

EGF, TGF-α, and EGF-Rs are believed to be involved in different stages of embryogenesis including preimplantation, the time during which the cell lineages are set up, and the beginning of organogenesis (Werb, 1990; Adamson, 1993). Later during secondary palate development, the role of EGF, TGF-α, and EGF-R has also been investigated (Pratt, 1987; Dixon *et al.*, 1991,1993). Pratt (1987) speculated that TGF-α, produced by the epithelium of the palatal shelves, stimulates epithelial proliferation in the early stages. Upon fusion of the palatal shelves, the epithelial seam ceases production of TGF-α and undergoes a series of

events of programmed cell death. More recently, TGF-α and EGF-R were immunolocalized to the tips of the maxillary prominences in the early stages of the secondary palate formation (Dixon *et al.*, 1991). Upon fusion of the palatal shelves, TGF-α and EGF-R were intensely labelled in the fusion area and remained in the disrupted epithelial seam. EGF, on the other hand, was sparsely expressed at all stages examined. EGF and TGF-α were also found to be capable of stimulating production of a variety of extracellular matrix molecules by mouse embryonic palatal mesenchymal cells *in vitro* (Dixon *et al.*, 1993). Therefore, it was proposed that growth factors, especially TGF-α, and EGF-R participate in the secondary palate formation (Dixon *et al.*, 1991,1993).

Interestingly, the expression of the homeobox-containing genes encoding transcriptional factors, *Msx*-1 (Hox-7) and *Msx*-2 (Hox-8), was localized particularly in the mesenchyme and ectoderm of the tips of the facial prominences (see Table I) (Robert *et al.*, 1989; MacKenzie *et al.*, 1992; Nishikawa *et al.*, 1994), the regions where EGF, TGF-α, and EGF-R were also distributed during primary palate morphogenesis. *Msx*-1 and *Msx*-2 are believed to be important for epithelial-mesenchymal interactions in the developing organs. *Msx*-1, in particular, promotes cell proliferation and suppresses cell differentiation in myoblastic cell culture (Song *et al.*, 1992). Taken together, these findings imply relationships between *Msx*-1 and *Msx*-2 with EGF, TGF-α, and EGF-R especially in cell proliferation during the outgrowth of the primary palate. Recently, another growth factor member, FGF-8, was studied by *in situ* hybridization and also found to be localized to the facial prominences (Heikinheimo *et al.*, 1994; Ohuchi *et al.*, 1994; Crossley and Martin, 1995). At day 9.5, FGF-8 was expressed in the prospective nasal placode, the commissural plate

of the forebrain, and the surface ectoderm of the the maxillary and mandibular components of the first branchial arch. In later development (day 10.5), the expression of FGF-8 was found in ectodermal cells surrounding the nasal pits and surface ectoderm of the maxilla and mandible. It was suggested that FGF-8 may be a component of the epithelium-derived signal involved in regulating the outgrowth and patterning of the facial prominences (Heikinheimo *et al.*, 1994; Ohuchi *et al.*, 1994; Crossley and Martin, 1995). More recently, the FGF-8 isoforms were also immunolocalized to the similar regions of the mRNA expression (MacArthur *et al.*, 1995). Collectively, the multiple expressions of several growth and regulatory factors in the developing primary palate suggest that morphogenesis of the primary palate is a complex process that needs specific interactions between tissues and molecules produced at the precise locations and times.

The expression of multiple growth factors has also been investigated during secondary palate morphogenesis. For example, before elevation of the palatal shelf, PDGF-AA isoform was localized only to the palatal epithelia while the PDGFα-receptor was present in the palatal mesenchyme, nasal and medial edge epithelia by immunohistochemistry (Qiu and Ferguson, 1995). During palatal midline epithelial seam formation and disruption, both PDGF-AA and PDGFα-receptor were colocalized in the nasal and midline seam epithelia. However, the staining of PDGF-BB/β receptor were sparse or absent at all stages examined. IGF-II transcripts and peptides were localized to the mesenchyme of the horizontal prefusion palate (Ferguson *et al.*, 1992). Subsequently, IGF-II transcripts were found to be absent during palatal fusion. IGF-II peptides, on the other hand, were localized to the nasal and medial edge epithelia. The expression patterns of TGF-β2, in particular, was found to be remarkably similar to that of

IGF-II during secondary palatal formation (Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1990). The expression of TGF-β3 was first present in the epithelial component of the vertical palatal shelf. Subsequently, TGF-β3 was co-localized with TGF-β1 to the medial edge epithelium of the horizontal palatal shelf and midline epithelial seam during palatal fusion (Fitzpatrick *et al.*, 1990). Similarly, aFGF and bFGF were found to be expressed mainly in the midline epithelial seam and remain in the epithelium of the degenerating seam (Sharpe *et al.*, 1993). These data indicate an interactive network between different growth factors and their receptors in controlling the complex and rapid differentiation process during palatal adhesion, fusion, midline epithelial seam disruption and mesenchymal consolidation (Qiu and Ferguson, 1995). Depletion of TGF-β3, but not TGFβ1 and TGFβ2, was found to inhibit palatal fusion in cultures by using antisense oligonucleotides and neutralizing antibodies (Brunet *et al.*, 1995). This inhibition could be rescued by exogenouse TGF-β3. More recently, TGF-β3 null mutant mice have been generated by gene-targeting and found to have impaired palatal fusion leading to cleft palate and delayed lung development (Proetzel *et al.*, 1995; Kaartinen *et al.*, 1995). Other tissues including the primary palate, craniofacial complex and heart appeared to have normal development. Indeed, the palatal shelves of TGF-β3 null mutants grew and elevated normally but failed to fuse. These findings suggested that TGF-β3 acts specifically on the medial edge epithelial cells and may mediate epithelial-mesenchymal interactions during secondary palate fusion (Kaartinen *et al.*, 1995; Proetzel *et al.*, 1995).

To determine whether there is any relationship between the expression of EGF, TGF-α, and EGF-R and the sites of cell proliferation, cell proliferation was studied by using BrdU and PCNA as the markers (Gratzner, 1982; Leibovici *et al.*,

1992). The distribution patterns of BrdU and PCNA were almost identical and most pronounced in the similar regions to those of the growth factors and their receptor. These findings suggest that EGF and TGF-α may play a role in cell proliferation via EGF-R during primary palate morphogenesis. The expression of PCNA, in particular, may have a direct association with some growth factors *in vivo* since it was found to be inducible by EGF, PDGF, and FGF *in vitro* (Bravo and Macdonald-Bravo, 1984; Jaskulski *et al.*, 1988).

In the present study, the labelling of BrdU and PCNA showed differential distribution during primary palate morphogenesis in which cells at the tips and peripheral regions of the facial prominences particularly the lateral nasal prominence showed more intense staining than those in the deeper regions and the nasal epithelium. In more advanced stages, the labelling was generally reduced but remained pronounced at the peripheral regions of the prominences. These results indicate that the rate of cell proliferation remains stable in the peripheral regions of the facial prominences, while it is reduced in the midline regions as the primary palate development progresses. These findings are consistent with previous studies (Minkoff and Martin, 1984; Minkoff, 1991; Gui *et al.*, 1993; Diewert *et al.*, 1993b). It was suggested that a differential rate in the decline of cell proliferation may be operative as a morphogenetic mechanism during enlargement and union of the facial prominences (Gui *et al.*, 1993) and may be associated with expression of a differentiated phenotype such as chondrogenic differentiation in the roof of the stomodeum (Minkoff and Martin, 1984).

Regional differences of growth rates within the mesenchyme was studied in the maxillary prominence of chick embryos during stages 19-29 by [3]H-thymidine injection (Bailey *et al.*, 1988). Four different regions; including medial

side of the maxilla, ventral tip of the maxilla, lateral side of the maxilla, and central portion of the maxilla; were characterized. It was found that growth rate of the maxillary mesenchyme differed based on its proximity to the overlying epithelium. The rate of cell proliferation in the facial prominences was higher in the subepithelial zone of the mesenchyme compared to that of cell populations which were located further away from the epithelium. Differences of growth rate among the regions, on the other hand, was not detected in the later stages (stages 28-29). These data suggested that the epithelium may exert a growth regulating effect such as growth factors on the subjacent mesenchyme and this effect may be related to the stage of development (Bailey *et al.*, 1988).

Collectively, the localization of the growth factors and their receptor at the tips of the prominences prior to fusion and at the fusion area upon fusion; and the absence of their expression in the disrupted epithelial seam implicate that EGF, TGF-α, and EGF-R may selectively induce tissue proliferation resulting in successful fusion of the prominences. In addition, the intense localization of the growth factors and their receptor as well as BrdU and PCNA at the peripheral regions of the facial prominences suggests that EGF, TGF-α, and EGF-R may participate in the outgrowth and enlargement of the primary palate.

Nonsyndromic cleft lip with or without cleft palate (CL/P) is one the most common malformations of the human structure that is believed to involve unsuccessful fusion of the facial prominences during the primary palate formation (Diewert and Wang, 1992). Since CL/P tends to aggregate within families, genetic factors are believed to play an important role in its cause (Feng *et al.*, 1994). Based on population association, several investigations have indicated that an allele of the TGF-α locus, or a gene closely linked to it, is a risk factor for the CL/P malformation in human (Ardinger *et al.*, 1989; Chenevix-

Trench *et al.*, 1991, 1992; Holder *et al.*, 1992; Sassani *et al.*, 1993; Feng *et al.*, 1994). In the present study, TGF-α was found to be present in the facial prominences during the time of the fusion of the facial prominences. Together, these findings suggest that TGF-α may be important for the fusion of the facial prominences. Recently, knock-out mice with mutant TGF-α gene were generated and found to have normal primary palate development (Mann *et al.*, 1993; Luetteke *et al.*, 1993). However, these data cannot completely preclude the importance of TGF-α in primary palate development, since the mutant mice may obtain compensatory effects from other growth factors for example EGF which is also expressed during primary palate development in the present study.

Although EGF and TGF-α were found to be co-localized during primary palate morphogenesis, only EGF was present in the developing lung bud. These data suggest that some tissues may not be able to fully compensate for lack of a gene product. A future study of transgenic mice lacking EGF would provide support for the role of EGF in lung development.

In summary, the temporo-spatial expression of EGF, TGF-α, and EGF-R was present during primary palate morphogenesis. The three molecules were shown to have similar distribution patterns at all stages examined. The distribution patterns of BrdU and PCNA were similar and most pronounced at the similar regions to EGF, TGF-α, and EGF-R. These findings suggest that EGF, TGF-α, and EGF-R may stimulate cell proliferation, which in turn contribute to the fusion of the facial prominences and outgrowth of the primary palate.

CHAPTER 4: Distribution of 72-kDa gelatinase (MMP-2) in the developing craniofacial complex of the mouse embryo

Introduction

Craniofacial development is complex and involves many basic biological processes including tissue interactions, cell migration, proliferation, and differentiation. As a result, many organ systems; for example the primary and secondary palates, mandible, brain, nose, ear, and eye; are formed. Remodelling of the extracellular matrix (ECM) was suggested to be important for cell migration, cell-cell interactions, embryo expansion, uterine implantation, and tissue invasion during mammalian embryogenesis (Werb *et al.*, 1992). However, the mechanisms of tissue remodelling in the developing craniofacial complex remain unclear.

Matrix metalloproteinases (MMPs) are believed to play an important role in physiological matrix degradation (reviewed by Overall, 1994). The MMP family is composed of four enzyme classes; the collagenases, type IV collagenases/gelatinases, stromelysins, and membrane-type MMPs (MT-MMPs). Interstitial collagenase specifically degrades types I-III collagens to produce thermally unstable degradation fragments which then denature to form gelatin, which is further degraded by other MMPs including gelatinases and stromelysins (reviewed by Overall, 1994). The 72-kDa (MMP-2) and 92-kDa gelatinases (MMP-9) also degrade native types IV, V (Fessler *et al.*, 1984), VII (Seltzer *et al.*, 1989), and X collagens (Welgus *et al.*, 1990). Although 72-kDa gelatinase can degrade fibronectin and laminin, 92-kDa gelatinase does not (Okada *et al.*, 1990).

The importance of 72-kDa gelatinase in tissue remodelling and embryogenesis is indicated from studies in which 72-kDa gelatinase has been detected in a wide variety of embryonic tissues. During tooth morphogenesis, 72-kDa gelatinase was found to be associated with enamel development (Overall and Limeback, 1988) and dental basement membrane degradation in mice (Sahlberg *et al.*, 1992) and humans (Heikinheimo and Salo, 1995). EGF and TGF-α can induce 72-kDa gelatinase activity during branching morphogenesis of the lung (Ganser *et al.*, 1991). 72-kDa gelatinase is also associated with breakdown of the basement membrane during mammary gland involution (Talhouk *et al.*, 1992; Dickson and Warburton, 1992), development of the mandibular condyle (Breckon *et al.*, 1994), neurite outgrowth (Muir, 1994), and myogenesis (Guerin and Holland, 1995).

The expression of 72-kDa gelatinase has been studied by *in situ* hybridization during mouse development and found to be localized to the mesenchyme of the first, second, and third branchial arches of 10- and 11-day-old embryos but different stages of primary palate formation were not examined (Reponen *et al.*, 1992). 72-kDa gelatinase was also expressed in the vitreous body and corneal stroma of the developing eye and osteogenic mesenchyme of the mandible in the 13-day-old embryo. More recently, MMPs, including 72-kDa gelatinase, were detected in the mouse facial prominences of 10-to-11-day-old mouse embryos by zymography (Da Silveira *et al.*, 1995), Western blot and reverse transcriptase polymerase chain reaction (RT-PCR) analyses (Da Silviera *et al.*, 1996). These results have confirmed the presence of 72-kDa gelatinase in the developing craniofacial complex and suggested its importance during craniofacial development. However, the temporo-spatial protein expression of 72-kDa gelatinase in the developing craniofacial complex has not been investigated.

The purpose of the study was to characterize relationships between 72-kDa gelatinase and early development of the mouse craniofacial complex by gelatin zymography and indirect immunofluorescence with conventional and confocal laser scan microscopy. In addition, the co-distribution of 72-kDa gelatinase and the growth factors, EGF and TGF-α, were also investigated by indirect immunofluorescence with confocal laser scan microscopy.

Materials and Methods

Tissue preparation

CD1 and CL/Fr mice were mated overnight and embryos were collected on days 10 and 11 (plug = day 0). The number of tail somites (TS) from the caudal edge of the hind limb was determined (Wang, 1992). The heads were fixed in 4% (w/v) paraformaldehyde in PBS for 24 hours and paraffin embedded. The specimens were serially sectioned frontally through the face as previously described (Diewert and Lozanoff, 1993) at a thickness of 7 μm. Thirty four CD1 mouse embryos were used for the facial prominences and seventeen CD1 mouse embryos for eye studies. A small number of CL/Fr mouse embryos which showed normal development of the primary palate were also used in these studies (see Appendix 4).

Immunofluorescence

Deparaffinized sections were incubated with 0.4% (w/v) pepsin in 0.02 M HCl for 5 min and then washed with PBS. No other proteolytic treatment was performed in these studies. Subsequently, the slides were incubated in normal goat serum for 30 min at room temperature and in an antipeptide antibody

against the C-terminal domain of 72-kDa gelatinase (Wallon and Overall, manuscript in preparation) at a concentration of 1:200 overnight at 4° C. Different concentrations were tried and found that 1:200 was the optimum concentration. Both non- and affinity-purified anti-72-kDa gelatinase antibodies were used and showed similar results. Therefore, the results from these two antibodies were used in the present studies. After washing with PBS, the slides were incubated in rhodamine-conjugated secondary antibody (Sigma, St. Louis, MO, goat anti-rabbit IgG crystalline TRITC conjugate, T-6778) at a concentration of 1:200 for 1 hour at 4° C and washed. The slides were observed and photographed with a Zeiss photomicroscope equipped with a filter for rhodamine excitation.

The co-localization of 72-kDa gelatinase with the growth factors, EGF and TGF-α, was determined by using the adjacent consecutive sections of the primary palate of the 10-to-11-day-old mouse embryos under the same conditions of immunostaining. Sections from embryonic day 12 were also included. The immunofluorecence studies of EGF, TGF-α, and 72-kDa gelatinase and confocal laser scan microscopy were performed as described in Chapters 3 and 4A. For semiquantitative study, three embryos from each stage were included (see Table V). Intensity of the fluorescence staining was recorded under the epifluorescence microscope as the following: +++ = strongly positive staining; ++ = moderately positive staining; + = weakly positive staining; - = negative staining. The modal values were recorded and shown in Table V.

The anti-72-kDa gelatinase antibody was an antipeptide antibody raised in rabbits against peptide coupled to keyhole limpet hemocyanin. The peptide sequence was selected from an analysis of the primary sequence of the C-terminal domain of human and murine 72-kDa gelatinase which indicated it to be solvent exposed. Subsequent analysis of the 3D structure of the C-terminal domain of

human 72-kDa gelatinase reported by Libson *et al.* (1995) confirmed the solvent accessibility of the peptide sequence which was located on the outer β-strand of the hemopexin-like module 3 of the C-terminal domain. Antipeptide antiserum was collected from whole bodybleed and then affinity purified against the peptide coupled to an Affigel-10 column (BioRad). The specificity against 72-kDa gelatinase was confirmed by Western blot analysis (Wallon and Overall, 1996, data not shown).

Sections of the developing primary palate were also studied with a polyclonal antibody against the whole molecule of human 72-kDa gelatinase (kindly provided by Dr. W. G. Stetler-Stevenson). The results showed immunolocalization in the similar regions to those stained with the antibody against the C-terminal domain of 72-kDa gelatinase (Wallon and Overall, 1996). However, the staining appeared to be also present in the extracellular matrix with high background suggesting nonspecific binding (data not shown). A small number of frozen sections of the primary palate were also immunostained with the antibody against the C-terminal domain of 72-kDa gelatinase. Although the staining was localized in the lateral edges of the nasal prominences and the maxillary tip, similar to paraformaldehyde-fixed sections, the results were inconclusive since tissue morphology was poorly preserved (data not shown).

Confocal Microscopy

Tissues of the craniofacial complex were examined with both epifluorescence and confocal microscopy using a 20X objective on the Confocal Laser Scan Microscope (Zeiss, Germany). A helium-neon laser (λ_{max}=543 nm) was used for rhodamine-labelled tissues. Tissues were sectioned at 1 or 2 μm intervals and images were printed using a video printer (UP-5000, Sony Canada).

Gelatin Zymography

72-kDa gelatinase activity was detected by utilizing a discontinuous-SDS-polyacrylamide-gel electrophoresis-system with L-methoxy-2,4-diphenyl-3(2H)furanone labelled (64958; Fluka, Switzerland) gelatin (G-6650, Sigma) as previously described (O'Grady *et al.*, 1984). By the use of this method, gelatin degradation could be visually monitored under long-wave ultraviolet light. Tissues from day 11 CD1 mouse heads were dissected, snap frozen, homogenized in SDS non-reducing electrophoresis buffer, and centrifuged. The supernatants were loaded onto a 7.5% SDS-PAGE gel co-polymerized with 1 mg/ml of labelled gelatin. After electrophoresis the gel was washed twice in 50 mM Tris buffer (pH 7.5) containing 0.02% NaN_3 and 2.5% Triton X-100. The second wash was then supplemented with 5 mM $CaCl_2$ and 1 μM $ZnCl_2$ and after incubation for 24 hours (37° C) in a 50 mM Tris buffer containing 5 mM $CaCl_2$, 200 nM NaCl, 1 μM $ZnCl_2$, and 0.02% NaN_3 (pH 7.5), the gel was viewed by long-wave ultraviolet light, photographed, and the gel then stained with 0.2% Coomassie Blue R-250.

Results

Immunofluorescence

Primary Palate

Morphogenesis of the primary palate can be classified into three stages; epithelial seam formation (7-11 TS), epithelial seam disruption (12-17 TS), and mesenchymal bridge enlargement (18-20 TS).

During epithelial seam formation (7-11 TS), in the anterior region of the developing mouse face, the staining of 72-kDa gelatinase was mainly present in epithelial and mesenchymal tissues particularly in the tips and peripheral

regions of the lateral and medial nasal prominences (Figs. 19A,B). Upon fusion of the medial nasal prominence with the lateral nasal and maxillary prominences, the staining was intensely localized to the fusion and the peripheral regions of the prominences (Figs. 19C,D). In the deeper region, the staining decreased in the nasal fin area but remained high in the peripheral regions of the facial prominences and the tip of the maxilla (Figs. 19E,F).

As the epithelial seam became disrupted with mesenchymal bridge formation (12-17 TS), the staining of 72-kDa gelatinase was strongly localized to the fusion and the peripheral regions of the nasal prominences (Figs. 20A,B). In the deeper region, the staining decreased to background levels in the nasal fin area but remained intense in the peripheral regions of the lateral nasal and maxillary prominences (Figs. 20C,D).

As the mesenchymal bridge enlarged (18-20 TS), the staining of 72-kDa gelatinase was generally reduced and mainly present in the peripheral regions of the lateral nasal and maxillary prominences (Figs. 20E,F). The midline region, brain, and nasal epithelium showed negative staining at all stages examined. A small number of CL/Fr mice were also stuidied and showed similar results to those of CD1 mice (data not shown).

Mandible and Second Branchial Arch

The immunolocalization of 72-kDa gelatinase was studied in the developing mandible and second branchial arch. At 9 day gestation, 72-kDa gelatinase staining in the mandible was at background levels (see Chapter 5, Fig. 27A). The data revealed a similar pattern of the distribution at embryonic days 10 and 11. In the anterior region of the mandible, the staining of 72-kDa gelatinase was localized to the epithelium and mesenchyme in the tip of the mandible (Figs. 21A,B). As the mandibular prominences merged, the distribution of 72-kDa

gelatinase was intensely present in the peripheral regions of the mandible and the superior aspect of the midline area. 72-kDa gelatinase was also labelled to the peripheral regions of the second branchial arch (Figs. 21C,D). The staining of 72-kDa gelatinase remained negative in the midline tissues at all stages examined.

Eye

During formation of the lens vesicle (embryonic day 10), in the region anterior to the lens, the staining of 72-kDa gelatinase was mainly localized to the surface ectoderm and the underlying mesenchyme (Figs. 22A,B). At the area where the surface ectoderm invaginated to form the lens vesicle (embryonic day 10), staining in the epithelium became negative (Figs. 22C,D). However, the surface ectoderm adjacent to the lens pit remained labelled. In the later stages (embryonic day 11), as the lens completely detached from the surface ectoderm, the staining of 72-kDa gelatinase reappeared in the developing corneal epithelium and mesenchyme (Figs. 22E,F). Blood cells in the vitreous capillaries revealed autofluorescence. The lens and the optic cup and stalk showed negative staining at all stages examined.

Gelatin Zymography

To confirm the expression of 72-kDa gelatinase in the developing mouse craniofacial complex, the gelatinase activity from day 11 embryos (16-18 TS) was analyzed by gelatin zymography. All six samples from embryonic mouse heads during 16-18 TS showed similar results. The results revealed a major gelatinase band corresponding to 72-kDa gelatinase and minor bands representing the activated form of 72-kDa gelatinase (Fig. 21E). In addition, a minor, higher molecular weight gelatinase band was also detected of unknown identity.

Co-distribution of 72-kDa gelatinase, EGF, and TGF-α

Sections from twelve CD1 mouse embryos were used in this series of experiments. The distribution patterns of EGF, TGF-α, and 72-kDa gelatinase in the developing primary palate from 10-day-old embryos through 12-day-old embryos were studied by the use of confocal laser scan microscope. The results were summarized in Table V and described below.

Epithelial seam formation (7-11 TS)

In the anterior region of the face, the staining of EGF, TGF-α and 72-kDa gelatinase was localized to the epithelial and mesenchymal tissues, particularly at the tips and peripheral regions of the lateral and medial nasal prominences (Figs. 24A,B,C). The staining of EGF, TGF-α and 72-kDa gelatinase appeared to decrease in the central area of the medial nasal prominence. In deeper regions, the labelling of EGF, TGF-α and 72-kDa gelatinase was intensely present in the peripheral regions of the facial prominences and the tip of the maxilla (data not shown). The midline tissue showed reduced staining. The nasal epithelium and brain showed minimal staining at all stages examined.

Epithelial seam disruption (12-17 TS)

As the epithelial seam became disrupted with a replacement of the mesenchymal bridge, in the anterior region to the fusion, the staining of EGF, TGF-α and 72-kDa gelatinase was localized to the tips and peripheral regions of the nasal prominences (data not shown). Upon fusion of the medial nasal prominence with the lateral nasal and maxillary prominences, the labelling of EGF, TGF-α and 72-kDa gelatinase was pronounced in the fusion and the

peripheral regions of the facial prominences and the tip of the maxilla (Figs. 25A,B,C). In the deeper region, the staining of EGF, TGF-α and 72-kDa gelatinase appeared to be generally decreased but remained in the peripheral regions of the facial prominences (data not shown).

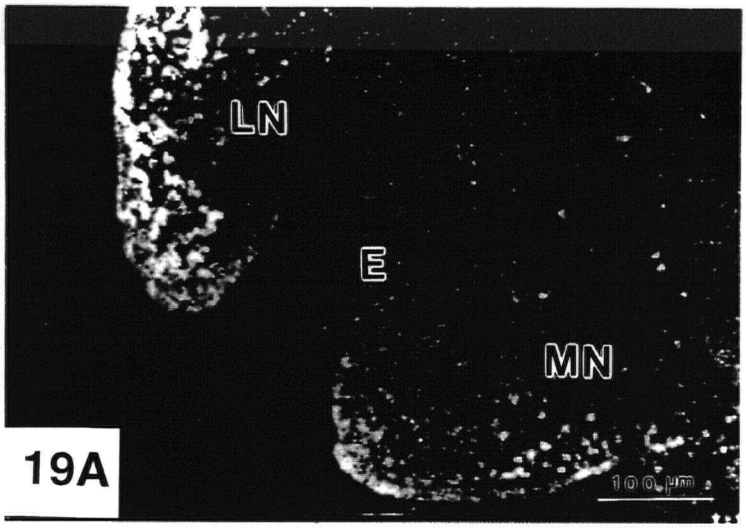
Mesenchymal bridge enlargement (18-20 TS)

As the mesenchymal bridge rapidly enlarged with the outgrowth of the primary palate, the staining of EGF, TGF-α and 72-kDa gelatinase became generally reduced but remained strongly distributed in the peripheral regions of the lateral nasal and maxillary prominences (Fig. 26A,B,C). The midline and deeper regions of the primary palate showed absent staining for EGF, TGF-α, and 72-kDa gelatinase. During embryonic day 12, the labelling of EGF, TGF-α, and 72-kDa gelatinase appeared negative in the primary palate.

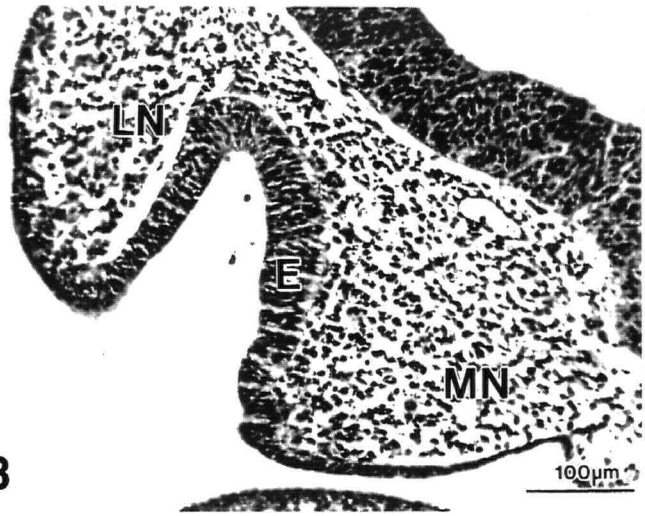
Table V: Distribution of 72-kDa gelatinase, EGF and TGF–α during primary palate morphogenesis.

	7-11 TS	12-17 TS	18-20 TS	day 12
72-kDa gelatinase	+	++	+	-
EGF	++	+++	++	-
TGF-α	+	++	+	-

+++ = strongly positive staining; ++ = moderately positive staining; + = weakly positive staining; - = negative staining



19A



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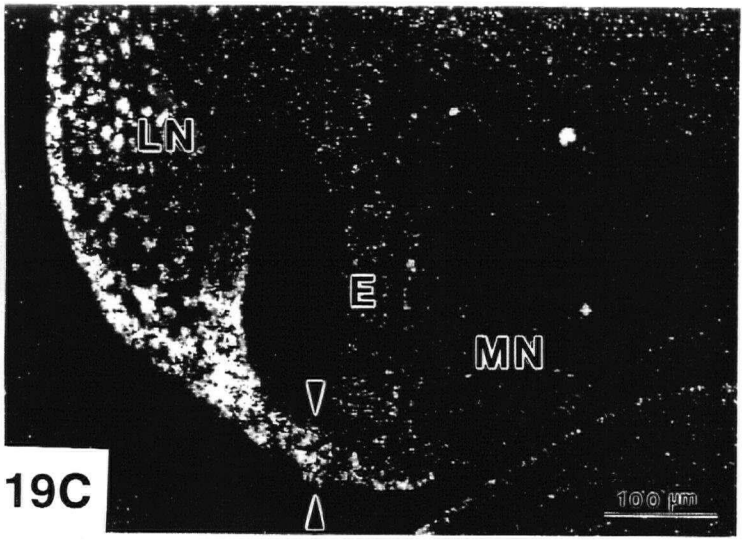

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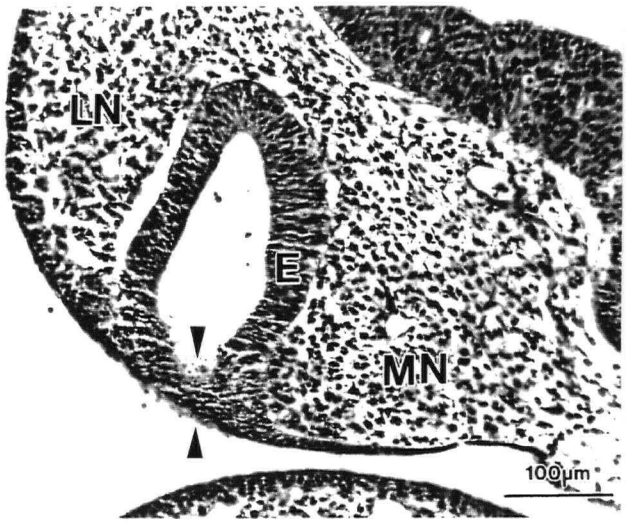
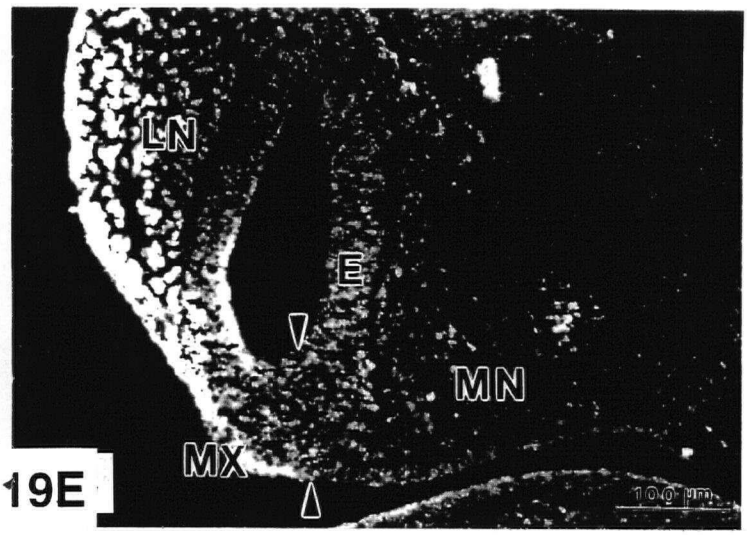


19D



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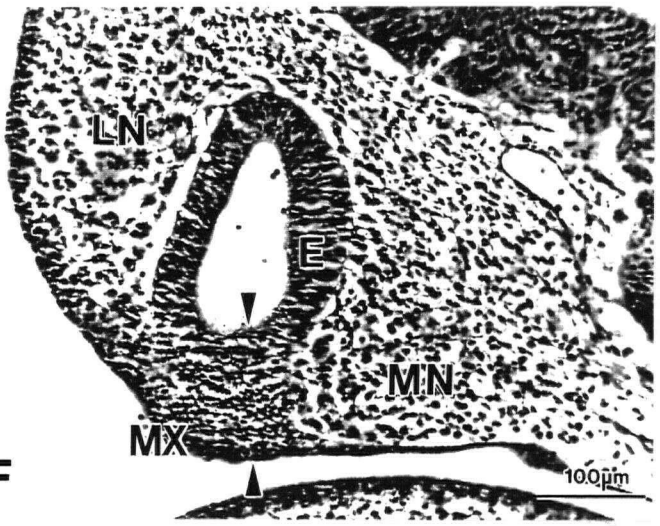

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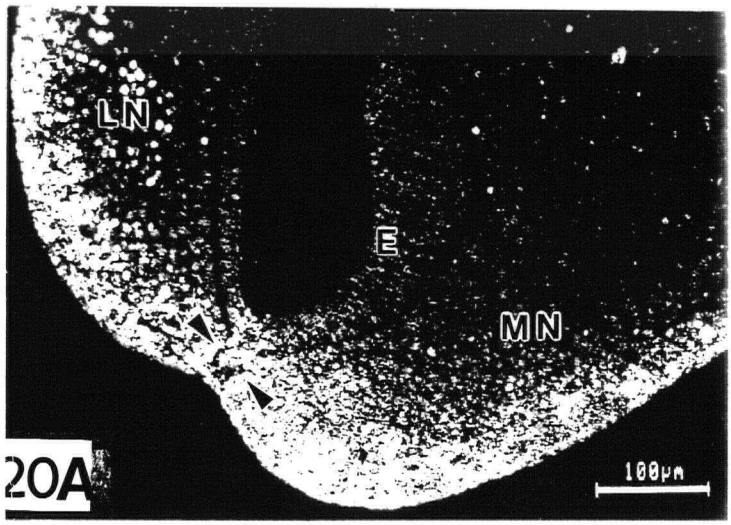
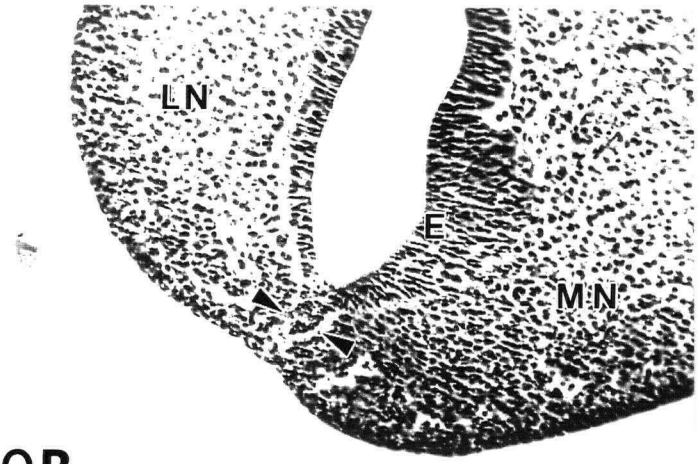
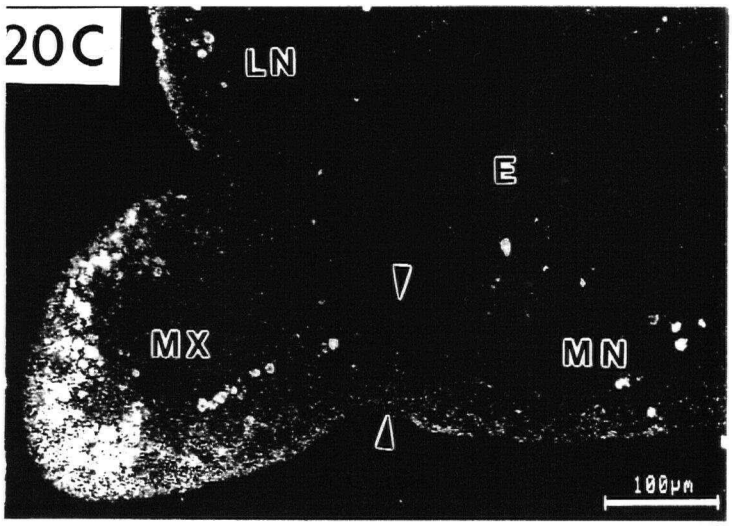


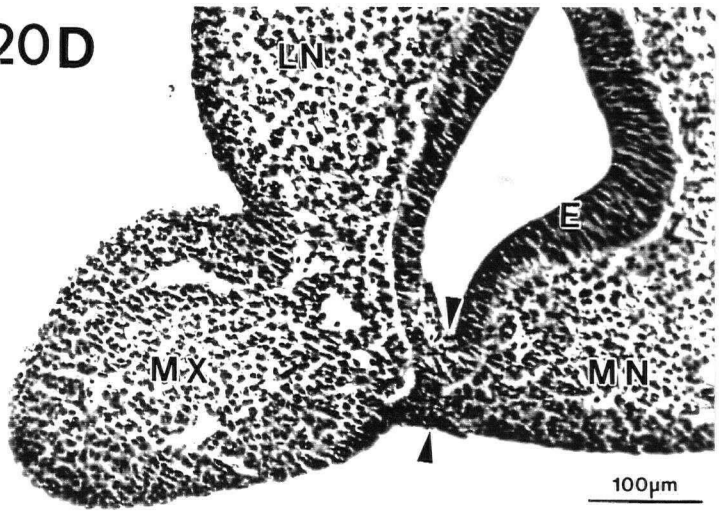
20A



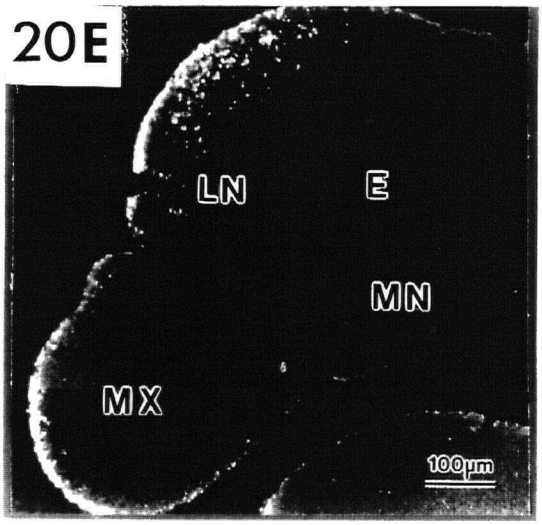
20B



20C



20D



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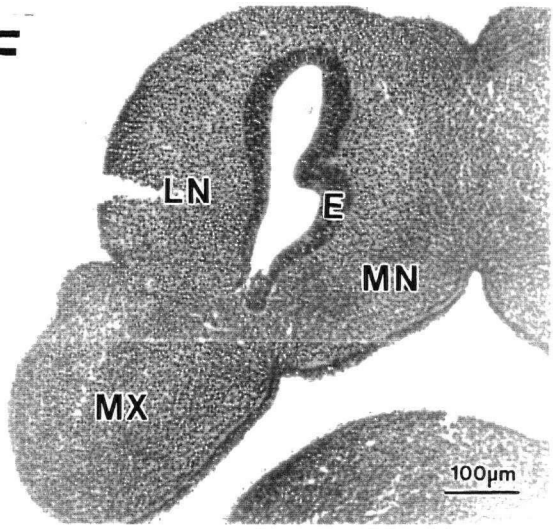
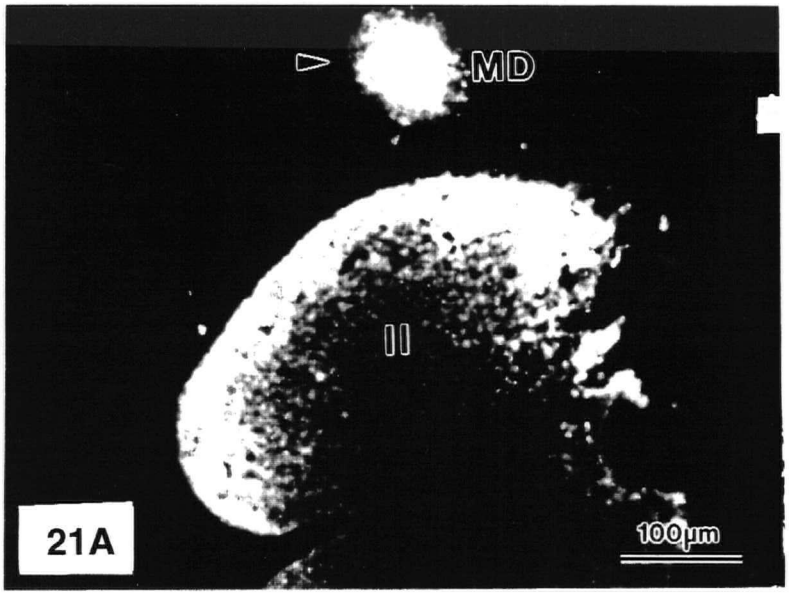
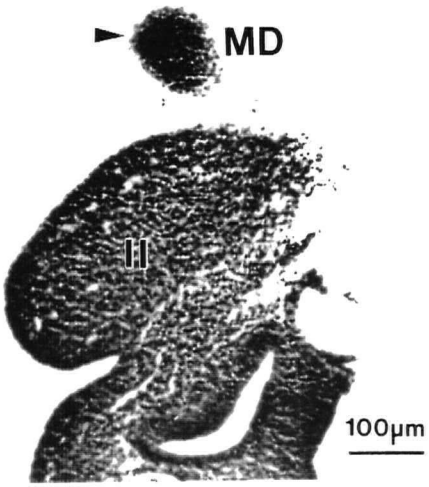


20F



21A



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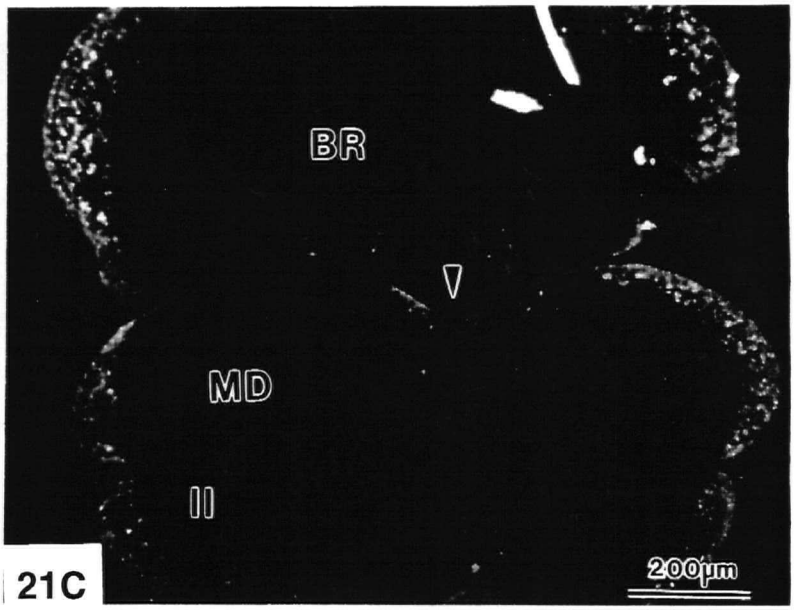

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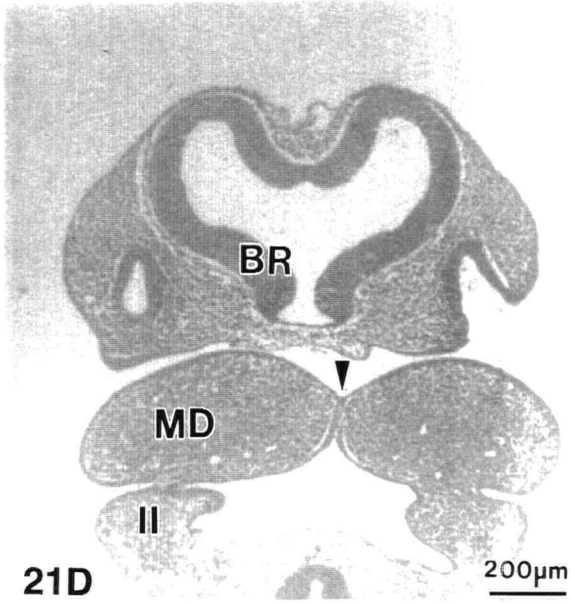

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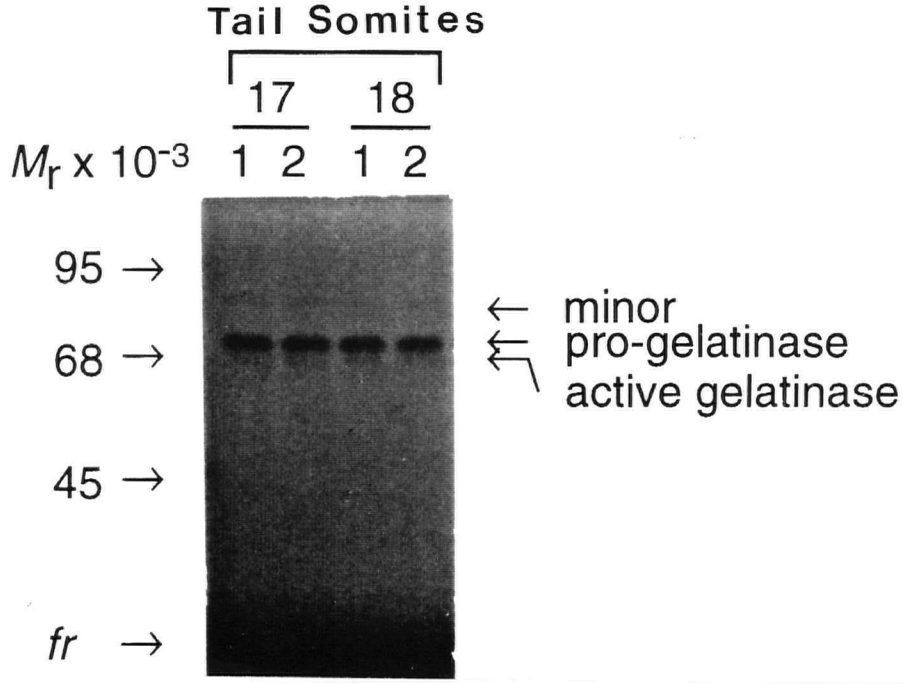

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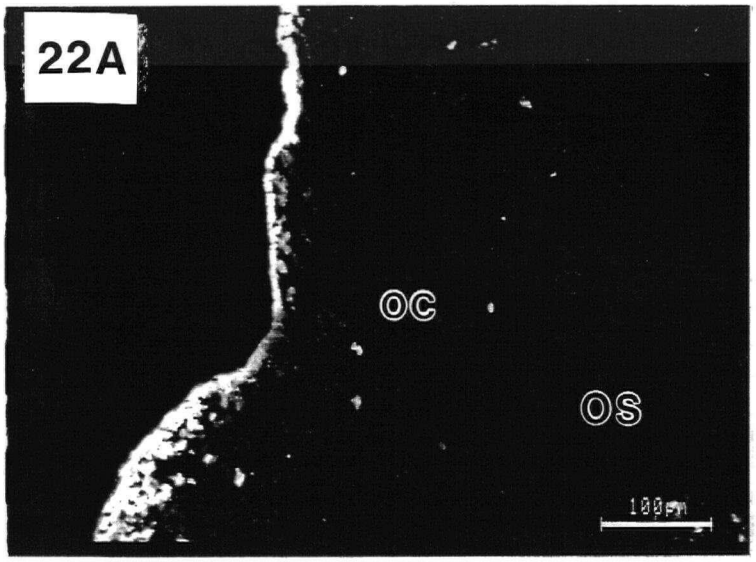
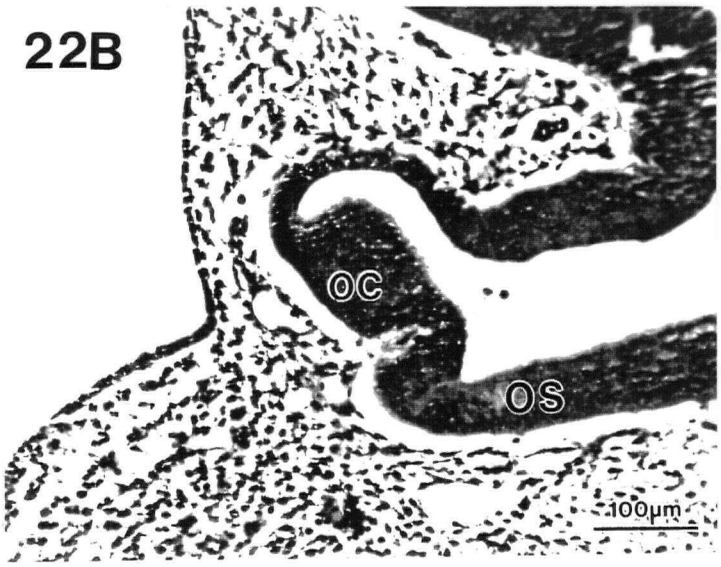

22A
OC
OS
100µm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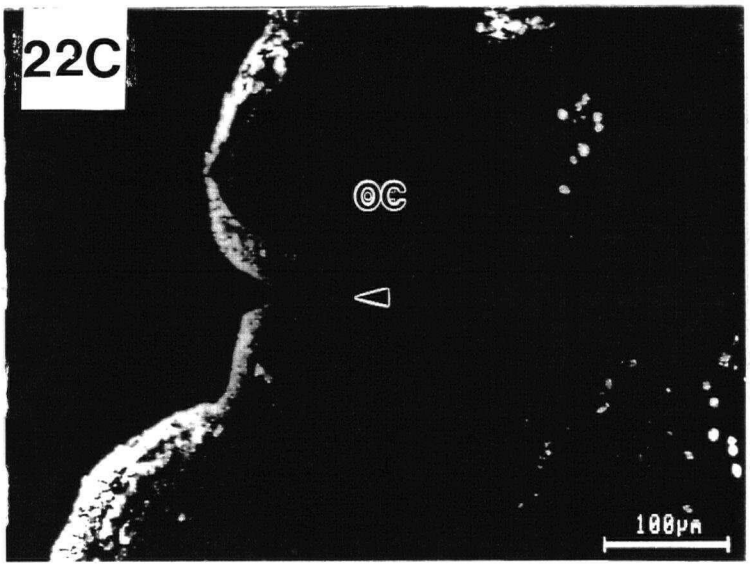
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OC
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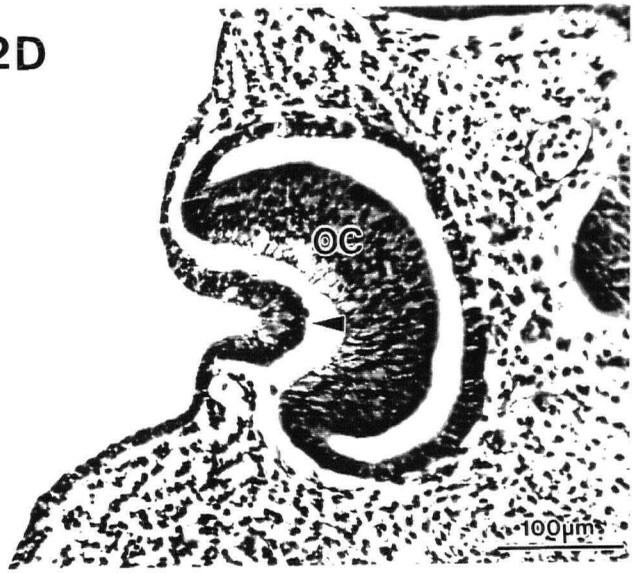
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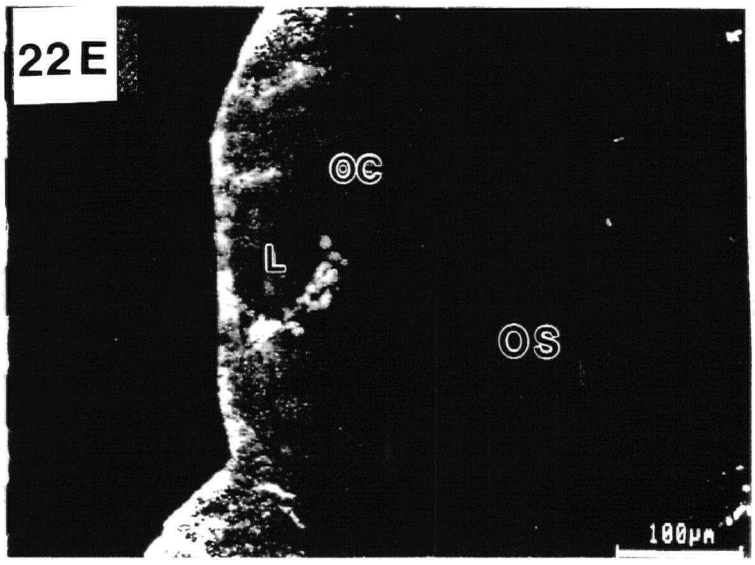
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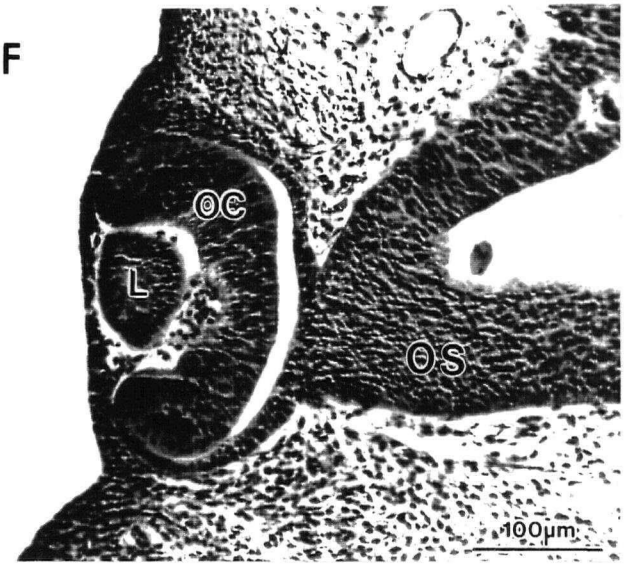
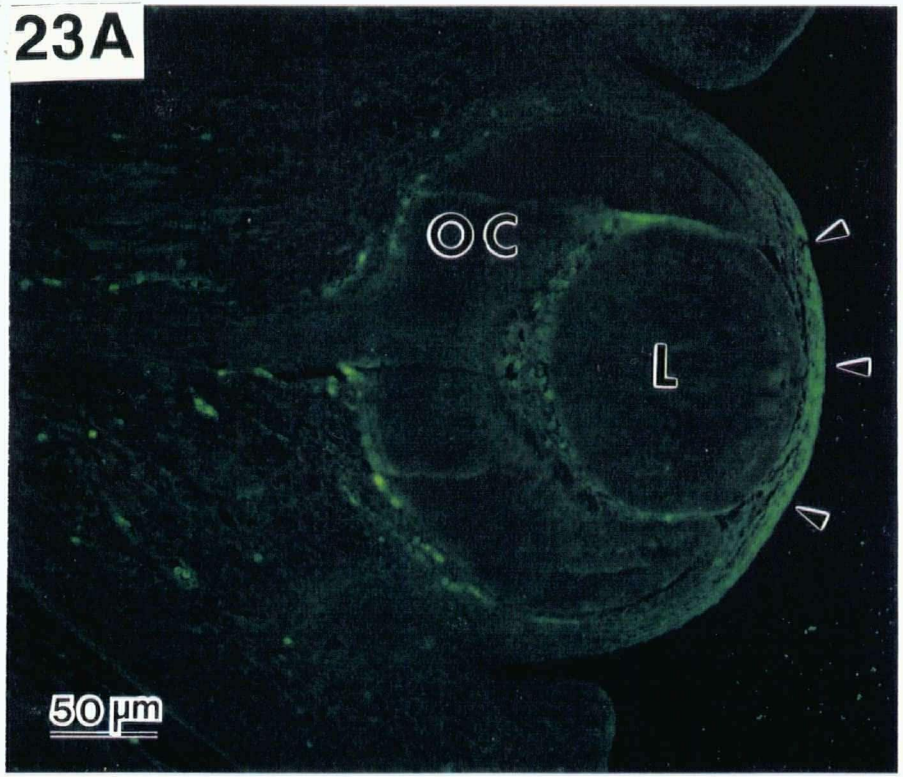
OC
L
OS
100µm


22F
OC
L
OS
100µm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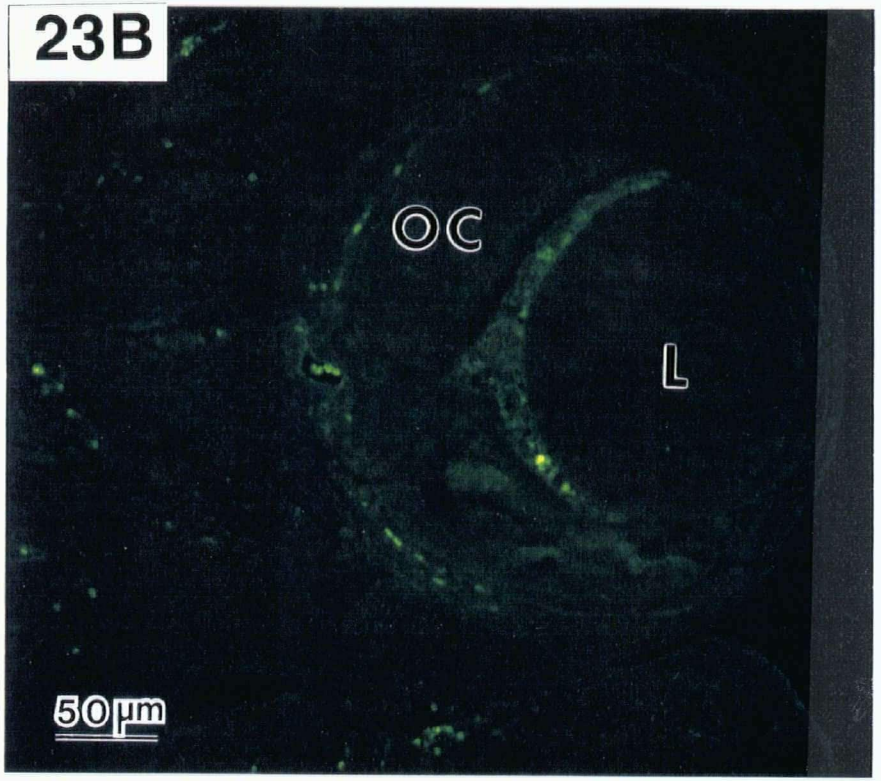
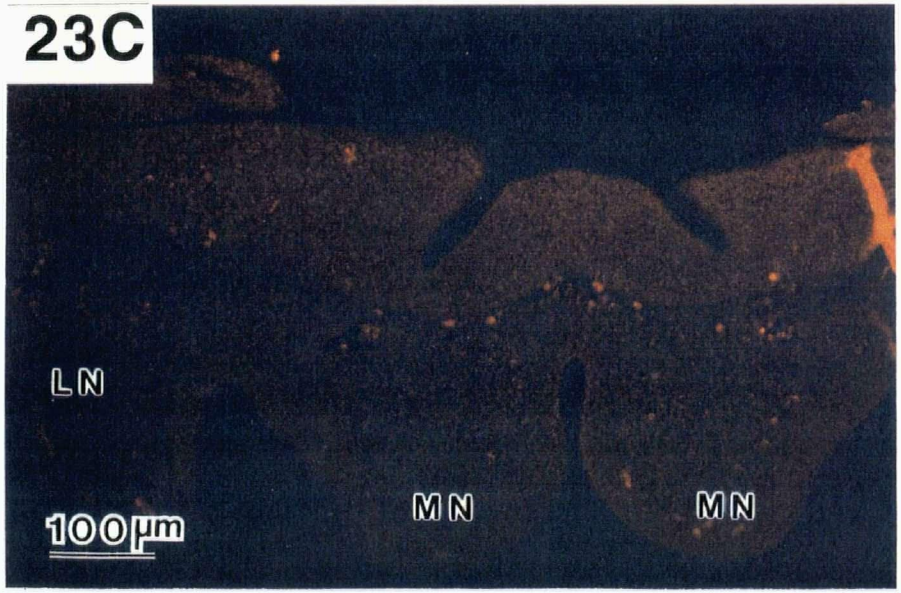
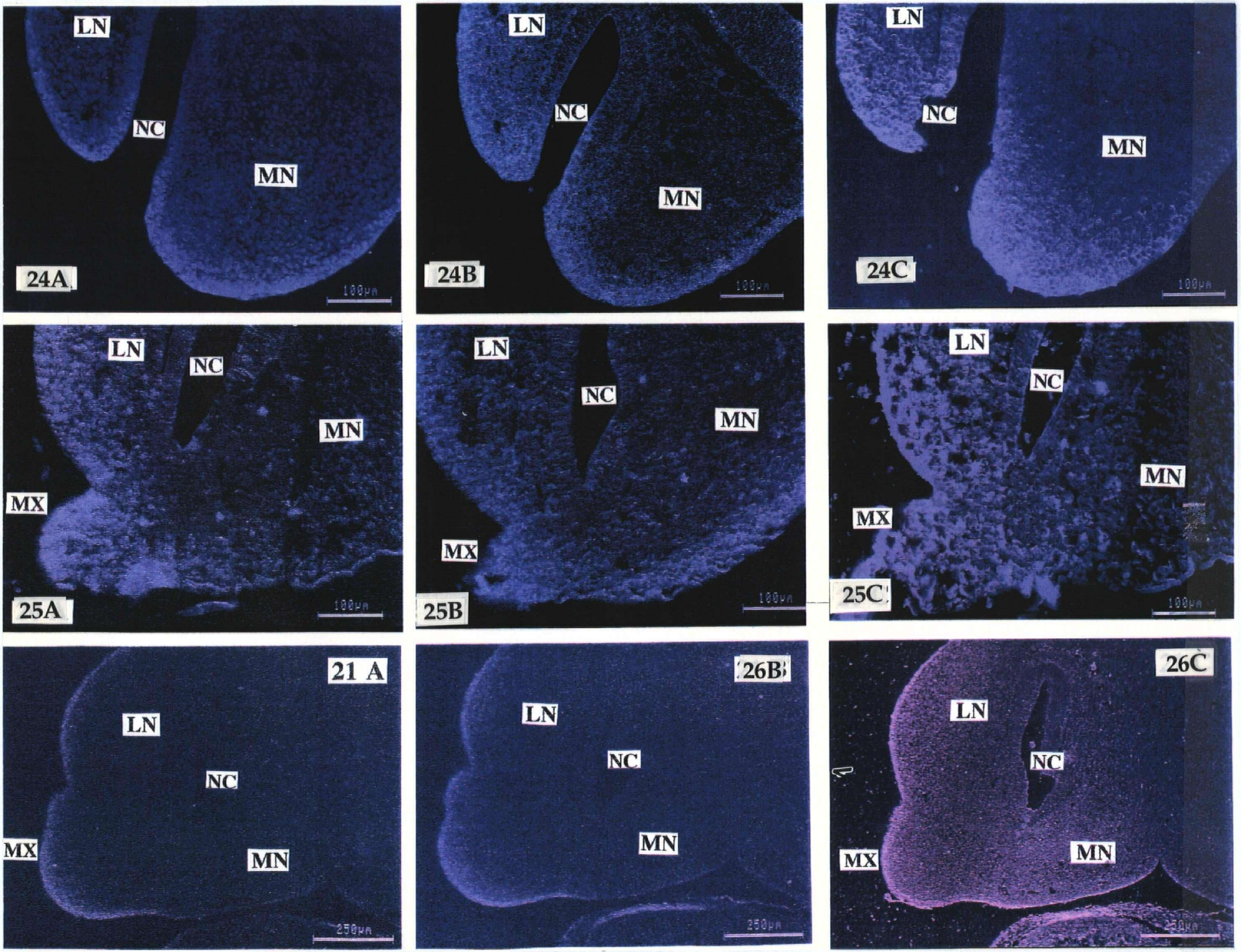
OC
L
50 µm


23B
OC
L
50µm


23C
LN
MN
MN
100µm


LN
NC
MN
24A
100µm
LN
NC
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24B
100µm
LN
NC
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24C
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LN
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MX
25A
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25B
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25C
100µm
21 A
LN
NC
MX
MN
250µm
26B
LN
NC
MN
250µm
26C
LN
NC
MX
MN
250µm

Figs. 19 A-F: Epithelial seam formation (embryonic day 10): The confocal micrographs of anterior frontal sections show the staining of 72-kDa gelatinase in the tips and peripheral regions of the lateral and medial nasal prominences (19A). Upon fusion of the facial prominences, the staining was intensely localized to the fusion (between arrowheads) and peripheral regions (19C). Posteriorly, the staining remained intense in the peripheral regions of the facial prominences. The nasal fin area showed negative staining (arrows) (19E). The corresponding hematoxylin and eosin (H&E) sections are shown (19B,D,F). LN, lateral nasal prominence; MN, medial nasal prominence; MX, maxillary prominence; E, nasal epithelium.

Figs. 20 A-F: Epithelial seam disruption (embryonic day 11): The confocal micrographs of frontal sections show intense staining of 72-kDa gelatinase at the epithelial seam (arrows) and the peripheral regions of the facial prominences (20A). Posteriorly, the staining remained pronounced in the peripheral regions of the facial prominences (20C). The nasal epithelium and nasal fin (arrows) were negative. During mesenchymal bridge enlargement, the staining of 72-kDa gelatinase was generally reduced but remained in the peripheral regions of the primary palate (4E). The corresponding H&E sections are shown (20B,D,F).

Figs. 21 A-E: At embryonic day 11, the staining of 72-kDa gelatinase was intensely localized in the tip of the mandible (arrow) and the peripheral region of the second branchial arch (21A). As the mandibular prominences merged, the staining was mainly present in the peripheral regions of the mandible and second branchial arch and the superior aspect of the merging area of the mandible (arrow) (21C). The corresponding H&E sections are shown (21B,D). Gelatinase activity of the mouse heads was detected by gelatin zymography (21E). Two heads from each 17 and 18 tail somite stages were analyzed by a 7.5% SDS-PAGE gel impregnated with gelatin labelled with L-methoxy-2,4-diphenyl-

3(2H)furanone. All lanes reveal similar results showing a major gelatinase band corresponding to 72-kDa gelatinase and minor bands representing activated forms of 72-kDa gelatinase. BR, brain; MD, mandible; II, second brachial arch.

Figs. 22 A-F: During lens development (embryonic day 10), the confocal micrographs show intense staining of 72-kDa gelatinase in the surface ectoderm and mesenchyme at the anterior region to the forming lens (22A). At the region where the surface ectoderm invaginated to form the lens vesicle, the staining became absent in the epithelium (arrow) (22C). At later stages (embryonic day 11), the staining reappeared in the surface ectoderm and mesenchyme as the lens completely formed (22E). The optic cup and stalk showed negative staining at all stages examined. The corresponding H&E sections are shown (22B,D,F). L, lens; OC, optic cup; OS, optic stalk.

Figs. 23 A-C: During the stage of lens differentiation (embryonic day 13), 72-kDa gelatinase was mainly localized to the developing corneal epithelium and mesenchyme (arrowheads) (23A). The staining appeared negative in the other structures of the developing eye. The negative control sections from the developing eye (23B) and primary palate (23C), incubated with normal serum as a replacement of the primary antibody, showed absent staining. Blood cells in capillaries revealed autofluorescence.

Figs. 24-26: Frontal sections through the developing mouse nasal region during formation of the primary palate. The sites of the fusion of the facial prominences are shown by the arrows. BR, brain; LN, lateral nasal prominence; MN, medial nasal prominence; MX, maxillary prominence; NC nasal cavity

Fig. 24: During the epithelial seam formation (7-11 TS), in the anterior region of the face, the confocal micrographs show intense staining of 72-kDa gelatinase (A), EGF (B) and TGF-α (C) in the epithelial and mesenchymal cells at the tips and peripheral regions of the lateral and medial nasal prominences.

Fig. 25: As the epithelial seam became disrupted (12-17 TS), the confocal micrographs show strong labelling of 72-kDa gelatinase (A), EGF (B) and TGF-α (C) in the epithelial and mesenchymal cells at the peripheral regions of the nasal and maxillary prominences and the tip of the maxilla.

Fig. 26: During enlargement of the mesenchymal bridge with the outgrowth of the primary palate (18-20 TS), the confocal micrographs show markedly reduced staining of 72-kDa gelatinase (A), EGF (B) and TGF-α (C) in the facial prominences. However, the labelling of 72-kDa gelatinase (A), EGF (B) and TGF-α (C) remained pronounced in the peripheral regions of the primary palate.

Discussion

The temporo-spatial distribution of 72-kDa gelatinase was demonstrated in early development of the mouse craniofacial complex, particularly in the primary palate, mandible, second branchial arch, and eye, by indirect immunofluorescence with conventional and confocal laser scan microscopy. Staining of 72-kDa gelatinase was found to be intensely localized in the peripheral regions and the tips of the primary palate, mandible, and second branchial arch. These regions of high expression, particularly of the primary palate, were consistent with the outgrowth regions characterized in previous studies (Diewert and Wang, 1992; Diewert and Lozanoff, 1993). It was found that the size of the maxillary region increases rapidly, particularly in the most distal regions, as the maxillary prominence grows forward to meet the medial nasal and lateral nasal prominences. Whereas, the size of the lateral nasal prominence increases with a predominantly lateral growth pattern. These outgrowth regions of the primary palate also show intense localization of fibronectin (see Chapter 2). Thus, adaptation of existing ECM and basement membrane to the expanded form of the rapidly growing facial proprominences appears to require adaptive remodelling by 72-kDa gelatinase. It was suggested that MMP function in tissue remodelling may be to disrupt ECM receptor-substrate contacts to mobilize cells or degrade the ECM in preparation for a change in composition (Werb *et al.*, 1992). Alternatively, the binding of cells to matrix components may reduce their proliferation (Martin and Sank, 1991). Therefore, the induction of MMPs by growth factors may serve to release the cell from its contacts with collagen and allow expression of the growth factor receptors required for cell proliferation. More specifically, the fibronectin degrading activity of 72-kDa gelatinase may release cells from this important cell attachment protein and facilitate alterations in cell shape and cell proliferation. Indeed, it was recently identified that a

fibronectin binding site on 72-kDa gelatinase localized to the hemopexin-like domain of the enzyme (Wallon and Overall, submitted) which may be involved in this process.

The results here are somewhat different from the previous *in situ* hybridization study of 72-kDa gelatinase expression by Reponen *et al.* (1992) where 72-kDa gelatinase mRNA was found mainly expressed in the mesenchyme with the surface ectoderm being completely negative. However, in the present study, it was clearly found that some regions of the surface ectoderm, particularly in the peripheral areas of the facial prominences, were strongly positive for 72-kDa gelatinase. Similar findings have also been reported for human breast (Monteagudo *et al.*, 1990; Soini *et al.*, 1994), colon (Levy *et al.*, 1991), and ovarian cancers (Autio-Harmainen *et al.*, 1993) where 72-kDa gelatinase protein has been localized to the cancer cells by immunohistochemistry, whereas the mRNA transcripts were found in the stromal cells adjacent to invading tumor cells of breast (Soini *et al.*, 1994), colon (Pyke *et al.*, 1993), and ovarian cancers (Autio-Harmainen *et al.*, 1993) by *in situ* hybridization. At these sites, it appears that 72-kDa gelatinase is synthesized by the stromal cells and exported to the ECM where binding to the tumor cells occurs *in trans*. However, in the present study, although 72-kDa gelatinase may be localized to cell surfaces on cells not expressing 72-kDa gelatinase mRNA, partly explaining the discrepant results to Reponen *et al.* (1992), cytoplasmic staining was also a prominent feature of the outer cell layers indicating cellular synthesis in these regions.

The presence of 72-kDa gelatinase in the craniofacial complex was confirmed by functional assays using gelatin zymography. The data revealed that 72-kDa gelatinase is the predominant gelatinase expressed during craniofacial development. In addition, an activated form of the enzyme was also detected showing that 72-kDa gelatinase is functional during craniofacial morphogenesis.

These results were consistent with those of previous investigations by gelatin zymography (Da Silveira *et al.*, 1995), Western blot and RT-PCR analyses (Da Silveira *et al.*, 1996) in which 72-kDa gelatinase was found to be expressed in the facial prominences during the primary palate formation. Taken together, these findings indicate that 72-kDa gelatinase may play a significant role in remodelling the ECM in the fast outgrowing regions of the primary palate, mandible, and second branchial arch.

During eye development, the staining of 72-kDa gelatinase was found to be absent in the invaginating epithelium during formation of the lens vesicles. In the later stages, as the lens completely detached from the surface ectoderm, the staining of 72-kDa gelatinase reappeared in the developing corneal epithelium and mesenchyme, whereas the lens and optic cup remained negative at all stages examined. These observations imply that in order to allow invagination of the surface ectoderm to form the lens vesicle, stabilization of the ECM in the invaginating areas may be needed. Indeed, it was found that there is a strong adhesion between the optic vesicle and the lens placode (McKeehan, 1971). Later immunohistochemical studies have also confirmed that the staining of types I and IV collagens, laminin, and fibronectin is intensely localized to the interspace between the lens and the optic vesicle throughout the invagination of the lens vesicle (Parmigiani and McAvoy, 1984; Hilfer and Randolph, 1993; Peterson *et al.*, 1995). Recently, Sheffield *et al.* (1992, 1994) have demonstrated participation of plaminogen activator and high molecular weight metalloproteinases, but not 72-kDa gelatinase, during histogenesis of the embryonic retina and suggested that these proteinases play a role in fiber outgrowth from retinal cells. Lastly, a further indication of the importance of MMPs in the development and maintenance of the ECM of the eye has been demonstrated by the presence of specific mutations of TIMP-3 gene in Sorsby's fundus dystrophy (Weber *et al.*,

1994) and the elevated expression of TIMP-3 mRNA in retinas affected by simplex retinitis pigmentosa (Jones *et al.*, 1994).

Other MMPs have also been implicated in developmental process in which epithelial invagination occurs. In branching morphogenesis of the salivary gland, collagenase inhibits cleft formation of the gland, whereas TIMP induces supernumerary clefts (Hayakawa *et al.*, 1992). Therefore, it was proposed that collagenase and TIMP regulate cleft formation through modulation of the ECM. Similarly, it was found that addition of purified mammalian collagenase to lung cultures inhibited epithelial branching and produced end bud enlargement (Ganser *et al.*, 1991). However, a more recent study showed that stromelysin-1 can induce hyperproliferation and differentiation of mammary epithelial cells in virgin female transgenic mice that express autoactivated isoforms of stromelysins-1 (Sympson *et al.*, 1994). During lactation, stromelysin-1 demonstrated lytic activities, disrupting basement membrane integrity and reducing mammary-specific function. Implantation of slow-release pellets containing TIMP-1, on the other hand, delayed mammary gland involution (Talhouk *et al.*, 1992). These findings suggest that epithelial invagination, like lens development, is complex and requires well-balanced expression between MMPs and TIMPs.

It is important that regulation of tissue remodelling be finely balanced to enable sufficient degradation of ECM to facilitate tissue proliferation while maintaining tissue integrity. Indeed, regulation of MMPs takes place in a complex but coordinated manner and at several levels. Regulation of MMPs can occur locally by TIMPs which form 1:1 complexes with activated MMPs to inhibit their activity (for review see Matrisian, 1992; Overall, 1994). Growth factors, cytokines, phorbol ester tumor promoters, and oncogene products can either induce or suppress expression of specific MMP genes (for review see Matrisian,

1992; Overall, 1994). For example, transforming growth factor-beta (TGF-β), a growth factor associated with developmental processes, can induce an increase in expression of 72-kDa gelatinase (Overall *et al.*, 1989, 1991). TGF-α and EGF proteins were also implicated in the induction of MMP activity, particularly 72-kDa gelatinase, during lung development (Ganser *et al.*, 1991). Gelatinase protein was also demonstrated in the blastocyst during mouse preimplantation development (Brenner *et al.*, 1989) and found to be induced by TGF-α (Dardik *et al.*, 1993). It was suggested that upon stimulation by TGF-α, gelatinases, secreted from the blastocyst, may be involved in blastocoel ECM remodelling and migration of the parietal endoderm cells (Dardik *et al.*, 1993). Indeed, we have also localized EGF and TGF-α expression within the similar regions of 72-kDa gelatinase expression shown in the present study during the primary palate formation (see Chapter 3). Taken together, these data indicate that EGF and TGF-α may be involved in the regulation of MMP expression during embryogenesis.

Since EGF, TGF-α, and 72-kDa gelatinase were localized to the similar regions of the developing primary palate at all stages examined in the present study, I hypothesized that EGF and TGF-α are inducers of 72-kDa gelatinase synthesis *in vivo* and participate in the regulation of cell proliferation and tissue outgrowth during primary palate morphogenesis. Further investigation is needed to be performed in order to study the induction of 72-kDa gelatinase by EGF and TGF-α. For example; EGF and TGF-α can be supplemented to organ cultures of primary palate, as was done in lung explants (Ganser *et al.*, 1991). If EGF and TGF-α are inducers of 72-kDa gelatinase, an increase of 72-kDa gelatinase expression in the primary palate will be detected. 72-kDa gelatinase

expression in culture can be evaluated by zymography, Western blots, and quantitative RT-PCR analyses.

In conclusion, the temporo-spatial distribution of 72-kDa gelatinase during early craniofacial development was found to be associated with the tips of growing facial prominences. These findings suggest that 72-kDa gelatinase may be involved, at least in part, in tissue remodelling during the early mouse craniofacial morphogenesis.

CHAPTER 5: Immunolocalization of 72-kDa Gelatinase and Extracellular Matrix Components during Mouse Cardiac Development

Introduction

The heart tube is initially formed by convergence of the two lateral cardiac primordia in the ventral midline at embryonic day 7 in mice (Sissman, 1970; Hopper and Hart, 1980). The heart tube is composed of two layers: an endothelium and a myocardium, which are separated by an acellular sleeve of extracellular matrix (ECM), the cardiac jelly or myocardial basement membrane (Markwald *et al.*, 1984; Kitten *et al.*, 1987). As the heart develops further, the myocardium, particularly in the ventricles, loses its epithelial organization and forms trabeculations at about embryonic day 9 in mice shortly after the first myocardial contractions (Sissman, 1970). These trabeculations subsequently contribute to the interventricular septum and papillary muscles (Pexieder and Janecek, 1984; Hay et al., 1984). In later development, the endothelial cells in the regions of the atrioventricular canal and outflow tract undergo transformation into mesenchymal cells (Manasek, 1976; Runyan and Markwald, 1983). They embed in the cardiac jelly forming endocardial cushion tissue. The ventral and dorsal endocardial cushions are first definable at embryonic day 10, unite at embryonic day 11 in mice (Sissman, 1970), and later give rise to septal and valvular structures (Van Mierop *et al.*, 1962; Manasek, 1976).

Extracellular matrix appears to play an important role in heart development, several components of which have been studied including fibronectin, laminin, type IV collagen, and elastin. Fibronectin is believed to regulate precardiac cell migration (Linask and Lash, 1988). Immunohistochemical studies on stages 8-18 of chick embryos revealed that

fibronectin was first found on the basal surfaces of the myocardium and endocardium during fusion of the the heart primordia. Later the intensity of fibronectin increases at the onset of trabeculations and then decreases as trabeculations are completed (Icardo and Manasek, 1983). Similar findings were found in rat heart embryos during 4-16 somite stages (Tuckett and Morriss-Kay, 1986). In addition, to being important for trabecular formation, fibronectin was also found to be closely associated with surfaces of migrating mesenchymal cells in the cardiac jelly of the endocardial cushion tissues (Icardo and Manasek, 1984). The distribution of fibronectin was demonstrated as a gradient extending from the myocardium towards the endocardium and appeared to contact endothelial cells just prior to epithelial-mesenchymal transformation (Mjaatvedt *et al.*, 1987). This suggests the importance of fibronectin in epithelial-mesenchymal transformation during heart development. Moreover, transgenic homozygous mouse embryos lacking fibronectin showed various defects in heart development including abnormal fusion of the heart primordia, thickened myocardium, deficient cardiac jelly, and abnormal or absent endocardium (George *et al.*, 1993). The results in the transgenic mouse study also support the importance of fibronectin in heart development.

Immunolocalization revealed that laminin first appears in the cardiac jelly at stage 15 of chick embryos (Little *et al.*, 1989). Later laminin was localized to the basal surfaces of the myocardium and endocardium at stage 17 of chick embryos (Kitten *et al.*, 1987) and during 8-16 somite stages of rat embryos (Tuckett and Morriss-Kay, 1986). On the other hand, type IV collagen was first detected in ECM between the splanchnic mesoderm and endoderm of pretubular-heart chick embryos (stage 9) (Drake *et al.*, 1990) and found on the basal surfaces of the myocardium and endocardium at the stage 17 of chick embryos (Kitten *et al.*, 1987). More recently, elastin was immunolocalized to the outflow tract and the

base of the atrioventricular cushion during stages 22-29 of embryonic chick embryos (Hurle *et al.*, 1994). In later development, elastin was found in the developing valvular apparatus, subendocardial space of the atria, and epicardium. Thus, the distribution of these ECM molecules changes throughout heart development. Accordingly, understanding the processes of tissue remodelling and the proteinases involved in the developing heart is central to the understanding of heart morphogenesis and may provide insight into the remodelling processes that occur later in life during heart diseases.

During cardiac development, rat interstitial collagenase-3 (Nakagawa *et al.*, 1992) and plasminogen activator (McGuire and Orkin, 1992) have been localized to embryonic heart tissues and suggested to be important for heart morphogenesis. MMPs have also been found to be involved with tissue remodelling in the normal adult (Tyagi *et al.*, 1993) and diseased hearts (Takahashi *et al.*, 1990) and very recently a novel tissue inhibitor of metalloproteinases (TIMP-4) was cloned from a human heart cDNA library (Eric Shi, personal communication) indicating the importance of MMPs in the development and maintenance of the myocardial structures. Although 92-kDa gelatinase mRNA has not been detected in embryonic heart tissue (Reponen *et al.*, 1994; Canete-Soler *et al.*, 1995), the expression of 72-kDa gelatinase was detected in the heart tissue of 9-day-old mice by *in situ* hybridization and newborn and adult mice by Northern analysis (Reponen *et al.*, 1992). However, the temporo-spatial expression of 72-kDa gelatinase protein during early heart morphogenesis has not been investigated in detail.

Collectively, remodelling of the ECM is a major event during heart morphogenesis and may be associated with MMP activity. In the present study, the expression of 72-kDa gelatinase protein was investigated and correlated with changes in the distribution of substrates of the enzyme: type IV collagen,

laminin, and fibronectin during early morphogenesis of the mouse heart using indirect immunohistochemistry with conventional and confocal laser scan microscopy.

Materials and Methods

Tissue preparation

CD1 mice were mated overnight and embryos were collected on days 9-13 (plug = day 0), fixed in 4% (w/v) paraformaldehyde in PBS for 24 hours, and paraffin embedded. The specimens were serially sectioned sagittally (9- and 10-day-old embryos) and coronally (10- to 13-day-old embryos) at a thickness of 7 μm. Sections from at least 60 mouse embryos were used in these studies (see Appendix 4).

Immunohistochemistry

The immunolocalization of 72-kDa gelatinase and the ECM components was studied on the embryonic heart from day 9 when the ventricular trabeculation begins (Sissman, 1970) until day 13 when the ventricles are largely composed of trabeculae carneae (Kaufman, 1992). The avidin-biotin-immunoperoxidase technique was used as previously described (Hsu *et al.*, 1981). Deparaffinized sections were incubated with 3% (v/v) hydrogen peroxide for 20 min to eliminate endogenous peroxidases. After washing with PBS, the slides were incubated with normal goat serum for 20 min at room temperature and then with an affinity purified anti-72-kDa gelatinase antibody (Wallon and

Overall, 1996) overnight at 4° C. After washing with PBS, the slides were incubated with biotin-conjugated secondary antibody (Sigma, St. Louis, MO, goat anti-rabbit biotin conjugate, B-8895) and then with StreptABComplex (Dakopatts, Santa Barbara, CA, K377) both at the concentration of 1:100 for 30 min at room temperature. Dark brown staining was developed by using 3,3'-diaminobenzidine as a substrate. The sections were counter stained with methyl green. Heart tissues were also studied by indirect immunofluorescence techniques with a non-affinity purified anti-72-kDa gelatinase antibody. The results showed similar patterns of labelling to those of an affinity purified anti-72-kDa gelatinase with the ABC technique at all stages examined.

To investigate the ECM components, deparaffinized sections were digested with 0.4% (w/v) pepsin (Sigma, St. Louis, MO) in 0.02M HCl for 15 min, washed with PBS and preincubated with normal goat serum for 30 min. Subsequently, sections were incubated overnight at 4° C with rabbit anti-laminin (Sigma, St. Louis, MO, rabbit anti-EHS mouse sarcoma, L-9393), rabbit anti-type IV collagen (Chemicon, Temecula, CA, rabbit anti-mouse, AB756), or rabbit anti-fibronectin (Dako, Glostrup, Denmark, rabbit anti-human, A 245) antiserum at concentrations of 1:100, 1:50, and 1:100, respectively. The slides were incubated with fluorescein-conjugated (Sigma, St. Louis, MO, sheep anti-rabbit IgG FITC conjugate, F-7512) or rhodamine-conjugated (Sigma, St. Louis, MO, goat anti-rabbit IgG crystalline TRITC conjugate, T-6778) secondary antibody at concentrations of 1:100 and 1:200, respectively for 1 hour at 4° C and washed. The slides were observed and photographed with the Zeiss photomicroscope equipped with the appropriate filters for rhodamine and fluorescein excitation.

The specificity of the anti-fibronectin antibody has been previously characterized (Van Helden *et al.*, 1985). Pre-absorption of anti-laminin and anti-type IV collagen antibodies with an excess of laminin and type IV collagen, respectively, abolished immunoreactivity on heart tissue sections (data not shown). Experimental slides were incubated in both the primary and secondary antibodies. Within each group of the sections, the negative control slides were incubated with normal goat serum as a replacement for the primary antibody. The results showed no staining of the negative control. The mouse mandibular bone forming tissue was used as positive control tissue for the antibody against 72-kDa gelatinase (Reponen *et al.*, 1992) which was strongly positive as predicted. Anti-type IV collagen, anti-laminin, and anti-fibronectin antisera showed intense immunoreactivity at the basement membrane of the surface ectoderm of the primary palate, which was used as the positive control for type IV collagen, laminin, and fibronectin (see Chapter 2).

Confocal Microscopy

The 10-to-11-day-old embryonic heart tissues were studied by indirect immunofluorescence technique. The endocardial cushion tissue at the atrioventricular canal was examined with both epifluorescence and confocal microscopy using a 20X objective on the Confocal Laser Scan Microscope (Zeiss, Germany). A Helium-Neon laser (λ_{max}=543 nm) was used for rhodamine-labelled tissues. Tissues were sectioned at 1 or 2 μm intervals and images were printed using a video printer (UP-5000, Sony Canada, Richmond, British Columbia).

Results

9-Day-Old Embryo

During this developmental stage, the staining of 72-kDa gelatinase was generally present in the cytoplasm and on the surfaces of the myocytes and endothelial cells of the ventricular and atrial walls (Figs. 27A,B). The body (thoracic) wall overlying the pericardial cavity and mandible showed only weak staining of 72-kDa gelatinase. Of note, the staining of 72-kDa gelatinase in the cardiac jelly of the heart and the neural tissues appeared negative. The immunofluorescent staining also showed similar patterns of distribution of 72-kDa gelatinase (Fig. 27C).

The staining of type IV collagen (Fig. 29A), laminin (Fig. 29B), and fibronectin (Fig. 29C) was mainly localized to the basal surfaces of the endothelial cells and myocytes of the ventricular and atrial walls. Type IV collagen, laminin, and fibronectin were also intensely stained in the basement membrane of the body wall.

10-Day-Old Embryo

During this developmental stage, staining of 72-kDa gelatinase was uniformly present in the cytoplasm and on the surfaces of the myocytes and endothelial cells of the ventricular and atrial walls by the ABC (Figs. 27D,E) and immunofluorescent techniques (Fig. 27F). 72-kDa gelatinase was also distributed on the surface ectoderm of the facial prominences. The endocardial cushion tissues appeared less intensely positive for 72-kDa gelatinase in comparison with the myocardial tissues when viewed with epifluorescence microscopy (Fig. 27G). However, confocal microscopy revealed that 72-kDa gelatinase was strongly expressed in many endocardial cushion endothelial and mesenchymal cells (Fig.

27H). The less intense labelling observed by epifluorescence microscopy was related to the low cell density and high ECM content of endocardial cushion tissue.

Type IV collagen (Fig. 29D), laminin (Fig. 29E), fibronectin (Fig. 29F) were strongly and uniformly labelled on the basal surfaces of the endothelial cells and myocytes of the ventricular and atrial walls and in the basement membrane of the body wall. Fibronectin also showed intense localization in the mesenchyme of the body wall and endocardial cushion tissue at the atrioventricular canal (Fig. 29F).

11-Day-Old Embryo

During this developmental stage, the ventricular walls are markedly thickened and have become trabeculated, whereas the atrial walls appear to be thinner and more expanded. 72-kDa gelatinase was found to be strongly labelled in the cytoplasm and on the surfaces of the endothelial cells and myocytes of the ventricular and atrial walls Figs. 28A-C). On the other hand, the endocardial cushion tissue at the atrioventricular canal showed decreased labelling for 72-kDa gelatinase (Figs. 28A,C) compared to 9- and 10-day-old embryos.

The staining of type IV collagen (Fig. 29G), laminin (Fig. 29H), and fibronectin (Fig. 29I) was mainly localized to the basal surfaces of the endothelial cells and myocytes of the ventricular and atrial walls particularly in the ventricular trabeculation. The distribution of type IV collagen, laminin, and fibronectin was markedly decreased in the interventricular septum.

12-Day-Old Embryo

As the ventricular walls became more thickened, the labelling of 72-kDa gelatinase remained intense in the ventricular trabeculations (Fig. 28E) and atrial

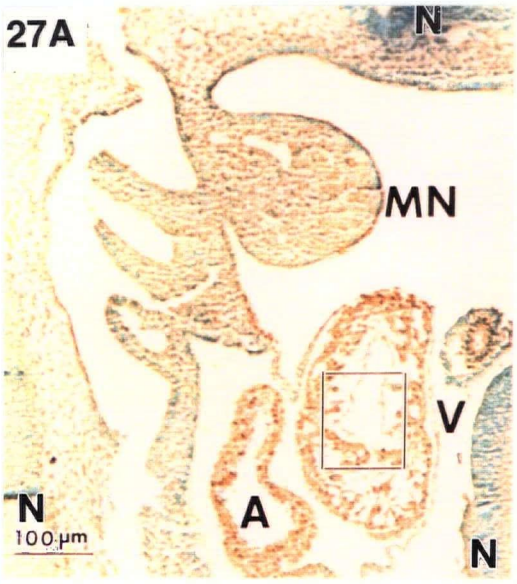
walls (Fig. 28F). However, the distribution of 72-kDa gelatinase was dramatically reduced in the ventricular walls and the interventricular septum.

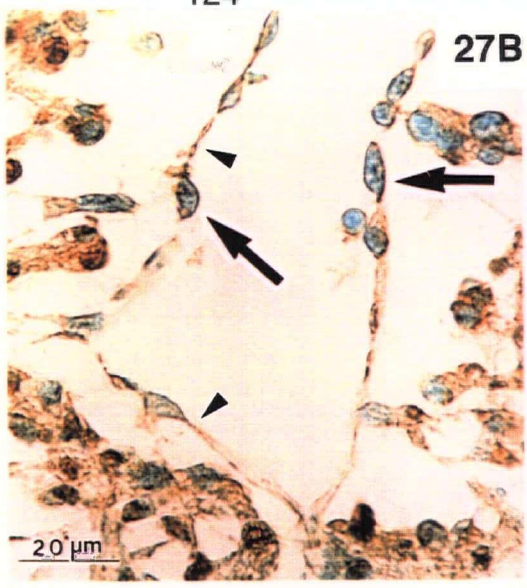
The distribution of type IV collagen (Fig. 30A), laminin (Fig. 30B), and fibronectin (Fig. 30C) was pronounced in the ventricular trabeculations. However, the staining of type IV collagen, laminin, and fibronectin was decreased in the ventricular wall and the interventricular septum.

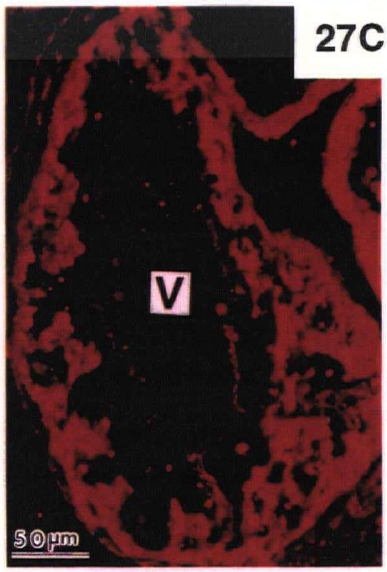
13-Day-Old Embryo

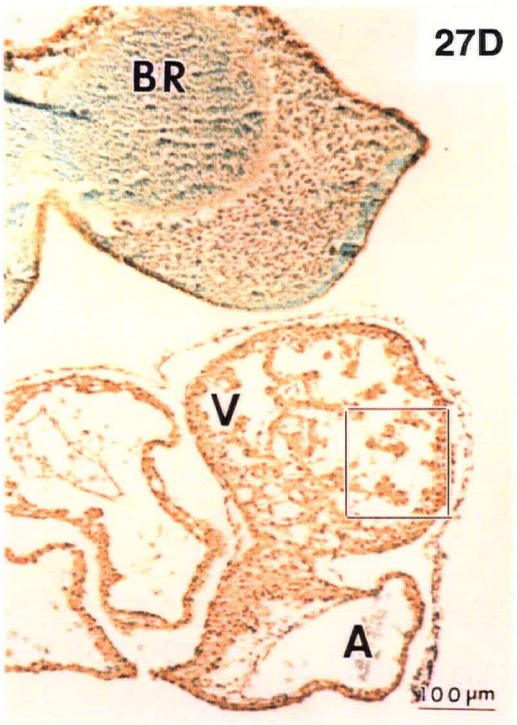
As the ventricular and atrial walls and the trabeculations matured, the staining of 72-kDa gelatinase dramatically decreased. However, there was strong labelling of 72-kDa gelatinase in the ventricular trabeculations (Fig. 28G).

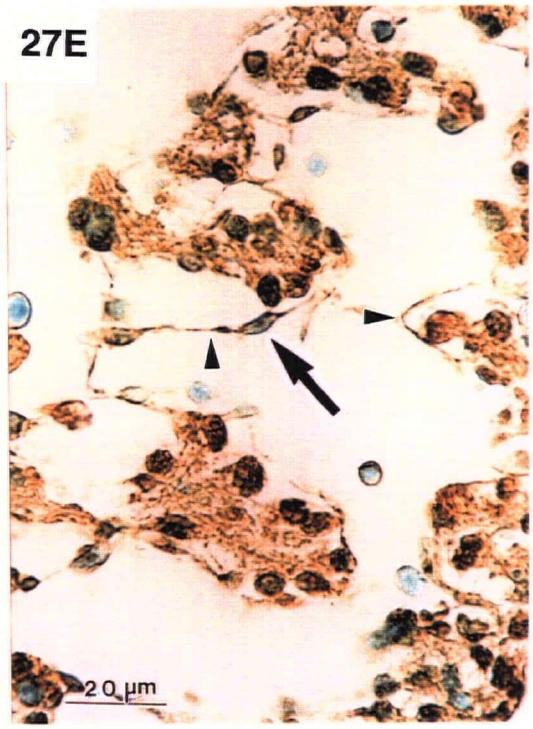
The intensity of the staining of type IV collagen (Fig. 30D), laminin (Fig. 30E), and fibronectin (Fig. 30F) was also generally decreased in the heart tissue, particularly in the ventricular and atrial walls and the interventricular septum. The labelling of type IV collagen, laminin, and fibronectin appeared reduced in the ventricular trabeculations in comparison with the previous stages.

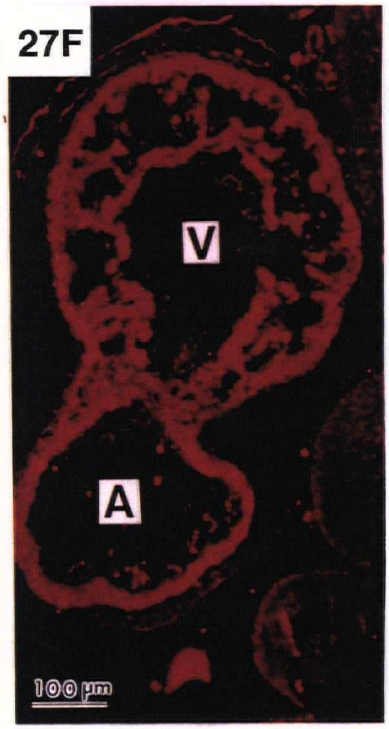

27A
N
MN
V
N
A
N
100 µm

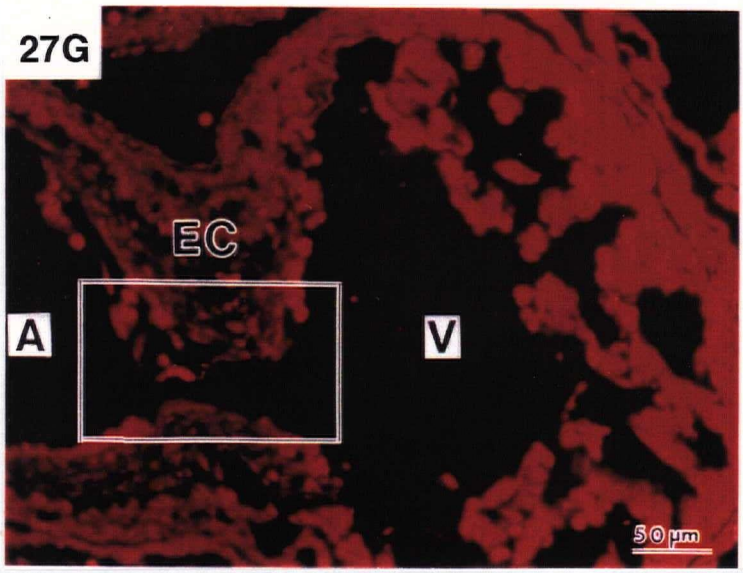

27B
20 µm

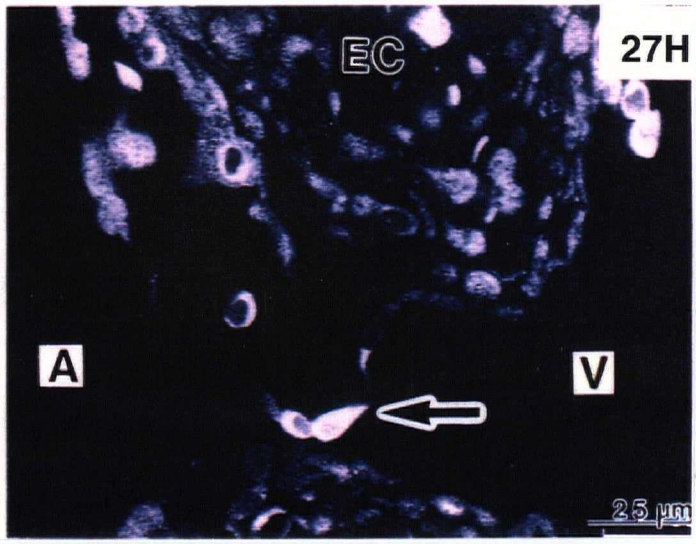

27C
V
50 µm


27D
BR
V
A
100 µm

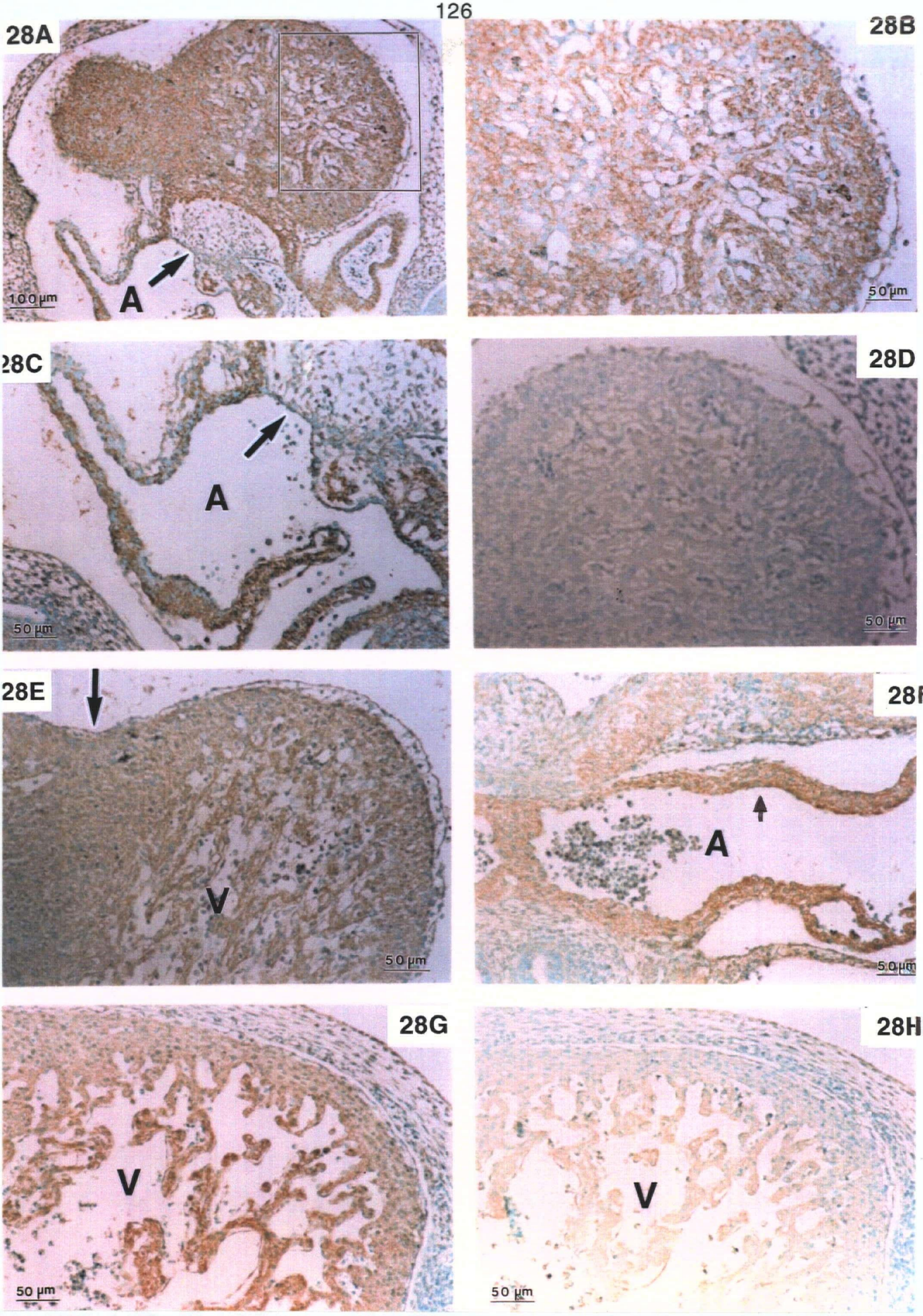

27E
20 µm


27F
V
A
100 µm


27G
EC
A
V
50 µm


EC
27H
A
V
25 µm

Figs. 27 A-H: Sagittal and coronal sections through the embryonic heart from days 9-10. A, atrial chamber; BR, brain, EC, endocardial cushion tissue; MN, mandible; N, neural tissues; V, ventricular chamber. At the 9-day-old stage of heart development, the staining of 72-kDa gelatinase was mainly present in the ventricular and atrial walls, while the staining in the mandible and neural tissues was slightly above background (Fig. 27A). High magnification of the ventricle reveals labelling in the myocytes and on cell surfaces of the endothelial cells (arrows) and their cytoplasmic processes (arrowheads) (Fig. 27B). Similar patterns of immunofluorescent labelling was also found in the ventricular wall (Fig. 27C). At the 10-day-old stage, the staining of 72-kDa gelatinase was now intensely and uniformly expressed in the ventricular and atrial walls (Fig. 27D). High magnification also shows the staining in the myocytes and on cell surfaces of the endothelial cells (arrow) and their cytoplasmic processes (arrowheads) (Fig. 27E). The immunofluorescent staining revealed similar patterns of distribution in the ventricular and atrial walls (Fig. 27F). The endocardial cushion tissue at the atrioventricular canal (inset) showed labelling of 72-kDa gelatinase (Fig. 27G). By using confocal microscopy, the endocardial cushion tissue revealed intense cytoplasmic staining of 72-kDa gelatinase particularly in the endothelial (arrow) and mesenchymal cells (Fig. 27H).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28C
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28D
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28E
V
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28F
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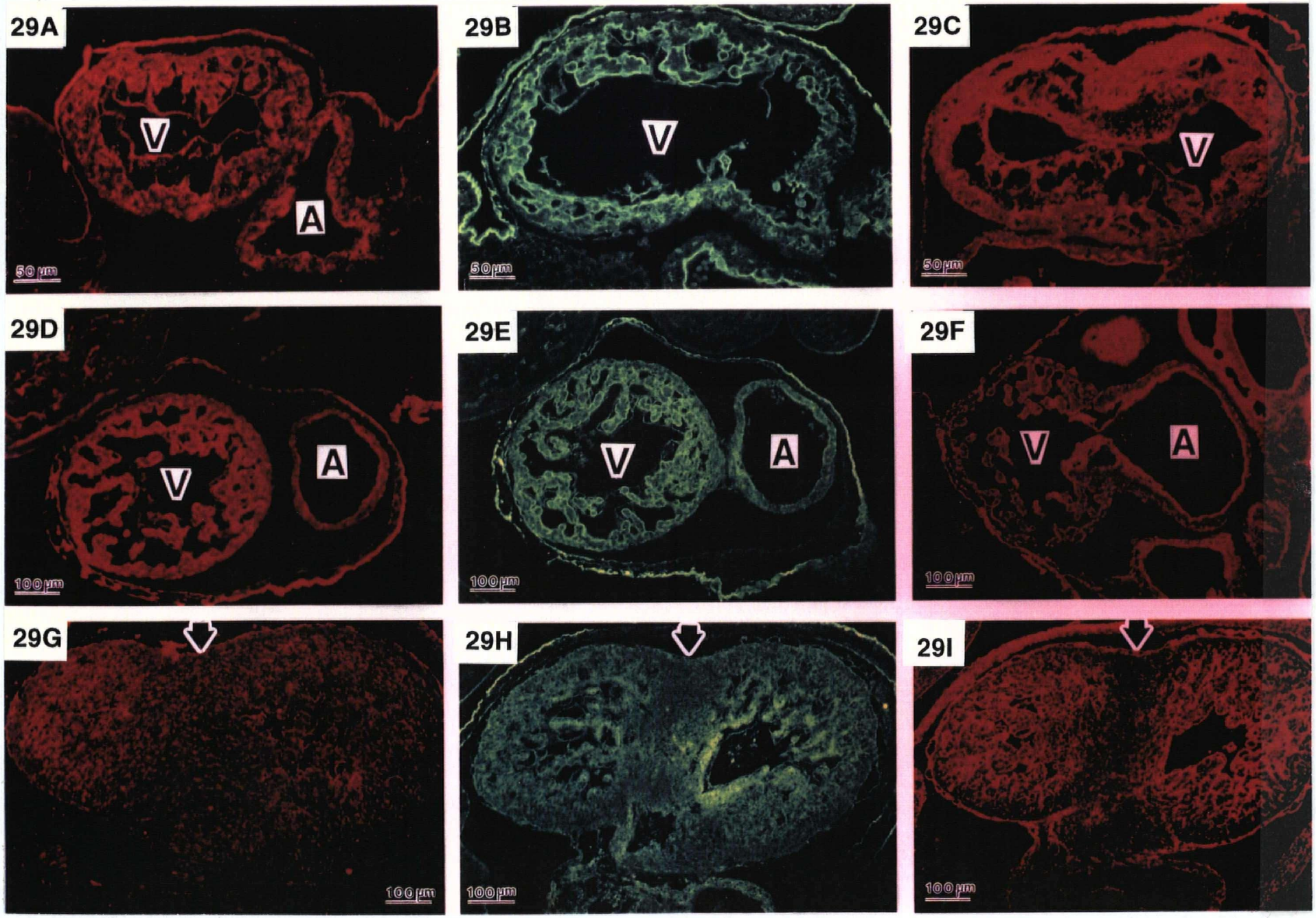
28G
V
50 µm
28H
V
50 µm

Figs. 28 A-H: Coronal sections through the embryonic heart from days 11-13. A, atrial chamber; V, ventricular chamber. At the 11-day-old stage, the staining of 72-kDa gelatinase remained intense in the ventricular and atrial walls (Fig. 28A). Higher magnification shows the labelling in the myocytes and endothelial cells in the ventricular (Fig. 28B) and atrial walls (Fig. 28C). The endocardial cushion tissue (arrow) showed decreased staining of 72-kDa gelatinase (Figs. 28A,C). The negative control section, incubated with normal serum as a replacement for the primary antibody, showed methyl green counterstaining and a light brown background staining (Fig. 28D). At the 12-day-old stage, 72-kDa gelatinase was strongly labelled in the trabeculations of the ventricle (Fig. 28E) but its intensity appeared reduced in the outer ventricular wall and interventricular septum (arrow) (Fig. 28E). The atrial walls also showed intense labelling (arrow) (Fig. 28F). At the 13-day-old stage, the staining of 72-kDa gelatinase generally decreased but remained intense in the ventricular trabeculations (Fig. 28G). The negative control section, incubated with normal serum as a replacement for the primary antibody, showed methyl green counterstaining and a light brown background staining (Fig. 28H).


29A
V
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29B
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29G
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29H
100 µm
29I
100 µm

Figs. 29 A-H: Selected sagittal and coronal sections through the embryonic heart from days 9-11. A, atrial chamber; V, ventricular chamber. At the 9-day-old stage, the staining of type IV collagen (Fig. 29A), laminin (Fig. 29B), and fibronectin (Fig. 29C) were localized to the basal surfaces of the myocytes and endothelial cells in the ventricular and atrial walls. At the 10-day-old stage, type IV collagen (Fig. 29D), laminin (Fig. 29E), and fibronectin (Fig. 29F) were now uniformly and intensely stained in the myocytes and endothelial cells in the ventricular and atrial walls. Fibronectin was also labelled in the cardiac jelly at the atrioventicular canal (Fig. 29F). At the 11-day-old stage, the staining of type IV collagen (Fig. 29G), laminin (Fig. 29H), and fibronectin (Fig. 29I) remained pronounced in the ventricular walls but the intensity appreared reduced in the interventricular septa (arrows).

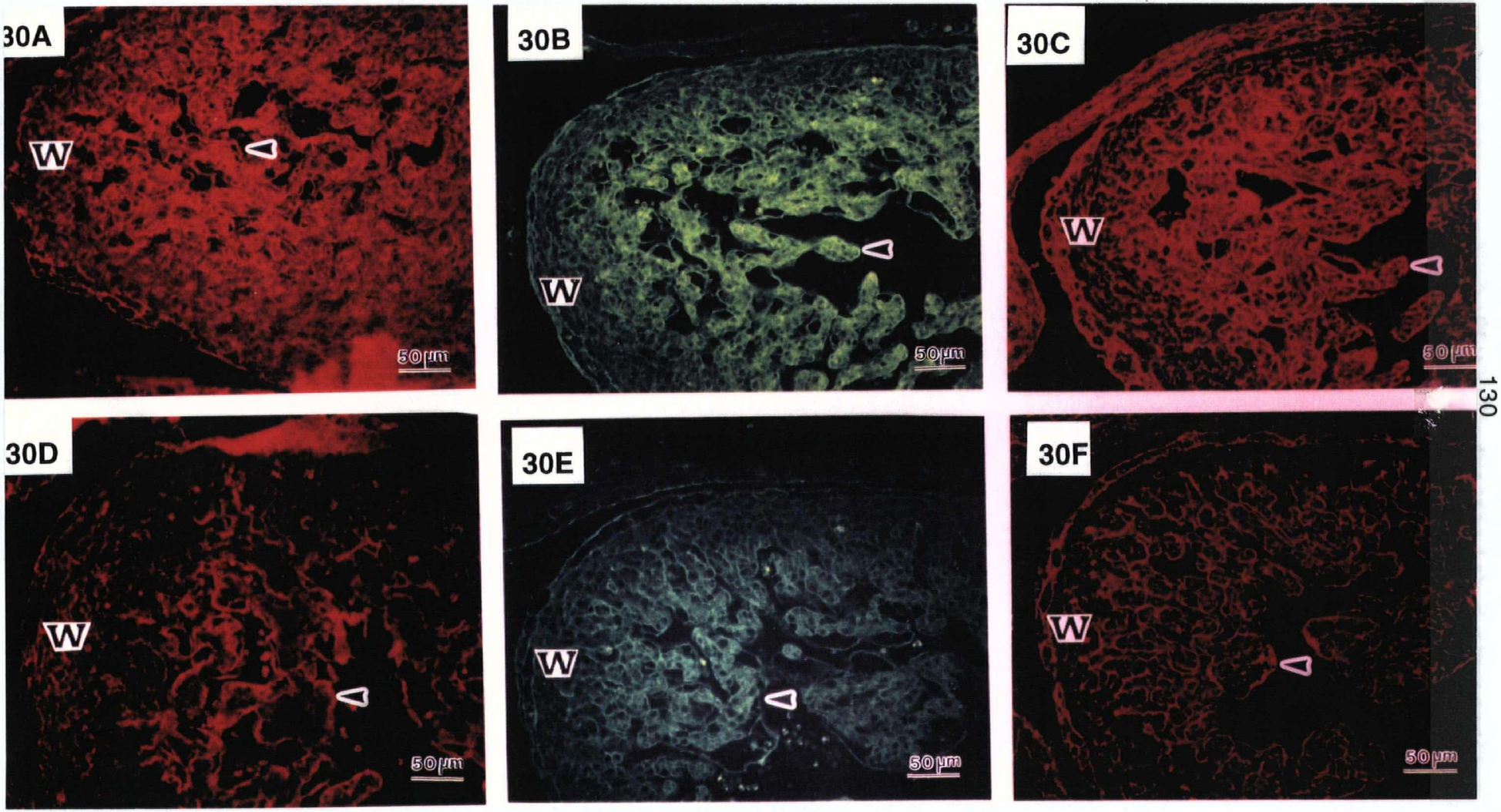
30A
W
50 µm
30B
W
50 µm
30C
W
50 µm
30D
W
50 µm
30E
W
50 µm
30F
W
50 µm

Fig. 30 A-F: Selected coronal sections through the embryonic heart from days 12 and 13. W, ventricular wall. At the 12-day-old stage, the labelling of type IV collagen (Fig. 30A), laminin (Fig. 30B) and fibronectin (Fig. 30C) was mainly localized to the ventricular trabeculations (arrowheads) but the intensity appeared decreased in the outer ventricular wall. At the 13-day-old stage, the staining of type IV collagen (Fig. 30D), laminin (Fig. 30E) and fibronectin (Fig. 30F) appeared to generally decrease in the heart tissue but remained in the ventricular trabeculations (arrowheads).

Discussion

In the present study, we have undertaken a comprehensive analysis of the temporo-spatial distribution of 72-kDa gelatinase as well as type IV collagen, laminin and fibronectin, ECM molecules that are substrates of 72-kDa gelatinase. The expression and activity of 72-kDa gelatinase in heart tissues reported here is consistent with a role for the enzyme in the remodelling of these components. The temporo-spatial distribution of type IV collagen, laminin, and fibronectin, the major basement membrane components, was highly correlated with that of 72-kDa gelatinase during days 9-13 of heart morphogenesis. Indeed, it was shown that the fibronectin type II-like repeats of 72-kDa gelatinase bind native and denatured type I collagen and elastin (Steffensen *et al.*, 1995) whereas the C-terminal domain of the enzyme binds fibronectin and heparin (Wallon and Overall, 1996), indicating a possible mechanism for tissue localization of the enzyme.

Type IV collagen, laminin and fibronectin were found to be uniformly and intensely labelled on the basal surfaces of the myocytes and endothelial cells during the early stages of development. As the heart matured, the staining of type IV collagen, laminin, and fibronectin was retained in the ventricular trabeculation, but showed reduced intensity particularly in the ventricular walls. Previous studies on the distribution of fibronectin (Icardo and Manasek, 1983; Tuckett and Morriss-Kay, 1986) also showed reduced staining in later stages of heart development, which with our data indicate that in addition to fibronectin, remodelling of type IV collagen and laminin occurs throughout early heart morphogenesis.

72-kDa gelatinase was also uniformly present in the myocytes and endothelial cells in the ventricular and atrial walls at the early stages. At later

stages, the staining of 72-kDa gelatinase was dramatically reduced in the walls and mainly localized to the trabeculated ventricles. Taken together, these results indicate that 72-kDa gelatinase may be coexpressed with the ECM molecules and then participates in the remodelling of the ECM during the rapid formation of the ventricular trabeculations. The expression of MMPs has also been observed during organ development where fast growth occurs. For example, the distribution of 72-kDa gelatinase was found to be correlated with outgrowth formation of the developing craniofacial complex at mouse embryonic days 10 and 11 (see Chapter 4). Moreover, 72-kDa gelatinase colocalized with epidermal growth factor and transforming growth factor-α during the outgrowth of the developing mouse primary palate (see Chapters 3 and 4). Nerve growth factor upregulated 72-kDa gelatinase expression by chick dorsal root ganglionic neurons in culture and was thought to play an important role in ECM degradation during neurite outgrowth (Muir, 1994). MMPs, including 72-kDa gelatinase, have also been found to be expressed during mouse blastocyst outgrowth (Brenner *et al.*, 1989; Behrendtsen *et al.*, 1992; Harvey *et al.*, 1995). Indeed, elevation and co-expression of ECM components (fibronectin, type I collagen, and SPARC) with 72-kDa gelatinase by transforming growth factor-β1 (TGF-β1), a potent growth factor involved in tissue morphogenesis, has been detected in human fibroblast cells (Overall *et al.*, 1989; 1991). At that time, it was proposed that 72-kDa gelatinase may not only be involved in the remodelling of newly deposited matrix, but may also perform a "quality control" role by degrading misfolded or denatured collagens in the provisional matrix.

In the present study, the decreased expression of 72-kDa gelatinase and the ECM components in the interventricular septum and ventricular wall in the later stages was evident. These data indicate that reduction of the 72-kDa

gelatinase expression in those regions may occur possibly by a passive rundown of the inducing signal or an active down-regulation by growth factors or hormones. Indeed, in areas showing reduced 72-kDa gelatinase staining, isolated cells still showed intense 72-kDa gelatinase labelling. This indicates that although a tissue may exhibit a general down-regulation of 72-kDa gelatinase expression, individual cell responsiveness varies. In addition, tissue inhibitors of metalloproteinases (TIMPs) may also play an important role in local repression of 72-kDa gelatinase activity in those regions. Indeed, the expression of TIMP-3 was intensely localized to the developing mouse heart at day 12.5 and reduced at day 14.5 (Apte *et al.*, 1994). However, TIMP-1 was not detected in the developing heart (Nomura *et al.*, 1989) and there is no available information on TIMP-2 expression during heart development. The distribution of TIMP-4 in the embryonic heart is currently under study in our laboratories.

72-kDa gelatinase may play a significant role in cell migration during an epithelial-mesenchymal transformation by degrading the basement membrane components, since the expression of 72-kDa gelatinase was found in the endothelial and mesenchymal cells of the endocardial cushion tissue. Support for an important role of the enzyme in cell migration and invasion also comes from the cell surface distribution of 72-kDa gelatinase reported here on the myocytes and endothelial cells. This is consistent with the cell surface distribution of endogenously activated enzyme upon Concanavalin-A stimulation (Overall and Sodek, 1990) that was later found to be associated with the cell membrane (Ward *et al.*, 1994). TGF-β1, a transcriptional inducer of 72-kDa gelatinase (Overall *et al.*, 1991), was also localized to the endothelial cells in the atrioventricular canal during formation of the endocardial cushion tissue (Akhurst *et al.*, 1990). In addition, TGF-β1, in combination with ventricular

myocardium, can mediate an epithelial-mesenchymal transformation by cultured atrioventricular canal endothelial cells *in vitro* (Potts and Runyan, 1989). This mesenchyme containing invaded myocytes will eventually form heart septal and valvular structures (Van Mierop *et al.*, 1962; Manasek, 1976). Taken together, it is suggested that the endothelial cells in the atrioventricular canal may produce 72-kDa gelatinase, upon stimulation by TGF-β1, in order to degrade the basement membrane and other ECM molecules during invasion of the underlying tissue to form the heart mesenchyme. Involvement of 72-kDa gelatinase during endothelial invasion into the underlying tissue at the atrioventricular canal models a similar situation that may take place during invasion of the tumor cells into stromal tissue. Indeed, 72-kDa gelatinase is found to be produced by the culture explants of a highly metastatic murine tumor and shows a strong correlation with the metastatic potential of cancer cells (reviewed by Liotta *et al.*, 1980; Stetler-Stevenson *et al*, 1993). Various human cancers, for example breast (Monteagudo *et al.*, 1990), colon (Levy *et al.*, 1991), and ovarian (Autio-Harmainen *et al.*, 1993) cancers demonstrate 72-kDa gelatinase in the tumor cells. Collectively, tumor cells and endocardial cushion endothelial cells may share similar mechanisms for tissue invasion that utilize 72-kDa gelatinase. Therefore, a better understanding of the mechanism of endothelial invasion of the underlying tissue in the atrioventricular canal may prove useful in understanding the mechanisms of invasion and metastasis of the tumors.

Recently, MMP-1 and TIMP-1 were found to be co-localized to the interstitial space between cardiac muscle bundles and within the endothelium and subendocardial space of the endocardium in normal adult rat heart (Tyagi *et al.*, 1995a). Northen blot results also showed the co-expression of MMP-1 and TIMP-1 in various adult tissues including the heart, skin, lung, liver, and kidney.

The expression of 72-kDa gelatinase, in particular, was specifically higher in the heart tissue (Tyagi *et al.*, 1995a). Upon induction by serum in cultures, human heart fibroblast and endothelial cells increase the production of MMP-1 and TIMP-1 at the mRNA and protein levels in a dose-dependent manner (Tyagi *et al.*, 1995b). Taken together, involvement of MMPs and TIMPs in normal adult heart tissue indicate their role in the integrity of cardiovascular structures. MMPs were also suggested to be associated with the pathogenesis of various kinds of heart diseases including aortic aneurysm, myocardial infarction, ischemia, and dilated cardiomyopathy (Cannon *et al.*, 1983; Sato *et al.*, 1983; Cleutjens *et al.*, 1995). More recently, increased expression of MMPs including neutrophil-type collagenase and 72-kDa and 92-kDa gelatinases was found to be associated with idiopathic dilated cardiomyopathy (Gunja-Smith *et al.*, 1996). 72-kDa gelatinase showed both latent and active forms. TIMP activity, on the other hand, was undetected. The newly-deposited collagen also showed poor cross-links. These changes are believed to contribute to weakening and dilatation of the ventricular wall.

In summary, the temporo-spatial expression of 72-kDa gelatinase was shown to be associated with type IV collagen, laminin, and fibronectin and correlated with the changes of this distribution pattern during early heart morphogenesis, particularly during the 9-to-13-day-old stages. Thus, these results indicate that 72-kDa gelatinase may play an important role in remodelling of the heart ECM components; type IV collagen, laminin, and fibronectin; which may be the *in vivo* substrates of 72-kDa gelatinase during early morphogenesis of the mouse heart. The current studies on the relationship of 72-kDa gelatinase expression in heart with TIMP-4 levels should also provide insight into the mechanisms of cardiac tissue remodelling in development and disease.

CHAPTER 6: GENERAL DISCUSSION

Collectively, the characterization of the growth and tissue remodelling during morphogenesis of the mouse primary palate in the present study suggests a hypothesis of the sequential events of molecular inductions and interactions (Diagram IV).

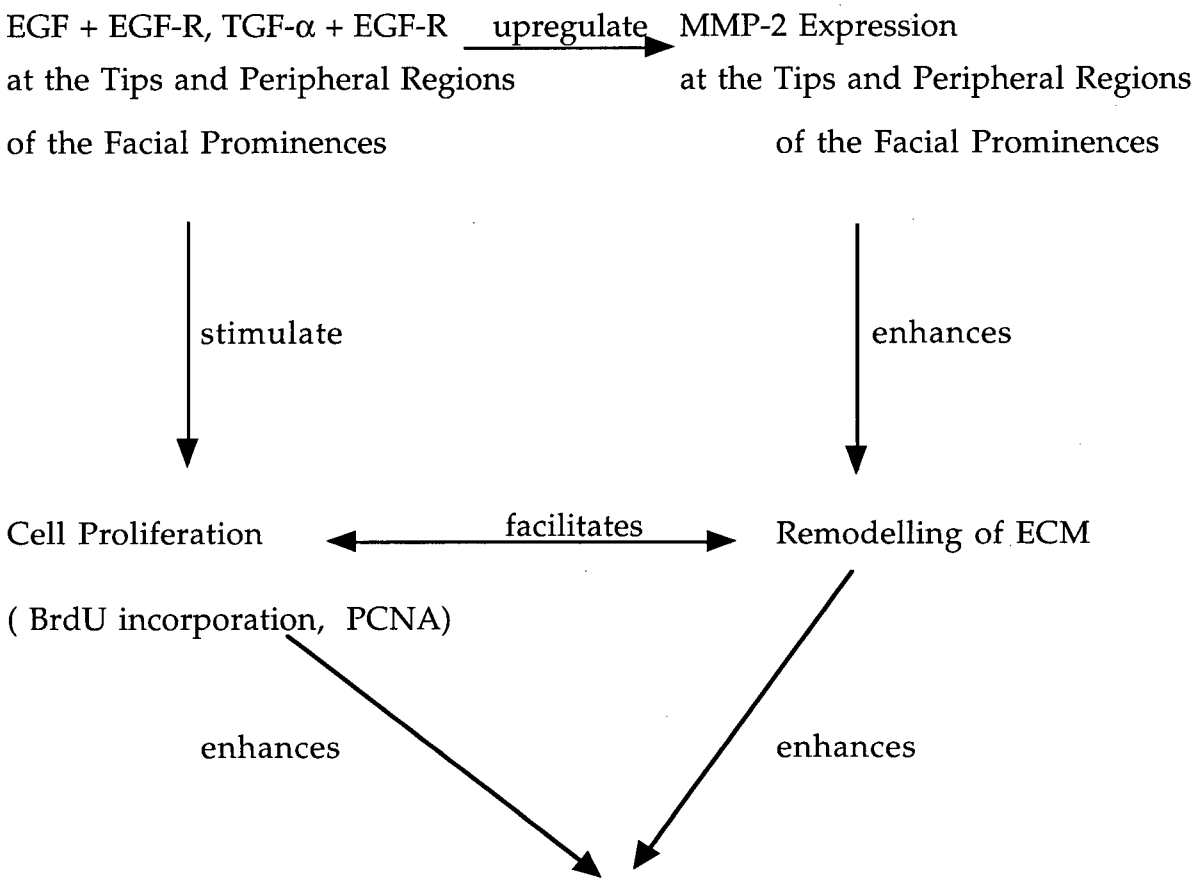

Diagram IV: Schematic model of the sequential events of molecular inductions and interactions during morphogenesis of the primary palate.

Craniofacial growth is involved with morphological changes of the facial prominences and the brain. The data from computer reconstructions and morphometric analyses showed that the size of the maxillary region increases rapidly as the maxillary prominence grows frontally to contribute to the primary palate (see review in Diewert and Wang, 1992; Diewert and Lozanoff, 1993). The size of the lateral nasal prominence also increases with a predominantly lateral growth pattern. In contrast, the size of the medial nasal prominence appears to increase only slightly. Since the facial prominences are physically attached to the brain, changes of the brain morphology affect growth of the face. It was found that the brain and the face become vertically separated as the brain becomes more superiorly positioned relative to the developing face. Rapid growth of the facial prominences to facilitate tissue contact plays an important role in primary palate morphogenesis. If a robust mesenchymal bridge is not well-established, failure of primary palate formation that leads to cleft lip malformation may take place (Wang, 1992; Diewert and Wang, 1992; Wang *et al.*, 1995).

Based on the proposed model shown in Diagram IV, a cascade of molecular signals with the expression of EGF, TGF-α, and EGF-R and 72-kDa gelatinase is suggested. EGF, TGF-α, and EGF-R were mainly expressed by the epithelium and the adjacent underlying mesenchyme of the maxillary and nasal prominences during 10-to-11-day-old mouse embryos. At the early stages (7-17 TS), the expression of EGF, TGF-α and EGF-R was intensely localized to the tips and peripheral regions of the facial prominences. At later stages (18-20 TS), the expression generally decreased but remained in peripheral regions of the primary palate. The uptake of BrdU and PCNA labelling, two cell proliferation markers, were most pronounced in regions with high levels of EGF, TGF-α, and EGF-R

expression. EGF and TGF-α act synergistically via EGF-Rs and may stimulate proliferation of epithelial and mesenchymal cells. As a result, the facial prominences enlarged and grew out from the base of the head.

The distribution of EGF and TGF-α in both epithelial and mesenchymal cells in the study is interesting and indicates that EGF and TGF-α are not tissue or cell type specific, but rather are expressed in temporal and spatial manners during primary palate morphogenesis. These findings are similar to those that occur during embryonic lung development in which EGF and EGF-R were co-localized to a specific position-restricted distribution in the epithelium and mesenchyme of the primitive airways (Warburton *et al.*, 1992). In the present study, an autocrine mode of regulation is speculated since their receptor, EGF-R was localized to the similar regions to EGF and TGF-α at all stages examined. Indeed, an autocrine mode of regulation of EGF and TGF-α was found to be important for embryonic development (Shum *et al.*, 1993; Johnson *et al.*, 1994; Chia *et al.*, 1995) and tumorigenesis (Derynck *et al.*, 1987; Ju *et al.*, 1991; Barton *et al.*, 1991; Christensen *et al.*, 1993). In addition, transgenic mice with overexpression of TGF-α developed liver (Jhappan *et al.*, 1990) and breast (Matsui *et al.*, 1990; Sandgren *et al.*, 1990) neoplasia. These neoplastic tissues also expressed EGF-R in their epithelial components. Alternatively, a paracrine mode of regulation may also be operative since the strongest signals of EGF and TGF-α appeared to be at the surface ectoderm. The growth factors may diffuse into the underlying mesenchyme through the basement membrane, bind to EGF-R at the surfaces of the mesenchymal cells and stimulate cell proliferation. As a result, a differential proliferation of the mesenchymal cells occurred. Decreased staining of BrdU and PCNA as well as EGF, TGF-α and EGF-R in the midline tissues and

deeper regions of the primary palate in the present study suggested that relative growth reduction takes place in certain regions of the primary palate and may contribute to further tissue differentiation and specialization in those regions. Previous cell proliferation study by Minkoff and Martin (1984) showed similar results and suggested chondrogenic differentiation at the roof of the stomodeum.

Regional differences of the labelling indices were also detected in the maxillary prominence by using [3]H-thymidine injection in chick embryos (Bailey *et al.*, 1988). It was found that growth rate of the maxillary mesenchyme differed based on its proximity to the overlying epithelium. The rate of cell proliferation in the facial prominences was higher in the subepithelial zone of the mesenchyme compared to that of cell populations which were located further away from the epithelium. These results are consistent with the present study and suggest that the epithelium may have growth regulating effect by secreting morphogens or growth factors into the subjacent mesenchyme (Bailey *et al.*, 1988).

72-kDa gelatinase was found to co-localize with EGF and TGF-α, particularly in outgrowth regions of the primary palate, and in the tips and peripheral regions of the facial prominences. At advanced stages of primary palate formation, expression of 72-kDa gelatinase, EGF and TGF-α became dramatically reduced. These findings indicate that there may be an interaction between 72-kDa gelatinase and the two growth factors. Indeed, EGF and TGF-α have been found to stimulate synthesis of MMPs in a variety of cells and tissues (Kerr *et al.*, 1988; Shima *et al.*, 1993; Lyons *et al.*, 1993; Chen *et al.*, 1993). For example, upon being activated by TGF-α and EGF, rat mucosal keratinocytes, under permanently serum-free conditions, increased the production of MMP-1 and MMP-9 (Lyons *et al.*, 1993). Increased synthesis of 72-kDa gelatinase was also

found to be induced by EGF and TGF-α during *in vitro* branching morphogenesis of the lung (Ganser *et al.*, 1991). The protein expression of gelatinases, including 72-kDa gelatinase, was also demonstrated in the blastocyst during mouse pre-implantation development (Brenner *et al.*, 1989) and found to be induced by TGF-α (Dardik *et al.*, 1993). In differentiating human epidermal raft cultures, TGF-α was found to induce keratinocytes to secrete type I collagenase and gelatinase (Turksen *et al.*, 1991). As a result, invasion of basal cells into the collagen matrix took place. Taken together, EGF and TGF-α may regulate the expression of 72-kDa gelatinase during primary palate morphogenesis.

72-kDa gelatinase, upon being locally induced by the growth factors, is probably synthesized into the ECM as a latent, inactive enzyme. The mechanism of activation of 72-kDa gelatinase *in vivo* was suggested to be involved with MT-MMPs (Sato *et al.*, 1994) and take place on the cell surface (Ward *et al.*, 1994). These data are consistent with the present study since the immunostaining of 72-kDa gelatinase was intensely localized on the cell surfaces of the myocytes and endothelial cells in the heart tissue (see Chapters 5). More recently, MT-MMP-2 was identifed and found to induce processing of progelatinase A into the activated forms, like MT-MMP-1 (Takino *et al.*, 1995). Taken together, MT-MMPs may be *in vivo* activators for 72-kDa gelatinase during primary palate morphogenesis. Further investigation of MT-MMP expression in the developing primary palate will support this mode of activation of 72-kDa gelatinase *in vivo*. The active enzyme may enhance and facilitate cell proliferation by remodelling the ECM in the tips and peripheral regions of the primary palate. Indeed, the expression of MMPs and TIMPs have been found to be associated with cell proliferation in many studies. For example, serum can induce cell proliferation and the expression of MMP-1 and TIMP-1 in human heart fibroblast and

endothelial cells in cultures (Tyagi *et al.*, 1995b). 72-kDa and 92-kDa gelatinases have been suggested to be involved in basement membrane and matrix degradation in balloon-injured carotid arterial wall during the period of smooth muscle cell proliferation and migration (Zempo *et al.*, 1994). EGF, PDGF, and insulin can induce cell proliferation and the production of MMP-1 and MMP-3 in rheumatoid synovial fibroblasts in cultures (Hiraoka *et al.*, 1992). TIMP-1 and TIMP-2 can directly stimulate cell proliferation in a Burkitt lymphoma cell line (Hayakawa, 1994; Hayakawa *et al.*, 1994). Collectively, 72-kDa gelatinase may play be involved in primary palate morphogenesis by facilitating cell proliferation at the outgrowth regions.

72-kDa gelatinase appears to be a common molecule for tissue remodelling during embryonic development (Ganser *et al.*, 1991; Reponen *et al.*, 1992; Sahlberg *et al.*, 1992; Heikinheimo and Salo, 1995), wound healing (Salo *et al.*, 1994), and tumor invasion and metastasis (Liotta *et al.*, 1980; Monteagudo *et al.*, 1990; Levy *et al.*, 1991; Autio-Harmainen *et al.*, 1993; Soini *et al.*, 1994). The expression of 72-kDa gelatinase has also been detected in a wide variety of normal and transformed cell lines including fibroblast (Seltzer *et al.*, 1981), chondrocytes (Murphy *et al.*, 1989), keratinocytes (Salo *et al.*, 1991), endothelial cells (Kalebic *et al.*, 1983), and rheumatoid synovial cells (Okada *et al.*, 1990). However, the substrates for 72-kDa gelatinase *in vivo* remain unclear. In the present study, the association between the protein expression of 72-kDa gelatinase and the ECM components including type IV collagen, laminin and fibronectin was characterized in the developing primary palate and heart. Previous *in vitro* studies showed that type IV collagen (Fessler *et al.*, 1984), fibronectin and laminin (Okada *et al.*, 1990; Nagase *et al.*, 1991) are also substrates for 72-kDa gelatinase. Type IV collagen, in particular, has been found to be associated with 72-kDa gelatinase during mouse (Sahlberg *et al.*, 1992) and

human (Heikinkeimo and Salo, 1995) tooth development and mammary gland development (Dickson and Warburton, 1992). Taken together, the ECM components; particularly type IV collagen, laminin and fibronectin; may be substrates for 72-kDa gelatinase *in vivo* during embryonic development.

Recently, Overall (1994) has proposed a model for MMP and TIMP regulation in tissue remodelling. He proposed that gradients of growth factors or other regulatory molecules at sites of tissue remodelling may modulate the expression of MMPs, TIMPs, and ECM proteins. Normal connective tissue is characterized by low constitutive levels of TIMPs and 72-kDa gelatinase. At a remodelling site, a net resorptive cell phenotype may be expected at the focus of degradation, characterized by increased MMP and reduced TIMP expression. The surrounding normal tissue is likely protected from MMP activity by a "green belt" comprising cells that exhibit an active formative phenotype induced by "formative factors" such as TGF-β. This "green belt" region is marked by an up-regulation of TIMP, 72-kDa gelatinase, and ECM protein synthesis to both protect local tissue and to buffer adjacent normal tissue from inappropriate MMP activity diffusing from the focus of degradation (Overall, 1994).

The results from the present work suggest that gradients of EGF and TGF-α at the outgrowth regions, the remodelling sites, of the developing facial prominences are present and operative by inducing the expression of 72-kDa gelatinase. Decreased levels of 72-kDa gelatinase in the deeper and midline tissues also provide support for the model of tissue remodelling proposed by Overall (1994).

During primary palate morphogenesis, a number of growth factors, transcription factors, and oncogene product are expressed in the facial prominences (see Table I). This multiple expression of molecular factors

indicates that the mechanism of primary palate morphogenesis is complex and needs interactions and inductions between molecules. For example, *AP-2*, a transcriptional factor, was found to be present in the ectoderm and mesenchyme of the mouse facial prominences (Mitchell *et al.*, 1991). Recently, transgenic mouse embryos lacking *AP-2* gene function demonstrated exencephaly, midline clefts of the face, and absent or malformed sensory organs and cranial ganglia (Schorle *et al.*, 1996; Zhang *et al.*, 1996). These findings suggest that *AP-2* is important for craniofacial development. Interestingly, it was found that an *AP-2* binding site is located in the first exon of 72-kDa gelatinase gene and suggested to be a regulatory element for gene transcription (Huhtala *et al.*, 1990; Sato and Seiki, 1993). These data indicate that 72-kDa gelatinase may be a target gene regulated by *AP-2* since 72-kDa gelatinase was also expressed in the mouse facial prominences at similar stages (see Chapter 4). Some other transcriptional factors expressed in the facial prominences may be regulators of *AP-2*, particularly *Msx-1*, since the expression of *Msx-1* overlaps with that of *AP-2* (Mitchell *et al.*, 1991; Robert *et al.*, 1989; Brown *et al.*, 1993). *AP-2* mutants also showed abnormal expression of *Msx-1* in which it was reduced in the proximal portions of the facial prominences and was laterally displaced in the distal portions (Schorle *et al.*, 1996). However, *Pax-3* and *twist*, regulatory genes known to be required for cranial closure, appeared to be normally expressed in *AP-2* mutants (Schorle *et al.*, 1996). These indicate that defects in cranial closure in *AP-2* mutants are not caused by loss of *Pax-3* and *twist*.

CHAPTER 7: CONCLUSIONS AND FUTURE WORK

CONCLUSIONS

1. The major basement membrane components including laminin, type IV collagen and fibronectin, became disrupted and gradually disappeared in association with the regression of the epithelial seam and the mesenchymal bridge formation during morphogenesis of mouse primary palate.

2. EGF, TGF-α, and EGF-R were temporo-spatially co-distributed in the enlarging facial prominences of the developing primary palate, suggesting their role in outgrowth of the mouse primary palate tissues.

3. The distribution patterns of BrdU and PCNA were similar to those of EGF, TGF-α and EGF-R at similar stages of primary palate morphogenesis. These observations suggest that EGF, TGF-α and EGF-R may stimulate cell proliferation during outgrowth of the mouse primary palate.

4. Expression of 72-kDa gelatinase was associated with morphogenesis of the primary palate, mandible, eye, and heart. Based on co-localization of ECM components and 72-kDa gelatinase, I suggest that 72-kDa gelatinase may enhance ECM remodelling of those developing organs.

5. 72-kDa gelatinase was found to be localized to the same regions as was EGF and TGF-α at all stages examined during morphogenesis of the mouse primary palate, suggesting that EGF and TGF-α may regulate morphogenesis of the primary palate, at least in part, by inducing the expression of 72-kDa gelatinase.

FUTURE WORK

Based on the results of the present study, 72-kDa gelatinase appears to be important for development of many organ systems including the primary palate, mandible, eye, and heart because the temporo-spatial distribution of 72-kDa gelatinase was found to be expressed at increased levels at critical times during development of those organs. However, the mechanisms of 72-kDa gelatinase in organ development, especially the functional aspects, are unknown. EGF, TGF-α, and EGF-R were also shown to be involved with morphogenesis of the primary palate but their direct relationship with 72-kDa gelatinase and cell proliferation remains to be determined.

Therefore, I further hypothesize that

1. 72-kDa gelatinase is a major MMP involved in degradation of basement membranes during fusion and outgrowth of the facial prominences,

2. elevated levels of expression of other MMPs and TIMPs are present in the areas of fusion and outgrowth during primary palate morphogenesis,

3. growth factors, particularly TGF-α and EGF, are *in vivo* inducers for 72-kDa gelatinase expression during primary palate morphogenesis.

Approaches

1. To further determine involvement of 72-kDa gelatinase at the transcription level in tissue remodelling during primary palate morphogenesis, the mRNA expression of 72-kDa gelatinase can be studied by *in situ* hybridization technique (Reponen *et al.*, 1992; Wilkinson, 1993). This technique would allow us to investigate which type of cells, epithelial or mesenchymal cells, can synthesize 72-kDa gelatinase. *In situ* hybridization can be performed

either on tissue sections or whole mount embryos (Wilkinson, 1993). The mouse anti-sense 72-kDa gelatinase probe can be generated according to Reponen *et al.* (1992). The expression of 72-kDa gelatinase mRNA can also be confirmed by Northern blots and reverse transcription polymerase chain reaction (RT-PCR) analyses (Koopman, 1993). In order to quantitatively compare the amount of 72-kDa gelatinase mRNA from different stages of primary palate morphogenesis, the RNAase protection assay can be analyzed (Altaba, 1993).

To further understand the mechanisms of 72-kDa gelatinase in tissue remodelling during primary palate morphogenesis, functional tests for 72-kDa gelatinase can be investigated by several approaches.

a) A first approach is to perform loss of function experiments. Organ cultures can be used for this purpose (Morriss-Kay, 1993). The developing primary palate is dissected as one unit and cultured. To test whether 72-kDa gelatinase is required for tissue outgrowth and fusion of the facial prominences, the cultured explants will be incubated with a monoclonal anti-72-kDa gelatinase antibody specific for the catalytic domain of 72-kDa gelatinase at various times in comparison with the controls. If 72-kDa gelatinase is important for development of the primary palate, the cultured explants incubated with a monoclonal anti-72-kDa gelatinase antibody will show deficiency in growth and unsuccessful fusion of the facial prominences. Alternatively, a monoclonal anti-72-kDa gelatinase antibody can be replaced by TIMPs and synthetic MMP inhibitors (Fisher *et al.*, 1994). These approaches will provide not only a better understanding of the functions of 72-kDa gelatinase during development of the primary palate but also the therapeutic potential of MMP inhibitors in controlling 72-kDa gelatinase-dependent pathological processes such as malignant tumors and inflammatory diseases (Birkedal-Hansen, 1995).

b) A second approach is to perform whole embryo culture (Morriss-Kay, 1993). Similar to the organ cultures, the whole embryo culture will allow us to examine the effects of a monoclonal anti-72-kDa gelatinase antibody, TIMPs, synthetic MMP inhibitors or the other molecules of interest; such as EGF and TGF-α; to the development of the whole embryo by introducing the antibody or the growth factors into the amniotic fluid at various times. By introducing an anti-72-kDa gelatinase antibody, TIMPs, or synthetic MMP inhibitors in the culture, growth of the primary palate may be abnormal. On the other hand, by adding EGF or TGF-α in the culture, growth of the primary palate and the expression of 72-kDa gelatinase may be stimulated.

c) A third approach is to generate transgenic mice with mutant or deleted 72-kDa gelatinase gene. The homozygous mouse embryos with mutant or deleted 72-kDa gelatinase gene can be generated by two techniques, pronuclear injection (Schnieke *et al.*, 1983) or gene targeting in embryonic stem (ES) cells (Joyner, 1991; Robertson and Martin, 1993). These techniques will allow us to directly examine functional effects of 72-kDa gelatinase during multiple organ development of the mouse embryo by comparing the homozygous embryos with the heterozygous and normal embryos.

2. To determine whether the other MMPs; such as interstitial collagenase, stromelysins, 92-kDa gelatinase, and MT-MMPs; and TIMPs; such as TIMP-1, -2, -3, and -4; are involved in tissue remodelling during primary palate morphogenesis, co-localization of the proteins and mRNAs of MMPs and TIMPs can be studied on tissue sections or whole mount embryos by employing sequential immunohistochemistry and *in situ* hybridization technique (Stern, 1993). In addition, the functional analyses of MMPs and TIMPs can be performed *in vitro* similar to the above experiments.

3. To test whether the growth factors; for example, EGF and TGF-α can induce the production of 72-kDa gelatinase from epithelial cells or mesenchymal cells, the epithelial cells or mesenchymal cells can be removed from the primary palate and cultured separately under appropriate conditions (Dixon *et al.*, 1993). Subsequently, EGF or TGF-α will be added to the culture medium at various times in comparison with the controls. Then the culture medium is collected and the expression of 72-kDa gelatinase is studied by using gelatin zymography. The experimental results can be semiquantitatively compared with the controls by using densitometry on the zymograms.

BIBLIOGRAPHY

Abbott, B.D., and Pratt, R.M. (1988): EGF receptor expression in the developing tooth is altered by exogenous retinoic acid and EGF. Dev. Biol. 128:300-304.

Adamson, E.D. (1990): EGF receptor activities in mammalian development. Mol. Reprod. Dev. 27:16-22.

Adamson, E.D. (1993): Activities of growth factors in preimplantation development. J. Cell Biochem. 53:280-287.

Adler, R.R., Brenner, C.A., and Werb. Z. (1990): Expression of extracellular matrix-degrading metalloproteinases and metalloproteinase inhibitors is developmentally regulated during endoderm differentiation of embryonal carcinoma cells. Development 110:211-220.

Ahumada, G.C., Rennard, S.I., Figueroa, A.A., and Silver, M.H. (1981): Cardiac fibronectin: Developmental distribution and quantitative comparison of possible sites of synthesis. J. Mol. Cell Cardiol. 13:667-678.

Akhurst, R.J., Lehnert, S.A., Faissner, A., and Duffie, E. (1990): TGF beta in murine morphogenetic processes: the early embryo and cardiogenesis. Development 108:645-656.

Alexander, C.M., and Werb, Z. (1991): Extracellular matrix degradation. In: Hay E. (ed.) "Cell Biology of Extracellular Matrix." Plenum Press, New York. pp.255-302.

Altaba, A.R.i. (1993): RNAase protection assays. In: Stern, C.D., and Holland, P.W.H. (eds.) "Essential Developmental Biology." Oxford University Press, Oxford. pp.213-222.

Andermarcher, E., Surani, M.A., and Gherardi, E. (1996): Co-expression of the HGF/SF and c-met genes during early mouse embryogenesis precedes reciprocal expression in adjacent tissues during organogenesis. Dev. Genetics 18:254-266.

Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Rahmsdorf, H.J., Jonat, C., Herrlich, P., and Karin, M. (1987): Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. Cell 49:729-739.

Apte, S.S., Hayashi, K., Seldin, M.F., Mattei, M.-G., Hayashi, M., and Olsen, B.R. (1994): Gene encoding a novel murine tissue inhibitor of metalloproteinases (TIMP), TIMP-3, is expressed in developing mouse epithelia, cartilage, and muscle, and is located on mouse chromosome 10. Dev. Dynamics 200:177-197.

Ardinger, H.H., Buetow, K.H., Bell, G.I., Bordach, J., VanDemark, D.R., and Murray, J.C. (1989): Association of genetic variation of the transforming growth factor alpha gene with cleft lip and palate. Am. J. Hum. Genet. 45:348-353.

Autio-Harmainen, H., Karttunen, T., Hurskainen, T., Hoyhtya, M., Kauppila, M., and Tryggvason, K. (1993): Expression of 72- kilodalton type IV collagenase (gelatinase A) in benign and malignant ovarian tumors. Lab. Invest. 69:312-321.

Bailey, L.J., Minkoff, R., and Koch, W.E. (1988): Relative growth rates of maxillary mesenchyme in the chick embryo. J Craniofac Genet Dev. Biol. 8:167-177.

Bancroft, M., and Bellairs, R. (1977): Placodes of the chick embryo studied by SEM. Anat. Embryol. 151:97-108.

Barsky, S.H., Siegal, G.P., Jannotta, F., and Liotta, L.A. (1983): Loss of basement membrane components by invasive tumors but not by their benign counterparts. Lab. Invest. 49:140-147.

Barton, C.M., Hall, P.A., Huges, C.M., Gullick, W.J., and Lemoine, N.R. (1991): Transforming growth factor alpha and epidermal growth factor in human pancreatic cancer. J. Pathol. 163:111-116.

Beerstecher, H.J., Huiskens-Van Der Meij, C., and Warnaar, S.O. (1988): An immunohistochemical study performed with monoclonal and polyclonal antibodies to mouse epidermal growth factor. J. Histochem. Cytochem. 36:1153-1160.

Behrendtsen, O., Alexander, C.M., and Werb, Z. (1992): Metalloproteinases mediate extracellular matrix degradation by cells from mouse blastocyst outgrowths. Development 114:447-456.

Bernfield, M. (1981): Organization and remodelling of the extracellular matrix in morphogenesis. In: Connelly, T.G. (ed.) "Morphogenesis and Pattern Formation." Raven Press, New York pp.139-162.

Bernfield, M., Banerjee, S.D., Koda, J.E., and Rapraeger, A.C. (1984): Remodelling of the basement membrane as a mechanism of morphogenetic tissue interaction. In: Trelstad R.L. (ed.) "The Role of Extracellular Matrix of Development." Alan R Liss Inc., New York. pp.545-572.

Birkedal-Hansen, H. (1995): Proteolytic remodelling of extracellular matrix. Cur. Opin. Biol. 7:728-735.

Birkedal-Hansen, H., Moore, W.G.I., Bodden, M.K., Windsor, L.J., Birkedal-Hansen, B., DeCarlo, A., and Engler, J.A. (1993): Matrix metalloproteinases: a review. Crit. Rev. Oral Biol. Med. 4(2):197-250.

Bravo, R., and Macdonald-Bravo, H. (1984): Induction of the nuclear protein 'cyclin' in quiescent mouse 3T3 cells stimulated by serum and growth factors. Correlation with DNA synthesis. E.M.B.O. J. 3:3177-3181.

Breckon, J.J., Hembry, R.M., Reynolds, J.J., and Meikle, M.C. (1994): Regional and temporal changes in the synthesis of matrix metalloproteinases and TIMP-1 during development of the rabbit mandibular condyle. J. Anat. 184:99-110.

Brenner, C.A., Adler, R.R., Rappolee, D.A., Pedersen, R.A., and Werb, Z. (1989): Genes for extracellular matrix-degrading metalloproteinases and their inhibitor, TIMP, are expressed during early mammalian development. Genes Dev. 3:848-859.

Brinkley, L., Du, Y., and Morris-Wiman, J. (1995): Matrix-degrading enzymes are present during palatal morphogenesis. J. Dent. Res. 74:66

Bronner-Fraser, M. (1986): An antibody to the receptor for fibronectin and laminin perturbs cranial neural crest development *in vivo*. Dev. Biol. 117:528-536.

Brown, J.M., Wedden, S.E., Millburn, G.H., Robson, L.G., Hill, R.E., Davidson, D.R., and Tickle, C. (1993): Experimental analysis of the control of expression of the homeobox-gene *Msx-1* in the developing limb and face. Development 119:41-48.

Brunet, C.L., Sharpe, P.M., and Ferguson, M.W.J. (1995): Inhibition of TGF-β3 (but not TGF-β1 or TGF-β2) activity prevents normal mouse embryonic palate fusion. Int. J. Dev. Biol. 39:345-355.

Burgeson, R.E. (1993): Dermal-epidermal adhesion in skin. In: Rohrbach, D.H., and Timpl, R. (eds.) "Molecular and Cellular Aspects of Basement Membrane." Academic Press, Inc., San Diego. pp.49-66.

Burgeson, R.E., Chiquet, M., Deutzmann, R., Ikblom, P., Engel, J., Kleinman, H., Martin, G.R., Ortonne, J.-P., Paulsson, M., Sanes, J., Timpl, R., Tryggvason, K., Yamada, Y., and Yurchenco, P.D. (1994): A new nomenclature for laminins. Matrix Biol. 14:209-211.

Burk, D.T. (1993): Distribution of glycosaminoglycans in the developing facial region of mouse embryos. J. Craniofac. Genet. Dev. Biol. 3:339-349.

Canete-Soler, R., Gui, Y.-H., Linask, K.K., and Muscher, R.J. (1995): Developmental expression of MMP-9 (gelatinase B) mRNA in mouse embryos. Dev. Dynamics 204:30-40.

Cannon, R.O., Butany, J.W., McManus, B.M., Speir, E., Kravitz, A.B., Boli, R., and Ferrans, V.J. (1983): Early degradation of collagen after acute myocardial infarction in the rat. Am. J. Cardiol. 52:390-395.

Carette, M.J.M., and Ferguson, M.W.J. (1992): The fate of medial edge epithelial cells during palatal fusion *in vitro*: an analysis by DiI labelling and confocal microscopy. Development 114:379-388.

Carpenter, G., and Wahl, M.I. (1991): The epidermal growth factor family. In: Sporn, M.B., Roberts, A.B. (eds.) "Peptide Growth Factors and Their Receptors I." Springer Verlag, New York. pp.69-171.

Cartlidge, S.A., and Elder, J.B. (1989): Transforming growth factor beta and epidermal growth factor levels in normal gastrointestinal mucosa. Br. J. Cancer 60:657-660.

Casasco, A., Giordano, M., Danova, M., Casaco, M., Conaglia, I., Calligaro, A. (1993): PC10 monoclonal antibody to proliferating cell nuclear antigen as probe for cycling cell detection in developing tissues. Histochem. 99:191-199.

Cawood, A.H., and Savage, J.R. (1983): A comparison of the use of bromodeoxyuridine and [3H]thymidine in studies of the cell cycle. Cell Tissue Kinet. 16:51-57.

Cawston, T.E. (1986): Protein inhibitors of metalloproteinases. In: Barrett, A.J. and Salvesen, G. (eds.) "Proteinase Inhibitors." Elsevier Science, Amsterdam. pp.589-610.

Celis, J.E. and Celis, A. (1985): Cell cycle-dependent variations in the distribution of the nuclear protein cyclin proliferating cell nuclear antigen in cultured cells: subdivision of S phase. Proc. Nat. Acad. Sci. U.S.A. 82:3262-3266.

Chapman, H.A., Reilly, J.J., and Kobzik, L. (1988): Role of plasminogen activator in degradation of extracellular matrix protein by live human alveolar macrophages. Am. Rev. Res. Dis. 137:412-419.

Chegini, N. and Williams, R.S. (1992): Immunocytochemical localization of transforming growth factors (TGFs) TGF-α and TGF−β in human ovarian tissues. J. Clin. Endocrinol. Metab. 74:973-980.

Chen, L.L., Narayanan, R., Hibbs, M.S., Benn, P.A., Clawson, M.L., Lu, G., Rhim, J.S., Greenberg, B., and Mendelsohn, J. (1993): Altered epidermal growth factor signal transduction in activated Ha-ras-transformed human keratinocytes. Biochem. Biophys. Res. Comm. 193:167-174.

Chenevix-Trench, G., Jones, K., Green, A.C., Duffy, D.L., and Martin, N.G. (1992): Cleft lip with or without cleft palate: associations with transforming growth factor and retinoic acid receptor loci. Am. J. Hum. Genet. 51:1377-1385.

Chenevix-Trench, G., Jones, K., Green, A.C., and Martin, N.G. (1991): Further evidence for an association between genetic variation in transforming growth factor alpha and cleft lip and palate. Am. J. Hum. Genet. 48:1012-1013.

Chia, C.M., Winston, R.M.L., and Handyside, A.H. (1995): EGF, TGF-α and EGFR expression in human preimplantation embryos. Development 121:299-307.

Christensen, M.E., Therkildsen, M.H., Poulsen, S.S., and Bretlau, P. (1993): Immunoreactive transforming growth factor alpha and epidermal growth factor in oral squamous cell carcinomas. J. Pathol. 169:323-328.

Clark, S.D., Kobayashi, D.K., and Welgus, H.G. (1987): Regulation of the expression of tissue inhibitor of metalloproteinases and collagenases by retinoids and glucocorticoids in human fibroblasts. J. Clin. Invest. 80:1280-1288.

Cleutjens, J.P.M., Kandala, J.C., Guarda, E., Guntaka, R.V., and Weber, K.T. (1995): Regulation of collagen degradation in the rat myocardium after infarction. 27:1281-1292.

Coffey, R.J., Derynck, R., Wilcox, J.N., Bringman, T.S., Goustin, A.S., Moses, H.L., and Pittelkow, M.R. (1987): Production and autoinduction of transforming growth factor-a in human keratinocytes. Nature 328:817-820.

Cohen, S. (1962): Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. J. Biol. Chem. 237:1555-1562.

Cohen, S., Carpenter, G., and King, L.J. (1980): Epidermal growth factor-receptor-kinase interactions. Co-purification of receptor and epidermal growth factor-enhanced phosphorylation activity. J. Biol. Chem. 255:4834-4842.

Cooper, A.R., and MacQueen, H.A. (1983): Subunits of laminin are differentially synthesized in mouse eggs and early embryos. Dev. Biol. 96:467-471.

Corliss, C.E. (1976): Patten's Human Embryology. Elements of Clinical Development. McGraw-Hill Book Company: New York. pp.68.

Crossley, P.H., and Martin, G.R. (1995): The mouse *Fgf8* gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. Development 121:439-451.

Curry, T.E. Jr., Mann, J.S., Estes, R.S., and Jones, P.B.C. (1990): α2-Macroglobulin and tissue inhibitor of metalloproteinases: collagenase inhibitors in human preovulatory ovaries. Endocrinology 127:63-68.

Dardik, A., Doherty, A.S., and Schultz, R.M. (1993): Protein secretion by the mouse blastocyst: Stimulatory effect on secretion into the blastocoel by transforming growth factor-α. Mol. Reprod. Dev. 34:396-401.

Da Silveira, A., Du, Y., and Brinkley, L. (1995): Appearance of metalloproteinases and serine proteases during murine facial development. J. Dent. Res. 74:65.

Da Silveira, A., Du, Y., and Brinkley, L. (1996): Occurrence of MMPs, TIMPs, and their mRNAs during murine facial morphogenesis. J. Dent. Res. 75:226.

Dean D.D., Muniz, O.E., Berman, I., Pita, J.C., Carreno, M.R., Woessner, J.F. Jr., and Howell D.S. (1985): Localization of collagenase in the growth plate of rachitic rats. J. Clin. Invest . 76:716-722.

Derynck, R. (1992): The physiology of transforming growth factor-α. Adv. Cancer Res. 58:27-52.

Derynck, R., Goeddel, D.V., Ullrich, A., Gutterman, J.U., Williams, R.D., Bringman, T.S., and Berger, W.H. (1987): Synthesis of messenger RNAs for transforming growth factor α and β and the epidermal growth factor receptor by human tumors. Cancer Res. 47:707-712.

Derynck, R., Roberts, A.B., Winkler, M.E., Chen, E.Y., and Goeddel, D.V. (1984): Human transforming growth factor-alpha: Precursor structure and expression in *E. coli.* Cell 38:287-297.

Dickson, M.C., Slager, H.G., Duffie, E., Mummery, C.L., and Akhurst, R.J. (1993): RNA and protein localizations of TGFβ2 in the early mouse embryo suggest an involvement in cardiac development. Development 117:625-639.

Dickson, S.R., and Warburton, M.J. (1992): Enhanced synthesis of gelatinase and stromelysin by myoepithelial cells during involution of the rat mammary gland. J. Histochem. Cytochem. 40:697-703.

Diewert, V.M., and Lozanoff, S. (1993): Growth and morphogenesis of the human embryonic midface during primary palate formation analyzed in frontal sections. J. Craniofac. Genet. Dev. Biol. 13:162-183.

Diewert, V.M., and Shiota, K. (1990): Morphological observations in normal primary palate and cleft lips embryos in the Kyoto collection. Teratology 41:663-667.

Diewert, V.M., and Van der Meer, D. (1991): Growth of the human primary palate. J. Dent. Res. 69:156.

Diewert, V.M., and Wang, K.-Y. (1992): Recent advances in primary palate and midface morphogenesis research. Crit. Rev. Oral Biol. Med. 4(1):111-130.

Diewert, V.M., Wang, K.-Y., and Tait B. (1993a): A morphometric analysis of cell densities in facial prominences of the rhesus monkey embryo during primary palate formation. J. Craniofac. Genet. Dev. Biol. 13:236-249.

Diewert, V.M., Wang, K.-Y., and Tait, B. (1993b): A new threshold model for cleft lip in mice. Ann. N.Y. Acad. Sci. 678:341-343.

Dixon, M.J., and Ferguson, M.W.J. (1992): The effects of epidermal growth factor, transforming growth factors alpha and beta and platelet-derived growth factor on murine palatal shelves in organ culture. Arch. Oral Biol. 37:395-410.

Dixon, M.J., Foreman, D., Schor, S., and Ferguson, M.W.J. (1993): Epidermal growth factor and transforming growth factor alpha regulate extracellular matrix production by embryonic mouse palatal mesenchymal cells cultured on a variety of substrata. Roux's Arch. Dev. Biol. 203:140-150.

Dixon, M.J., Garner, J., and Ferguson M.W.J. (1991): Immunolocalization of epidermal growth factor (EGF), EGF receptor and transforming growth factor alpha (TGF-α) during murine palatogenesis *in vivo* and *in vitro*. Anat. Embryol. 184:83-91.

Dolbeare, F. (1995): Bromodeoxyuridine: a diagnostic tool in biology and medicine, Part I: Historic perspectives, histochemical methods and cell kinetics. Histochem. J. 27:339-369.

Dolle, P., Ruberte, E., Leroy, P., Morriss-Kay, G., and Chambon, P. (1990): Retinoic acid receptors and cellular retinoid binding proteins. I. A systematic study of their differential pattern of transcription during mouse organogenesis. Development 110:1133-1151.

Drake, C.J., Davis, L.A., Walters, L., Little, C.D. (1990): Avian vasculogenesis and the distribution of collagens I, IV, laminin, and fibronectin in the heart primordia. J. Exp. Zool. 255:309-322.

Du, Y., Morris-Wiman, J., Da Silveira, A., and Brinkley, L. (1996): Occurrence of TIMPs during palatal morphogenesis *in vivo* and *in vitro*. J. Dent. Res. 75:225.

Duband, J.L., and Thiery, J.P. (1982a): Distribution of fibronectin in the early phase of avian cephalic neural crest cell migration. Dev. Biol. 93:308-323.

Duband, J.L., and Thiery, J.P. (1982b): Appearance and distribution of fibronectin during chick embryo gastrulation and neurulation. Dev. Biol. 94:337-350.

Duband, J.L., and Thiery, J.P. (1987): Distribution of laminin and collagens during avian neural crest development. Development 101:461-478.

Durham, P.L., and Snyder, J.M. (1995): Characterization of α1, β1, and γ1 laminin subunits during rabbit fetal lung development. Dev. Dynamics 203:408-421.

Dziadek, M., and Timpl, R. (1985): Expression of nidogen and laminin in basement membranes during mouse embryogenesis and in teratocarcinoma cells. Dev. Biol. 111:372-382.

Ede, D.A., Flint, O.P., and Teague, P. (1975): Cell proliferation in the developing wing-bud of normal and talpid mutant chick embryos. J. Embryol. Exp. Morphol. 34(3): 587-607.

Edwards, D.R., Heath, J.K., Hogan, B.L.M., Nomura, S., and Wills, A.J. (1992): Expression of TIMP in fetal and adult mouse tissues studied by *in situ* hybridization. In: Birkedal-Hansen, H., Werb, Z., Welgus, H.G., and Van Wart, H.E. (eds.) "Matrix Metalloproteinases and Inhibitors." Matrix. Spec. Suppl. No1. Gustav Fischer, Stuttgart. pp.286-293.

Edwards, D.R., Murphy, G., Reynolds, J.J., Whitman, S.E., Docherty, A.J.P., Angel, P., and Heath, J.K. (1987): Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. EMBO J. 6:1899-1904.

Ekblom, M., Klein, G., Mugrauer, G., Fecker, L., Deutzmann, R., Timpl, R., and Ekblom, P. (1990): Transient and locally restricted expression of laminin A chain mRNA by developing epithelial cells during kidney organogenesis. Cell 60:337-346.

Ekblom, P. (1981): Formation of basement membranes in the embryonic kidney: an immunohistological study. J. Cell Biol. 91:1-10.

Ekblom, P. (1993): Basement membranes in development. In: Rohrbach, D.H. and R. Timpl, R. (eds.) "Molecular and Cellular Aspects of Basement Membranes." Academic Press, Inc., San Diego. pp.359-383.

Elsasser, H.P., Biederbick, A., and Kern, H.F. (1994): Growth of rat pancreatic acinar cells quantitated with a monoclonal antibody against the proliferating cell nuclear antigen. Cell Tissue Res. 276:603-609.

Farrall, M., and Holder, S. (1992): Familial recurrence-pattern analysis of cleft lip with or without cleft palate. Am. J. Hum. Genet. 50:270-277.

Feng, H., Sassani, R., Bartlett, S.P., Lee, A., Hecht, J.T., Malcolm, S., Winter, R.M., Vintiner, G.M., Buetow, K.H., and Gasser, D.L. (1994): Evidence from family studies, for linkage disequilibrium between TGFA and a gene for nonsyndromic cleft lip with or without cleft palate. Am. J. Hum. Genet. 55:932-936.

Ferguson, M.W.J. (1988): Palate formation. Development 103(Suppl):41-60.

Ferguson, M.W.J., Sharpe, P.M., Thomas, B.L., and Beck, F. (1992): Differential expression of insulin-like growth factors I and II (IGF-I and II) mRNA, peptide and binding protein I during mouse palate development: comparison with TGF-β peptide distribution. J. Anat. 181:219-238.

Fessler, L.I., Duncan, K.G., Fessler, J.H., Salo, T., and Tryggvason, K. (1984): Characterization of procollagen IV cleavage products by a specific tumor collagenase. J. Biol. Chem. 259:9783-9789.

Filogamo, G., Corvetti, G., and Sisto Daneo, L. (1990): Differentiation of cardiac conducting cells from the neural crest cells. J. Autonomic Nervous System 30(Suppl):S55-57.

Fisher, C., Gilberton-Beadling, S., Powers, E.A., Petzold, G., Poorman, R., and Mitchell, M.A. (1994): Interstitial collagenase in required for angiogenesis *in vitro*. Dev. Biol. 162:499-510.

Fitch, J.M., and Linsenmayer, T.F. (1994): Interstitial basement membrane components in development. In: Yurchenco, P.D., Birk, D.E., and Mecham, R.P. (eds.) "Extracellular Matrix Assembly and Structure." Academic Press, Inc., San Diego. pp.441-462.

Fitzpatrick, D.R., Denhez, F., Kondaiah, P., and Akhurst, R.J. (1990): Differential expression of TGF beta isoforms in murine palatogenesis. Development 109:585-595.

Flenniken, A.M., and Williams, B.R.G. (1990): Developmental expression of the endogenous TIMP gene and a TIMP-lacZ fusion gene in transgenic mice. Genes Dev. 4:1094-1106.

Form, D.M., Pratt, B.M., and Madri, J.A. (1986): Endothelial cell proliferation during angiogeneis. *In vitro* modulation by basement membrane components. Lab. Invest. 55:521-530.

Fowler, K.J., Walker, F., Alexander, W., Hibbs, M.L., Nice, E.C., Bohmer, R.M., Mann, G.B., Thumwood, S., Maglitto, R., Danks, J.A., Chetty, R., Burgess, A.W., and Dunn, A.R. (1995): A mutation in the epidermal growth factor receptor in waved-2 mice has a profound effect on receptor biochemistry that results in impaired lactation. Proc. Natl. Acad. Sci. U.S.A. 92:1465-1469.

Francis-West, P.H., Tatla, T., and Brickell, P.M. (1994): Expression patterns of the bone morphogenetic protein genes *Bmp-4* and *Bmp-2* in the developing chick face suggest a role in outgrowth of the primordia. Dev. Dynamics 201:168-178.

Frommer, J., and Margolies, M.R. (1971): Contribution of Meckel's cartilage to ossification of the mandible in mice. J. Dent. Res. 50:1260-1267.

Fukiishi, Y., and Morriss-Kay, G.M. (1992): Migration of cranial neural crest cells to the pharyngeal arches and heart in rat embryos. Cell Tissue Res. 268:1-8.

Fukuda, Y., Masuda, Y., Kishi, J., Hashimoto, Y., Hayakawa, T., Nogawa, H., and Nahanishi, Y. (1988): The role of intersitial collagens in cleft formation of mouse embryonic submandibular gland during initial branching. Development 103:259-267.

Furthmayr, H. (1993): Basement membrane collagen: Structure, assembly, and biosynthesis. In: Zern, M.A., and Reid, L.M. (eds.) "Extracellular Matrix." Marcel Dekker, Inc., New York. pp.149-185.

Galloway, W.A., Murphy, G., Sandy, J.D., Gavrilovic, J., Cawston, T.E., and Reynolds, J.J. (1988): Purification and characterization of a rabbit bone metalloproteinase that degrades proteoglycan and other connective-tissue components. Biochem. J. 209:741-752.

Ganser, G.L., Stricklin, G.P., and Matrisian, L.M. (1991): EGF and TGF-α influence *in vitro* lung development by the induction of matrix-degrading metalloproteinases. Int. J. Dev. Biol. 35:1-8.

Garcia-Martinez, V., and Schoenwolf, G.C. (1993): Primitive-streak origin of the cardiovascular system in avian embryos. Dev. Biol. 159:706-719.

Gaunt, S.J., Blum, M., and De Robertis, E.M. (1993): Expression of the mouse *goosecoid* gene during mid-embryogenesis may mark mesenchymal cell lineages in the developing head, limbs and body wall. Development 117:769-778.

Gavin, B.J., McMahon, A.J., and McMahon, A.P. (1990): Expression of multiple novel Wnt-1/int-1-related genes during fetal and adult mouse development. Genes Dev. 4:2319-2332.

George, E.L., Georges-Labouesse, E.N., Patel-King, R.S., Rayburn, H., and Hynes, R.O. (1993): Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. Development 119:1079-1091.

Gessner, I.H., Lorincz, A.E., and Bostrom, H. (1965): Acid mucopolysaccharide content of the cardiac jelly of the chick embryo. J. Exp. Zool. 160:291-298.

Giudice, G.J., and Sternberg, M.S. (1981): A direct role for fibronectin in early heart development. J. Cell Biol. 91:151a.

Glass, R.H., Aggeler, J., Spindle, A., Pedersen, R.A., and Werb, Z. (1983): Degradation of extracellular matrix by mouse trophoblast outgrowths: A model for implantation. J. Cell Biol. 96:1108-1116.

Gonchoroff, N.J., Katzmann, J.A., Currie, R.M., Evans, E.L., Houck, D.W., Kline, B.C., Greipp, P.R., and Loken, M.R. (1986): S-phase detection with an antibody to bromodeoxyuridine. J. Immunol. Methods 93:97-101.

Gorza, L., Schiaffino, S., and Vitadello, M. (1988): Heart conduction system: a neural crest derivative? Brain Res. 457:360-366.

Goulding, M.D., Chalepakis, G., Deutsch, U., Erselius, J.R., and Cruss, R. (1991): Pax-3, a novel murine DNA binding protein expressed during early neurogenesis. EMBO J. 10:1135-1147.

Grant, D.S., Kleinman, H.K., and Martin, G.R. (1990): The role of basement membranes in vascular development. Ann. N. Y. Acad. Sci. 588:61-72.

Grant, M., Cutts, N.R., and Brody, J.S. (1983): Alterations in lung basement membrane during fetal growth and type 2 cell development. Dev. Biol. 97:173-183.

Gratzner, H.G. (1982): Monoclonal antibody to 5-Bromo- and 5-Iododeoxyuridine: A new reagent for detection of DNA replication. Science 218:474-475.

Greene, R.M., and Pratt, R.M. (1976): Developmental aspects of secondary palate formation. J Embryol Exp Morphol 36:225-245.

Gregory, H. (1975): Isolation and structure of urogastrone and its relationship to epidermal growth factor. Nature 257:325-327.

Griffith, C.M., and Hay, E.D. (1992): Epithelial-mesenchymal transformation during palatal fusion: carboxyfluorescein traces cells at light and electron microscopic levels. Development 116:1087-1099.

Grindley, J.C., Davidson, D.R., and Hill, R.E. (1995): The role of *Pax-6* in eye and nasal development. Development 121:1433-1442.

Grobstein, C. (1954): Tissue interaction in the morphogenesis of mouse embryonic rudiments *in vitro*. In: Rudnik, O. (ed.) "Aspects of Synthesis and order in Growth." Princeton University Press, Princeton. pp.233-256.

Gross J., and Bruschi, A.B. (1971): The pattern of collagen degradation in cultured tadpole tissues. Dev. Biol. 26:36-41.

Guerin, C.W., and Holland, P.C. (1995): Synthesis and secretion of matrix-degrading metalloproteinases by human skeletal muscle satellite cells. Dev. Dynamics 202:91-99.

Gui, T., Osumi-Yamashita, N., and Eto, K. (1993): Proliferation of nasal epithelial and mesenchymal cells during primary palate formation. J. Craniofac. Genet. Dev. Biol. 13:250-258.

Gullick, W.J., Marsden, J.J., Whittle, N., Ward, B., Bobrow, L., and Waterfield, M.D. (1986): Expression of epidermal growth factor receptors on human cervical, ovarian, and vulva carcinomas. Cancer Res. 46:285-292.

Gunja-Smith, Z., Morales, A.R., Romanelli, R., and Woessner, J.F. Jr. (1996): Remodeling of human myocardial collagen in idiopathic dilated cardiomyopathy. 148:1639-1648.

Guo, X., Johnson, J.J., and Kramer, J.M. (1991): Embryonic lethality caused by mutations in basement membrane collagen of *C. elegans*. Nature 349:707-709.

Hall, P.A., Levinson, D.A., Woods, A.L., Yu, C.C.W., Kellock, D.B., Watkins, J.A., Barnes, D.M., Gillett, C.E., Camplejohn, R., Dover, R., Waseem, J.A., and Lane, D.P. (1990): Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: and index of cell proliferation with evidence of deregulated expression in some neoplasms. J. Pathol. 162:285-294.

Harvey, M.B., Leco, K.L., Arcellana-Panlilio, M.Y., Zhang, X., Edwards, D.R., and Schultz, G.A. (1995): Proteinase expression in early mouse embryos is regulated by leukemia inhibitory factor and epidermal growth factor. Development 121:1005-1014.

Hay, E.D. (1991): Collagen and other matrix glycoproteins in embryogenesis. In: Hay, E.D. (ed.) "Cell Biology of Extracellular Matrix." Plenum Press, New York. pp. 419-462.

Hay, D.A., Markwald, R.R., and Fitzharris, T.P. (1984): Selected views of early heart development by scanning electron microscopy. In: Johari, O.M. (ed.) "Scanning Electron Microscopy." SEM Inc., Illinois. Vol. IV, pp.1983-1993.

Hayakawa, T. (1994): Tissue inhibitors of metalloproteinases and their cell growth-promoting activity. Cell Structure Function 19:109-114.

Hayakawa, T., Kishi, J.-I., and Nakanishi, Y. (1992): Salivary gland morphogenesis: possible involvement of collagenase. In: Birkedal-Hansen, H., Werb, Z., Welgus, H.G., and Van Wart, H.E. (eds.) "Matrix Metalloproteinases and Inhibitors." Matrix. Spec. Suppl. No1. Gustav Fischer, Stuttgart. pp.344-351.

Hayakawa, T., Yamashita, K., Ohuchi, E., and Shinagawa, A. (1994): Cell growth-promoting activity of tissue inhibitor of metalloproteinase-2 (TIMP-2). J. Cell Science 107:2373-2379.

Heath, J.K. (1993): Growth Factors. Oxford University Press, Oxford. pp.19-22.

Heikinheimo, K. (1993): Cell growth and differentiation of developing and neoplastic odontogenic tissues. Ph.D. Dissertation, University of Turku, Finland.

Heikinheimo, K., and Salo, T. (1995): Expression of basement membrane type IV collagen and type IV collagenases (MMP-2 and MMP-9) in human fetal teeth. J. Dent. Res. 74:1226-1234.

Heikinheimo, K., Voutilainen, R., Happonen, R.-P., and Miettinen, P.J. (1993): EGF receptor and its ligands, EGF and TGF-α, in developing and neoplastic human odontogenic tissues. Int. J. Dev. Biol. 37:387-396.

Heikinheimo, M., Lawshe, A., Shackleford, G.M., Wilson, D.B., and MacArthur, C.A. (1994): *Fgf-8* expression in the post-gastrulation mouse is localized to the developing face, limbs, and central nervous system. Mech. Dev. 48:129-138.

Heine, U.I., Munoz, E.F., Flanders, K.C., Ellingsworth, L.R., Lam, H.-Y.P., Thompson, N.L., Roberts, A.B., and Sporn, M.B. (1987): Role of transforming growth factor in the development of the mouse embryo. J. Cell Biol. 105:2861-2876.

Hernandez-Sotomayor, T.S.M., and Carpenter, G. (1992): Epidermal growth factor receptor: elements of intracellular communication. J. Membrane Biol. 128:81-89.

Hilfer, S.R., and Randolph, G.J. (1993): Immunolocalization of basal lamina components during development of chick otic and optic primordia. Anat. Rec. 235:443-452.

Hiraoka, K., Sasaguri, Y., Komiya, S., Inoue, A., and Morimatsu, M. (1992): Cell proliferation-related production of matrix metalloproteinase-1 (tissue collagenase) and -3 (stromelysin) by cultured human rheumatoid synovial fibroblasts. Biochem. Int. 27:1083-1091.

Holder, S.E., Vintiner, G.M., Farren, G., Malcolm, S., and Winter, R.M. (1992): Confirmation of an association between RFLPs at the transforming growth factor-alpha locus and nonsyndromic cleft lip and palate. J. Med. Genet. 29:390-392.

Hopper, A.F., and Hart, N.H. (1980): Foundations of Animal Development. Oxford University Press, Oxford.

Hormi, K., and Lehy, T. (1994): Developmental expression of transforming growth factor-a and epidermal growth factor receptor proteins in the human pancreas and digestive tract. Cell Tissue Res. 278:439-450.

Howard, E.W., Bullen, E.C., and Banda, M.J. (1991): Preferential inhibition of 72- and 92-kDa gelatinases by tissue inhibitor of metalloproteinases-2. J. Biol. Chem. 266:13070-13075.

Hsu, S.M., Raine, L., and Fanger, H. (1981): The use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase technique: A comparison between ABC and unlabelled antibody (PAP) procedures. J. Histochem. Cytochem. 29:577-580.

Hudson, B.G., Reeders, S.T., and Tryggvason, K. (1993): Type IV collagen: Structure, gene organization, and role in human diseases. J. Biol. Chem. 268:26033-26036.

Hudson, C.D., and Shapiro B.L. (1973): An autoradiographic study of deoxyribonucleic acid synthesis in embryonic rat palatal shelf epithelium with reference to the concept of programmed cell death. Archs. Oral Biol. 18:77-84.

Huhtala, P., Chow, L.T., and Tryggvason, K. (1990): Structure of the human type IV collagenase gene. J. Biol. Chem. 265:11077-11082.

Hurle, J.M., Kitten, G.T., Sakai, L.Y., Volpin, D., and Solursh, M. (1994): Elastic extracellular matrix of the embryonic chick heart: an immunohistological study using laser confocal microscopy. Dev. Dynamics 200:321-332.

Hurmerinta, K., and Thesleff, I. (1981): Ultrastructure of the epithelio-mesenchymal interface of the mouse tooth germ. J. Craniofac. Genet. Dev. Biol. 1:191-202.

Iamaroon, A., and Diewert V.M. (1996): Distribution of basement membrane components in the mouse primary palate. J. Craniofac. Genet. Dev. Biol. 16:48-51.

Iamaroon, A., Tait, B., and Diewert, V.M. (1996a): Cell proliferation and expression of EGF, TGF-α, and EGF-R in the developing primary palate. J. Dent. Res. (in press).

Iamaroon, A., Wallon, U.M., Overall, C.M., and Diewert, V.M. (1996b): Expression of 72-kDa gelatinase (matrix metalloproteinase 2) in the developing craniofacial complex of the mouse embryo. Arch. Oral Biol. (in press).

Iamaroon, A., Wallon, U.M., Overall, C.M., and Diewert, V.M. (1996c): Immunolocalization of 72-kDa gelatinase and extracellular matrix components during mouse cardiac development. J. Anat. (in revision).

Icardo, J.M., and Manasek, F.J. (1983): Fibronectin distribution during early chick embryo heart development. Dev. Biol. 95:19-30.

Icardo, J.M., and Manasek, F.J. (1984): An indirect immunofluorescence study of the distribution of fibronectin during the formation of the cushion tissue mesenchyme in the embryonic heart. Dev. Biol. 101:336-345.

Ikawa, H., Trelstad, R.L., Hutson, J.M., Manganaro, T.F., and Donahoe, P.K. (1984): Changing patterns of fibronectin, laminin, type IV collagen, and a basement membrane proteoglycan during rat Mullerian duct regression. Dev. Biol. 102:260-263.

Iruela-Arispe, M.L., and Sage, E.H. (1991): Expression of type VIII collagen during morphogenesis of the chicken and mouse heart. Dev. Biol. 144:107-118.

Jacobson, A.G. (1963a): The determination and positioning of the nose, lens and ear. I. Interactions within the ectoderm, and between the ectoderm and underlying tissues. J. Exp. Zool. 154:273-283.

Jacobson, A.G. (1963b): The determination and positioning of the nose, lens and ear. II. The role of the endoderm. J. Exp. Zool. 154:283-291.

Jaskoll, T.F., and Slavkin, H.C. (1984): Ultrastructural and immunofluorescence studies of basal-lamina alterations during mouse lung morphogenesis. Differentiation 28:36-48.

Jaskulski, D., Gatti, C., Travali, S., Calabretta, B., and Baserga, R. (1988): Regulation of the proliferating cell nuclear antigen cyclin and thymidine kinase mRNA levels by growth factors. J. Biol. Chem. 263:10175-10179.

Jhappan, C., Stahle, C., Harkins, R.N., Fausto, N., Smith, G.H., and Merlino, G.T. (1990): TGFα overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. Cell 61:1137-1146.

Johnson, S.E., Rothstein, J.L., and Knowles, B.B. (1994): Expression of epidermal growth factor family gene members in early mouse development. Dev. Dynamics 201:216-226.

Johnston, M.C. (1966): A radiographic study of the migration and fate of the craniofacial neural crest cells in the chick embryo. Anat. Rec. 156:143-155.

Johnston, M.C., and Bronsky, P.T. (1995): Prenatal craniofacial development: new insights on normal and abnormal mechanisms. Crit. Rev. Oral Biol. Med. 6:25-79.

Jones, C.M., Lyons, K.M., and Hogan, B.L.M. (1991): Involvement of *bone morphogenetic protein-4* (BMP-4) and *Vgl-1* in morphogenesis and neurogenesis in the mouse. Development 111:531-542.

Jones, S.E., Jomary, C., and Neal, M.J. (1994): Expression of TIMP3 mRNA is elevated in retinas affected by simplex retinitis pigmentosa. FEBS 352:171-174.

Joyner, A.L. (1991): Gene targeting and gene trap screens using embryonic stem cells: new approaches to mammalian development. BioAssays 13:649-656.

Ju, W.D., Velu, T.J., Vass, W.C., Papageorge, A.G., and Lowy, D.R. (1991): Tumorigenic transformation of NIH 3T3 cells by the autocrine synthesis of transforming growth factor a. New Biologist 3:380-388.

Juriloff, D.M. (1995): Genetic analysis of the construction of the AEJ. A congenic strain indicates that nonsyndromic CL(P) in the mouse is causes by two loci with epistatic interaction. J. Craniofac. Genet. Dev. Biol. 15:1-12.

Kaartinen, V., Voncken, J.W., Shuler, C., Warburton, D., Bu, D., Heisterkamp, N., and Groffen, J. (1995): Abnormal lung development and cleft palate in mice lacking TGF-β3 indicates defects of epithelial-mesenchymal interaction. Nature Genetics 11:415-421.

Kalebic, T., Garbisa, S., Glaser, B., and Liotta, L.A. (1983): Basement membrane collagen: degradation by migrating endothelial cells. Science 221:281-283.

Karkinen-Jaaskelainen, M. (1978): Permissive and directive interactions in lens induction. J. Embryol. Exp. Morphol. 44:167-179.

Kasselberg, A.G., Orth, D., Gray, M.E. and Stahlman, M.T. (1985): Immunohistochemical localization of human epidermal growth factor/urogastrone in several human tissues. J. Histochem. Cytochem. 33:315-322.

Kaufman, M.H. (1992): The atlas of mouse development. Academic Press, Harcourt Brace Jovanovich, Publishers: San Diego, CA pp.75-158.

Kaufman, M.H., and Navaratnam, V. (1981): Early differentiation of the heart in mouse embryos. J. Anat. 133:235-246.

Kerr, L.D., Miller, D.B., and Matrisian, L.M. (1990): TGF-β1 inhibition of transin/stromelysin gene expression is mediated through a fos-binding sequence. Cell 61:267-278.

Kerr, L.D., Holt, J.T., and Matrisian, L.M. (1988): Growth factors regulate transin gene expression by c-fos-dependent and c-fos-independent pathways. Science 242:1424-1427.

Kitten, G.T., Markwald, R.R., and Bolender, D.L. (1987): Distribution of basement membrane antigens in cryopreserved early embryonic hearts. Anat. Rec. 217:370-390.

Klein, G., Ekblom, M., Fecker, L., Timpl, R., and Ekblom, P. (1990): Differential expression of laminin A and B chains during development of embryonic mouse organs. Development 110:823-837.

Kleinman, H.K., Graf, J., Iwamoto, Y., Kitten, G.T., Ogle, R.C., Sasaki, M., Yamada, Y., Martin, G.R., and Luckenbill-Edds, L. (1987): Role of basement membranes in cell differentiation. Annal. NY. Acad. Sci. 513:134-145.

Kleinman, H.K., Weeks, B.S., Schnaper, H.W., Kibbey, M.C., Yamamura, K., and Grant, D.S. (1993): Tha laminins: a family of basement membrane glycoproteins important in cell differentiation and tumor metastases. Vitam. Horm. 47:161-186.

Kobayashi, Y. (1958): A genetic study of harelip and cleft palate. Jpn. J. Hum. Genet. 3:73-107.

Kondo, S., Hozumi, Y., Maejima, H., and Aso, K. (1992): Organ culture of psoriatic skin: effect of TGF-α and TGF-β on epidermal structure *in vitro*. Arch. Dermatol. Res. 284:150-153.

Koopman, P. (1993): Analysis of gene expression by reverse transcriptase-polymerase chain reaction. In: Stern, C.D., and Holland, P.W.H. (eds.) "Essential Developmental Biology." Oxford University Press, Oxford. pp.233-241.

Kosher, R.A., and Solursh, M. (1989): Widespread distribution of type II collagen during embryonic chick development. Dev. Biol. 131:558-566.

Kronmiller, J.E. (1995): Spatial distribution of epidermal growth-factor transcripts and effects of exogenous epidermal growth factors on the pattern of the mouse dental lamina. Arch. Oral Biol. 40:137-143.

Kronmiller, J.E., Upholt, W.B., and Kollar, E.J. (1991a): Expression of epidermal growth factor mRNA in the developing mouse mandibular process. Archs. Oral Biol. 36:405-410.

Kronmiller, J.E., Upholt, W.B., and Kollar, E.J. (1991b): Antisense EGF oligonucleotides inhibit odontogenesis in embryonic mouse mandible *in vitro*. Dev. Biol. 147:484-488.

Kronmiller, J.E., Upholt, W.B., and Kollar, E.J. (1993): Effects of retinol on the temporal expression of transforming growth factor-alpha mRNA in the embryonic mouse mandible. Arch. Oral Biol. 38:185-188.

Kurki, P., Vanderlaan, M., Dolbeare, F., Gray, J., and Tan, E.M. (1986): Expression of proliferating cell nuclear antigen (PCNA)/cyclin during the cell cycle. Exp. Cell Res. 166:209-219.

Kumar, V., Bustin, S.A., and McKay, I.A. (1995): Transforming growth factor alpha. Cell Biol. Int. 19:373-388.

Kwee, L., Baldwin, H.S., Shen, H.M., Stewart, C.L., Buck, C.A., and Labow, M.A. (1995): Defective development of the embryonic and extraembryonic circulatory systems in vascular cell adhesion (VCAM-1) deficient mice. Development 121:489-503.

Lammer, E.J., and Opitz, J.M. (1986): The DiGeorge anomaly as a developmental field defect. Am. J. Med. Genet. 2(Suppl):113-127.

de Larco, J.E., and Todaro, G.J. (1978): Growth factor from murine sarcoma virus-transformed cells. Proc. Natl. Acad. Sci. U.S.A. 75:4001-4005.

Lee, D.C. (1985): Cloning and sequence analysis of a cDNA for rat transforming growth factor-α. Nature 313:489-491.

Lee, D.C., and Hahn, K.M. (1991): Expression of growth factors and their receptors in development. In: Sporn, M.B. and Roberts, A.B. (eds.) "Peptide Growth Factors and Their Receptors II." Springer-Verlag, New York. pp.611-654.

Lefebvre, O., Regnier, C., Chenard, M.-P., Wendling, C., Chambon, P., Basset, P., and Rio, M.-C. (1995): Developmental expression of mouse stromelysin-3 mRNA. Development 121:947-955.

Leibovici, M., Monod, G., Geraudie, J., Bravo, R., and Mechali, M. (1992): Nuclear distribution of PCNA during embryonic development in Xenopus laevis: a reinvestigation of early cell cycles. J. Cell Sci. 102:63-69.

Leivo, I., Vaheri, A., Timpl, R., and Wartiovaara, J. (1980): Appearance and distribution of collagens and laminin in the early mouse embryo. Dev. Biol. 76:100-114.

Le Lievre, C., and Le Douarin, N.M. (1975): Mesenchymal derivatives of the neural crest: Analysis of chimeric quail and chick embryos. J. Embryol. Exp. Morphol. 34:125-154.

Lesot, H., Kubler, M.-D., Fausser, J.L., and Ruch, J.-V. (1990): A 165 kDa membrane antigen mediating fibronectin-vinculin interaction is involved in murine odontoblast differentiation. Differentiation 44:25-35.

Levy, A.T., Cioce, V., Sobel, M.E., Garbisa, S., Grigioni, W.F., Liotta, L.A., Stetler-Stevensen, W.G. (1991): Increased expression of the Mr 72,000 type IV collagenase in human colonic adenocarcinoma. Can. Res. 51:439-444.

Lewis, W.H. (1907): Lens formation from strange ectoderm in *Rana sylvatica*. Am. J. Anat. 7:145-169.

Libson, A.M., Gittis, A.G., Collier, I.E., Marmer, B.L., Goldberg, G.I., and Lattman, E.E. (1995): Crystal structure of the haemopexin-like C-terminal domain of gelatinase A. Nature Struct. Biol. 2:938-942.

Linask, K.K., and Lash, J.W. (1988): A role for fibronectin in the migration of avian precardiac cells. I. Dose-dependent effects of fibronectin antibody. Dev. Biol. 129:315-323.

Liotta, L.A., Abe, S., Gehron Robey, P., and Martin, G.R. (1979): Prerential digestion of basement membrane collagen by an enzyme derived from a metastatic murine tumor. Proc. Natl. Acad. Sci. USA 76:2268-2272.

Liotta, L.A., Rao, C.N., and Wewer, U.M. (1986): Biochemical interactions of tumor cells with the basement membrane. Ann. Rev. Biochem. 55:1037-1057.

Liotta, L.A., Tryggvason, K., Garbisa, S., Hart, I., Foltz, C.M., and Shafie, S. (1980): Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature 284:67-68.

Little, C.D., Piquet, D.M., Davis, L.A., Walters, L., and Drake, C.J. (1989): The distribution of laminin, collagen type IV, collagen type I and fibronectin in the cardiac jelly-basement membrane. Anat. Rec. 224:417-425.

Litvin, J., Montgomery, M., Gonzalez-Sanchez, A., Bisaha, J.G., and Bader, D. (1992): Commitment and differentiation of cardiac myocytes. Trends Cardiovas. Med. 2:27-32.

Lowry, R.B., and Renwick, D.H.G. (1969): Incidence of cleft lip and cleft palate in British Columbia Indians. J. Med. Genet. 6:67-69.

Luetteke, N.C., Qiu, T.H., Peiffer, R.L., Oliver, P., Smithies, O., and Lee, D. (1993): TGF-α deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. Cell 73:263-278.

Lumsden, A., Sprawson, N., and Graham, A. (1991): Segmental origin and migration of neural crest cells in the hindbrain region of the chick embryo. Development 113:1281-1292.

Lyons, J.G., Birkedal-Hansen, B., Pierson, M.C., Whitelock, J.M., and Birkedal-Hansen, H. (1993): Interleukin-1 beta and transforming growth factor-alpha/epidermal growth factor induce expression of M(r) 95,000 type IV collagenase/gelatinase and interstitial fibroblast-type collagenase by rat mucosal keratinocytes. J. Biol. Chem. 268:19143-19151.

Lyons, K.M., Pelton, R.W., and Hogan, B. (1990): Organogenesis and pattern formation in the mouse: RNA distribution patterns suggest a role for bone morphogenetic protein-2A (BMP-2A). Development 109:833-844.

MacArthur, C.A., Lawshe, A., Xu, J., Santos-Ocampo, S., Heikinheimo, M., Chellaiah, A.T., and Ornitz, D.M. (1995): FGF-8 isoforms activate receptor splice forms that are expressed in mesenchymal regions of mouse development. Development 121:3603-3613.

Machida, C.M., Scott, J.D., and Ciment, G. (1991): NGF-induction of the metalloproteinase transin/stromelysin in PC12 cells: involvement of multiple protein kinases. J. Cell Biol. 114:1037-1048.

MacKenzie, A., Ferguson, M.W.J., and Sharpe, P.T. (1992): Expression patterns of the homeobox gene, *Hox*-8, in the mouse embryo suggest a role in specifying tooth initiation and shape. Development 115:403-420.

MacNaul, K.L., Chartrain, N., Lark, M., Tocci, M.J., and Hutchinson, N.I. (1990): Discoordinate expression of stromelysin, collagenase and tissue inhibitor of metalloproteinase-1 in rheumatoid human synovial fibroblasts. Synergistic effects of interleukin-1 and tumor necrosis factor-α on stromelysin expression. J. Biol. Chem. 265:17238-17245.

Maden, M., Hunt, P., Eriksson, U., Kuroiwa, A., Krumlauf, R., and Summerbell, D. (1991): Retinoic acid-binding protein, rhombomeres and the neural crest. Development 111:35-44.

Manasek, F.J. (1976): Heart Development: Interactions involved in cardiac morphogenesis. In: Poste, G. and Nicholson, G.L. (eds.) "The Cell Surface in Animal Embryogenesis and Development." Elsevier, North Holland, Amsterdam. pp.545-598.

Manasek, F.J., Reid, M., Vinson, W., Seyer, J., and Johnson, R. (1973): Glycosaminoglycan synthesis by the early embryonic chick. Dev. Biol. 35:332-348.

Mann, G.B., Fowler, K.J., Gabriel, A., Nice, E.C., Williams, R.L., and Dunn, A.S. (1993): Mice with a null mutation of the TGF-α gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. Cell 73:249-261.

Mann, I. (1964): The development of the human eye. Grune & Stratton, New York.

Markwald, R.R., Krug, E.L., Runyan, R.B., Kitten, G.T. (1984): Proteins in cardiac jelly which induce mesenchyme formation. In: Ferrans, V.J., Rosenquist, G., Weinstein, C. (eds.) "Cardiac Morphogenesis." Elsevier, New York. pp.60-68.

Marquardt, H., Hunkapillar, M.W., Hood, L.E., and Todaro, G.J. (1984): Rat transforming growth factor type 1: structure and relation to epidermal growth factor. Science 223:1079-1082.

Martin, G.R., and Sank, A.C. (1991): Extracellular matrices, cells, and growth factors. In: Sporn, M.B. and Roberts, A.B. (eds) "Peptide Gowth Factors and Their Receptors II." Springer-Verlag, Berlin. pp.463-477.

Massague, J. (1983): Epidermal growth factor-like transforming growth factor. II. Interaction with epidermal growth factor receptors in human placenta membranes and A431 cells. J. Biol. Chem. 258:13614-13620.

Massague, J. (1990): Transforming growth factor-α. J. Biol. Chem. 265:21393-21396.

Matrisian, L.M. (1990): Metalloproteinases and their inhibitors in matrix remodeling. Trend Genet. 6:121-125.

Matrisian, L.M. (1992): The matrix-degrading metalloproteinases. Bioassays 14:455-463.

Matsui, Y., Halter, S.A., Holt, J.T., Hogan, B.L.M., and Coffey, R.J. (1990): Development of mammary hyperplasia and neoplasia in MMTV-TGFα transgenic mice. Cell 61:1147-1155.

Mawatari, M., Kohno, K., Mizoguchi, H., Matsuda, T., Asoh, K., van Damme, J., Welgus, H.G., and Kuwano, M. (1989): Effects of tumor necrosis factor and epidermal growth factor on cell surface morphology, cell surface receptors, and the production of tissue inhibitor of metalloproteinases and IL-6 in human microvascular endothelial cells. J. Immunol. 143:1619-1627.

McAvoy, J.W. (1980): Induction of the eye lens. Differentiation 17:137-149.

McGuire, P.G., and Orkin, R.W. (1992): Urokinase activity in the developing avian heart: A spatial and temporal analysis. Dev. Biol. 193:24-33.

McKeehan M.S. (1951): Cytological aspects of embryonic lens induction in the chick. J. Exp. Zool. 117:31-64.

Mercola, M., and Stiles, C.D. (1988): Growth factor superfamilies and mammalain embryogenesis. Development 102:451-460.

Miettinen, P.J. (1993): Transforming growth factor-alpha and epidermal growth factor expression in human fetal gastrointestinal tract. Ped. Res. 33:481-486.

Miettinen, P.J., and Heikinheimo, K. (1992): Transforming growth factor-alpha (TGF-α) and insulin gene expression in human fetal pancreas. Development 114:833-840.

Miettinen, P.J., Perheentupa, J., Otonkoski, T., Lahteenmaki, A., and Panula, P. (1989): EGF- and TGF-α-like peptides in human fetal gut. Ped. Res. 26:25-30.

Miller, K., and Rizzino, A. (1994): Developmental regulation and signal transduction pathways of fibroblast growth factors and their receptors. In: Nilsen-Hamilton, M. (ed.) "Growth Factors and Signal Transduction in Development." Wiley-Liss, Inc., New York. pp.19-49.

Millicovsky, G., and Johnston, M.C. (1981): Active role of embryonic facial epithelium: New evidence of cellular events in morphogenesis. J. Embryol. Exp. Morphol. 63:53-66.

Miner, J.H., and Sanes, J.R. (1994): Collagen IV α3, α4, and α5 chains in rodent basal laminae: sequence, distribution, association with laminins, and developmental switches. J. Cell Biol. 127:879-891.

Minkoff, R. (1980a): Regional variation of cell proliferation within the facial processes of the chick embryo: a study of the role of 'merging' during development. J. Embryol. Exp. Morphol. 57:37-49.

Minkoff, R. (1980b): Cell proliferation and migration during primary palate development. In: Pratt, R.M. and Christainsen, R.L. (eds.) "Current Research Trends in Prenatal Craniofacial Development." Elsevier, New York. pp.119-132.

Minkoff, R. (1991): Cell proliferation during formation of the embryonic facial primordia. J. Craniofac. Genet. Dev. Biol. 11:251-261.

Minkoff, R., and Kuntz, A.J. (1977): Cell proliferation during morphogenic change: analysis of frontonasal morphogenesis in the chick embryo employing DNA labelling indices. J. Embryol. Exp. Morphol. 40:101-113.

Minkoff, R., and Martin, R.E. (1984): Cell cycle analysis of facial mesenchyme in the chick embryo: II. Label dilution studies and developmental fate of slow cycling cells. J. Embryol. Exp. Morphol. 81:61-73.

Mitchell., L.E., and Risch, N. (1992): Mode of inheritance of nonsyndromic cleft lip with or without cleft palate: a reanalysis. Am. J. Hum. Genet. 51:323-332.

Mitchell, P.J., Timmons, P.M., Herbert, J.M., Rigby, P.W.J., and Tijan, R. (1991): Transcription factor AP-2 is expressed in neural crest cell lineages during mouse embryogenesis. Genes Dev. 5:105-119.

Mizuno, T. (1972): Lens differentiation *in vitro* in the absence of optic vesicle in the epiblast of chick blastoderm under the influence of skin dermis. J. Embryol. Exp. Morphol. 28:117-132.

Mjaatvedt, C.H., Lepera, R.C., and Markwald, R.R. (1987): Myocardial specificity for initiating endothelial-mesenchymal cell transition in embryonic chick heart correlates with a particulate distribution of fibronectin. Dev. Biol. 119:59-67.

Monteagudo, C., Merino, M.J., San-Juan, J., Liotta, L.A., and Stetler-Stevenson, W.G. (1990): Immunohistochemical distribution of type IV collagenase in normal, benign, and malignant breast tissue. Am. J. Pathol. 136:585-592.

Mori, C., Nakamura, N., Okamoto, Y., Osawa, M., and Shiota, K. (1994): Cytochemical identification of programmed cell death in the fusing fetal mouse palate by specific labelling of DNA fragmentation. Anat. Embryol. 190:21-28.

Morriss-Kay, G.M. (1993): Postimplantation mammalian embryos. In: Stern, C.D., and Holland, P.W.H. (eds.) "Essential Developmental Biology." Oxford University Press, Oxford. pp.55-66.

Morris-Wiman, J., and Brinkley, L. (1992): An extracellular matrix infrastructure provides support for murine secondary palatal shelf remodelling. Anat. Rec. 234:575-586.

Morris-Wiman, J., Du, Y., and Brinkley, L. (1996): Matrix-degrading enzymes during *in vitro* palatogenesis. J. Dent. Res. 75:225.

Muir, D. (1994): Metalloproteinase-dependent neurite outgrowth within a synthetic extracellular matrix is induced by nerve growth factor. Exp. Cell Res. 210:243-252.

Murphy, G., Hembry, R.M., McGarrity, A.M., and Reynolds, J.J. (1989): Gelatinase (type IV collagenase) immunolocalization in cells and tissues: use of an antiserum to rabbit bone gelatinase that identifies high and low M_r forms. J. Cell Sci. 92:487-495.

Nagase, H., Enghild, J.J., Suzuki, K., and Salvesen, G. (1990): Stepwise activation mechanisms of the precursor of matrix metalloproteinase 3 (stromelysin) by proteinases and (4-aminophenyl) mercuric acetate. Biochemistry 29:5783-5789.

Nagase, H., Ogata, Y., Suzuki, K., Enghild, J.J., and Salvesen, G. (1991): Substrate specificities and activation mechanisms of matrix metalloproteinases. Biochem. Soc. Trans. 19:715-718.

Nakagawa, M., Terracio, L., Carver, W., Birkedal-Hansen, H., and Borg, T.K. (1992): Expression of collagenase and IL-1α in developing rat hearts. Dev. Dynamics 195:87-99.

Nakanishi, Y., Sugiura, F., Kishi, J.-I., and Hayakawa, T. (1986): Collagenase inhibitor stimulates cleft formation during early morphogenesis of mouse salivary gland. Dev. Biol. 113:201-206.

Nanda, R., and Romeo, D. (1975): Differential cell proliferation of embryonic rat palatal processes as determined by incorporation of tritiated thymidine. Cleft Palate 12:436-443.

Neel, J.V. (1958): A study of major congenital defects in Japanese infants. Am. J. Hum. Genet. 10:398-445.

Nexo, E. and Kryger-Baggesen, N. (1989): The receptor for epidermal growth factor is present in human fetal kidney, liver and lung. Regul. Pept. 26:1-8.

Nichols, D.H. (1986): Formation and distribution of neural crest mesenchyme to the first pharyngeal arch region of the mouse embryo. Am. J. Anat. 176:221-231.

Nicholson, R., Murphy, G., and Breathnach, R. (1989): Human and rat malignant-tumor-associated mRNAs encode stromelysin-like metalloproteinases. Biochemistry 28:5195-5203.

Nishikawa, K., Nakanishi, T., Aoki, C., Hattori, T., Takahashi, K., and Taniguchi, S. (1994): Differential expression of homeo-containing genes *Msx*-1 and *Msx*-2 and homeoprotein Msx-2 expression during chick craniofacial development. Biochem. Mol. Biol. Int. 32:763-771.

Noden, D.M. (1988): Interactions and fates of avian craniofacial mesenchyme. Development 103: Suppl, 121-140.

Nomura, S., Hogan, B.L.M., Wills, A.J., Heath J.K., and Edwards, D.R. (1989): Developmental expression of tissue inhibitor of metalloproteinase (TIMP) RNA. Development 105:575-583.

O'Grady, R.L., Nethery, A., and Hunter, N. (1984): A fluorescent screening assay for collagenase using collagen labeled with L-methoxy-2,4-diphenyl-3(2H)furanone. Anal. Biochem. 140:490-494.

Ohuchi, H., Yoshioka, H., Tanaka, A., Kawakami, Y., Nohno, T., and Noji, S. (1994): Involvement of androgen-induced growth factor (Fgf-8) gene in mouse embryogenesis and morphogenesis. Biochem. Biophys. Res. Commun. 204:882-888.

Okada, Y., Morodomi, T., Enghild, J.J., Suzuki, K., Yasui, A., Nakanishi, I., Salvesen, G., and Nagase, H. (1990): Matrix metalloproteinase 2 from human rheumatoid synovial fibroblasts. Purification and activation of the precursor and enzymatic properties. Eur. J. Biochem. 194:721-730.

O'Rahilly, R., and Muller, F. (1987): Developmental Stages in Human Embryos. Carnegie Inst. Wash. Publ. 637: Carnegie Institute of Washington. pp.175-302.

Osumi-Yamashita, N., Ninomiya, Y., Doi, H., and Eto, K. (1994): The contribution of both forebrain and midbrain crest cells to the mesenchyme in the frontonasal mass of mouse embryos. Dev. Biol. 164:409-419.

Osumi-Yamashita, N., Noji, S., Nohno, T., Koyama, E., Doi, H., Eto, K., and Tanigushi, S. (1990): Expression of retinoic acid receptor genes in neural crest-derived cells during mouse facial development. FEBS 264:71-74.

Overall, C.M. (1994): Regulation of tissue inhibitor of matrix metalloproteinase expression. Ann. New York. Acad. Sci. 732:51-64.

Overall, C.M., and Limeback, H. (1988): Identification and characterization of enamel proteinases isolated from developing enamel. Biochem. J. 256:965-972.

Overall, C.M., and Sodek, J. (1990): Concanavalin-A produces a matrix-degradative phenotype in human fibroblasts. Induction/endogenous activation of collagenase, 72 kDa gelatinase, & Pump-1 is accompanied by suppression of tissue inhibitor of matrix metalloproteinases. J. Biol. Chem. 265:21141-21151.

Overall, C.M., Wrana, J.L., and Sodek, J. (1989): Independent regulation of collagenase, 72-kDa progelatinase, and metalloendoproteinase inhibitor expression in human fibroblasts by transforming growth factor-β. J. Biol. Chem. 264:1860-1869.

Overall, C.M., Wrana, J.L., and Sodek, J. (1991): Transcriptional and post-transcriptional regulation of 72-kDa gelatinase/type IV collagenase by transforming growth factor-β1 in human fibroblasts. J. Biol. Chem. 266:14064-14071.

Paria, B.C., Das, S.K., Huet-Hudson, Y.M., and Dey, S.K. (1994): Distribution of transforming growth factor alpha precursors in the mouse uterus during the preimplantation period and after steroid hormone treatments. Biol. Reprod. 50:481-491.

Parmigiani, C., and McAvoy, J. (1984): Localization of laminin and fibronectin during rat lens morphogenesis. Differentiation 28:53-61.

Partanen, A.M., Ekblom, P., and Thesleff, I. (1985): Epidermal growth factor inhibits morphogenesis and cell differentiation in cultured mouse embryonic teeth. Dev. Biol. 111:84-94.

Partanen, A.M. (1990): Epidermal growth factor and transforming growth factor-alpha in the development of epithelial-mesenchymal organs of the mouse. In: Nilsen-Hamilton, M. (ed.) "Growth Factors and Development." Academic Press, New York. pp.31-53.

Partanen, A.M., and Thesleff, I. (1987): Localization and quantitation of 125I-epidermal growth factor binding in mouse embryonic tooth and other embryonic tissues at different developmental stages. Dev. Biol. 120:186-197.

Paulsson, M. (1992): Basement membrane proteins: structure, assembly and cellular interactions. Crit. Rev. Biochem. Molec. Biol. 27:93-127.

Pellier, V., and Astic, L. (1994): Detection of apoptosis by electron microscopy and *in situ* labelling in the rat olfactory pit. NeuroReport 5:1429-1432.

Pelton, R.W., Hogan, B.L.M., Miller, D.A., and Moses, H.L. (1990): Differential expression of genes encoding TGFs β1, β2, and β3 during murine palate formation. Dev. Biol. 141:456-460.

Pendleton, N., Dixon, G.R., Burnett, H.E., Occleston, N.L., Myskow, M.W., and Green, J.A. (1993): Expression of proliferating cell nuclear antigen (PCNA) in dysplasia of the bronchial epithelium. J. Pathol. 170:169-172.

Peterson , P.E., Pow, C.S.T., Wilson, D.B., and Hendrickx, A.G. (1995): Localization of glycoproteins and glycosaminoglycans during early eye development in the macaque. J. Anat. 186:31-42.

Pexieder, T., and Janecek, P. (1984): Organogenesis of the human embryonic and early fetal heart as studied by microdissection and SEM. In: Nora, J.J. and Takao, A. (eds.) "Congenital Heart Disease: Causes and Processes." Futura Publishing Co., New York. pp.401-421.

Phillips, M.T., Kirby, M.L., and Forbes, G. (1987): Analysis of cranial neural crest distribution in the developing heart using quail chick chimeras. Circulation Res. 60:27-30.

Piatigorsky, J. (1981): Lens differentiation in vertebrates. A review of cellular and molecular features. Differentiation 19:134-153.

Pittman, R.N. (1985): Release of plasminogen activator and calcium-dependent metalloproteinases from cultured sympathetic and sensory neurons. Dev. Biol. 110:91-101.

Potts, J.D. and Runyan, R.B. (1989): Epithelial-mesenchymal cell transformation in the embryonic heart can be mediated, in part, by transforming growth factor β. Dev. Biol. 134:392-401.

Poulsen, S.S., Kryger-Baggesen, N., and Nexo, E. (1996): Immunohistochemical localization of epidermal growth factor in the second-trimester human fetuses. Histochem. Cell Biol. 105:111-117.

Poulsen, S.S., Nexo, E., Skov Olsen, P., Hess, J., and Kirkegaard, P. (1986): Immunohistochemical localization of epidermal growth factor in rat and man. Histochemistry 85:389-394.

Pratt, R.M. (1987): Role of epidermal growth factor in embryonic development. In: Sawyer, R.H. (ed.) "Current topics in developmental biology." Academic Press, New York. Vol. 22, pp.175-193.

Proetzel, G., Pawlowski, S.A., Wiles, M.V., Yin, M., Boivin, G.P., Howles, P.N., Ding, J., and Ferguson, M.W.J. (1995): Transforming growth factor-β3 is required for secondary palate fusion. Nature Genetics 11:409-414.

Pulkkinen, L., Christiano, A.M., Airenne, T., Haakana, H., Tryggvason, K., and Uitto, J. (1994): Mutations in the γ2 chain gene (LAM C2) of kalinin/laminin 5 in the junctional forms of epidermolysis bullosa. Nature Genet 6:293-297.

Pusztai, L., Lewis, C.E., Lorenzen, and McGee, J.O'D. (1993): Growth factors: regulation of normal and neoplastic growth. J. Pathol. 169:191-201.

Pyke, C., Ralfkiaer, E., Tryggvason, K., and Dano, K. (1993): Messenger RNA for two type IV collagenases is located in stromal cells in human colon cancer. Am. J. Pathol. 142:359-365.

Qiu, C.X., and Ferguson, M.W.J. (1995): The distribution of PDGFs and PDGF-receptors during murine secondary palate development. J. Anat. 186:17-29.

Ralphs, J.R., Wylie, L., and Hill, D.J. (1990): Distribution of insulin-like growth factor peptides in the developing chick embryo. Development 109:51-58.

Rappolee, D.A., Brenner, C.A., Schultz, R., Mark, D., and Werb, Z. (1988): Developmental expression of PDGF, TGF-α, and TGF-β genes in preimplantation mouse embryos. Science 241:1823-1825.

Reed, S.C. (1933): An embryological study of harelip in mice. Anat. Rec. 56:101-110.

Reponen, P., Leivo, I., Sahlberg, C., Apte, S.S., Olsen, B.R., Thesleff, I., and Tryggvason, K. (1995): 92-kDa type IV collagenase and TIMP-3, but not 72-kDa type IV collagenase or TIMP-1 or TIMP-2, are highly expressed during mouse embryo implantation. Dev. Dynamics 202:388-396.

Reponen, P., Sahlberg, C., Huhtala, P., Hurskainen, T., Thesleff, I., and Tryggvason, K. (1992): Molecular cloning of murine 72-kDa type IV collagenase and its expression during mouse development. J. Biol. Chem. 267:7856-7862.

Rhodes, J.A., Fitzgibbon, D.H., Macchiarulo, P.A., and Murphy, R.A. (1987): Epidermal growth factor-induced precocious incisor eruption is associated with decreased tooth size. Dev. Biol. 121:247-252.

Richman, J.M., and Diewert, V.M. (1987): An immunofluorescence study of chondrogenesis in murine mandibular ectomesenchyme. Cell Differentiation 21:161-173.

Richman, J.M., and Tickle, C. (1989): Epithelia are interchangeable between facial primordia of chick embryos and morphoogenesis is controlled by the mesenchyme. Dev. Biol. 136:201-210.

Richman, J.M., and Tickle, C. (1992): Epithelial-mesenchymal interactions in the outgrowth of limb buds and facial primordia in chick embryos. Dev. Biol. 154:299-308.

Riggott, M.J., and Moody, S.A. (1987): Distribution of laminin and fibronectin along peripheral trigeminal axon pathways in the developing chick. J. Comp. Neurol. 258:580-596.

Robert, B., Sassoon, D., Jacq, B., Gehring, W., and Buckingham, M. (1989): *Hox-7*, a mouse homeobox gene with a novel pattern of expression during embryogenesis. EMBO J. 8:91-100.

Robertson, E.J., and Martin, G.R. (1993): Embryonic stem cells and gene targeting. In: Stern, C.D., and Holland, P.W.H. (eds.) "Essential Developmental Biology." Oxford University Press, Oxford. pp.167-170.

Rogers, S.L., Edson, K.J., Letourneau, P.C., and McLoon, S.C. (1986): Distribution of laminin in the developing peripheral nervous system of the chick. Dev. Biol. 113:429-435.

Rosenquist, G.C., and De Haan, R.L. (1966): Migration of precardiac cells in the embryonic chick heart. Contrib. Embryol. 38:113-121.

Rowe, A., Richman, J.M., and Brickell, P.M. (1991): Retinoic acid treatment alters the distribution of retinoic acid receptor-b transcripts in the embryonic chick face. Development 111:1007-1016.

Rugh, R. (1968): The mouse. Its reproduction and development. Burgess Publishing Company: Minneapolis, MN pp.117-167.

Runyan, R.B., and Markwald, R.R. (1983): Invasion of mesenchyme into three dimensional collagen gels: A regional and temporal analysis of interaction in embryonic heart. Dev. Biol. 95:108-114.

Runyan, R.B., Potts, J.D., and Weeks, D.L. (1992): TGF-β3-mediated tissue interaction during embryonic heart development. Mol. Reprod. Dev. 32:152-159.

Saari, H., Suomalainen, K., Lindy, O., Konttinen, Y.T., and Sorsa, T. (1990): Activation of latent human nuetrophil collagenase by reactive oxygen species and serine proteases. Biochem. Biophys. Res. Commun. 3:979-987.

Saber, G.M., Parker, S.B., and Minkoff, R. (1989): Influence of epithelial-mesenchymal interaction on the viability of facial mesenchyme *in vitro*. Anat. Rec. 225:56-66.

Sage, E.H., and Iruela-Arispe, M.L. (1990): Type VIII collagen in murine development. Association with capillary formation *in vitro*. Ann. N.Y. Acad. Sci. 580:17-31.

Sahlberg, C., Reponen, P., Tryggvason, K., and Thesleff, I. (1992): Association between the expression of murine 72 kDa type IV collagenase and basement membrane degradation during mouse tooth development. Archs. Oral Biol. 37:1021-1030.

Salo, T., Liotta, L.A., Tryggvason, K. (1983): Purification and characterization of a murine basement membrane collagen-degrading enzyme secreted by metastatic tumor cells. J. Biol. Chem. 258:3058-3063.

Salo, T., Lyons, J.G., Rahemtulla, F., Birkedal-Hansen, H., and Larjava, H. (1991): Transforming growth factor-b1 upregulates type IV collagenase expression in cultured human keratinocytes. J. Biol. Chem. 266:11436-11441.

Salo, T., Makela, M., Kylmanieme, M., Autio-Harmainen, H., and Larjava, H. (1994): Expression of matrix metalloproteinas-2 and -9 during early human wound healing. Lab. Invest. 70:176-182.

Samuel, J.L., Farhadian, F., Sabri, A., Marotte, F., Robert, V., and Rappaport, L. (1994): Expression of fibronectin during rat fetal and postnatal development: an *in situ* hybridization and immunohistochemical study. Cardiovas. Res. 28:1653-1661.

Sanders, E.J., Varedi, M., and French, A.S. (1993): Cell proliferation in the gastrularating chick embryo: a study using BrdU incorporation and PCNA localization. Development 118:389-399.

Sanes, J.R., Engvall, E., Butkowski, R., and Hunter, D.D. (1990): Molecular heterogeneity of basal laminae: isoforms of laminin and collagen IV at the neuromuscular junction and elsewhere. J. Cell Biol. 111:1685-1699.

Sangren, E.P., Luetteke, N.C., Palmiter, R.D., Brinster, R.L., and Lee, D.L. (1990): Overexpression of TGF-α in transgenic mice: induction of epithelial hyperplasia, pancreatic metaplasia, and carcinoma of the breast. Cell 61:1121-1135.

Sassani, R., Bartlett, S.P., Feng, H., Golden-Sauve, A., Haq, A.K., Buetow, K.H., and Gasser, D.L. (1993): Association between alleles of the transforming growth factor-alpha locus and the occurrence of cleft lip. Am. J. Med. Genet. 45:565-569.

Sato, H., and Seiki, M. (1993): Regulatory mechanism of 92-kDa type IV collagenase gene expression which is associated with invasiveness of tumor cells. Oncogene 8:395-405.

Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki M. (1994): A Matrix metalloproteinase expressed on the surface of the invasive tumour cells. Nature 370:61-65.

Sato., S., Ashraf, M., Millard, R.W., Fujiwara, H., and Schwartz, A. (1983): Connective tissue changes in early ischemia of porcine myocardium: and ultrastructural study. J. Mol. Cell Cardiol. 15:261-275.

Schlessinger, J., Schreiber, A.B., Levi, A., Lax, I., Libermann, T., Yarden, Y. (1983): Regulation of cell proliferation by epidermal growth factor. C.R.C. Rev. Biochem. 14:93-111.

Schnieke, A., Harbers, K., and Jaenisch, R. (1983): Embryonic lethal mutant in mice induced by retrovirus insertion into the alpha 1(I) collagen gene. Nature 304:315-320.

Schorle, H., Meier, P., Buchert, M., Jaenisch, R., and Mitchell, P.J. (1996): Transcriptional factor AP-2 essential for cranial closure and craniofacial development. Nature 381:235-238.

Schreiber, A.B., Winkler, M.E., and Derynck, R. (1986): Transforming growth factor-α is a more potent angiogenic mediator than epidermal growth factor. Science 232:1250-1253.

Schuger, L., O'Shea, S., Rheinheimer, J., Varani, J. (1990): Laminin in lung development: Effects of anti-laminin antibody in murine lung morphogenesis. Dev Biol 137:26-32.

Schuger, L., Skubitz, A.P.N., O'Shea, K.S., Chang, J.F., and Varani, J. (1991): Identification of laminin domains involved in branching morphogenesis: Effects of anti-laminin monoclonal antibodies on mouse lung development. Dev. Biol. 146:531-541.

Seltzer, J.L., Adams, S.A., Grant, G.A., and Eisen, A.Z. (1981): Purification and properties of a gelatin-specific neutral protease from human skin. J. Biol. Chem. 256:4662-4668.

Seltzer, J.L., Eisen, A.Z., Bauer, E.A., Morris, N.P., Glanville, R.W., and Burgeson R.E. (1989): Cleavage of type VII collagen by interstitial collagenase and type IV collagenase (gelatinase) derived from human skin. J. Biol. Chem. 1264:3822-3826.

Senior, R.M., Griffin, G.L., Fliszar, C.J., Shapiro, S.D., Goldberg, G.I., and Welgus, H.G. (1991): Human 92- and 72-kilodalton type IV collagenases are elastases. J. Biol. Chem. 266:7870-7875.

Serbedzija, G.N., Bronner-Fraser, M., and Fraser, S.E. (1992): Vital dye analysis of cranial neural crest cell migration in the mouse embryo. Development 116:297-307.

Seth, R., Shum, L., Wu, F., Wuenschnell, C., Hall, F.L., Slavkin, H.C., and Warburton, D. (1993): Role of epidermal growth factor expression in early mouse embryo lung branching morphogenesis in culture: Antisense oligodeoxynucleotide inhibitory strategy. Dev. Biol. 158:555-559.

Sharpe, P.M., Brunet, C.L., and Ferguson, M.W.J. (1992): Modulation of the epidermal growth factor receptor of mouse embryonic palatal mesenchyme cells *in vitro* by growth factors. Int. J. Dev. Biol. 36:275-282.

Sharpe, P.M., Brunet, C.L., Foreman, D.M., and Ferguson, M.W.J. (1993): Localization of acidic and basic fibroblast growth factors during mouse palate development and their effects on mouse palate mesenchyme cells *in vitro*. Roux's Arch. Dev. Biol. 202:132-143.

Sheffield, J.B. (1992): Is there a role for metalloproteinases in chick neural retina development? In: Birkedal-Hansen, H., Werb, Z., Welgus, H.G., and Van Wart, H.E. (eds.) "Matrix Metalloproteinases and Inhibitors." Matrix. Spec. Suppl. No1. Gustav Fischer, Stuttgart. pp.391-392.

Sheffield, J.B., Krasnopolsky, V., and Dehlinger, E. (1994): Inhibitor of retinal growth cone activity by specific metalloproteinase inhibitors *in vitro*. Dev. Dynamics 200:79-88.

Sheng, Z., Pennica, D., Wood, W.I., and Chien, K.R. (1996): Cardiotrophin-1 displays early expression in the murine heart tube and promoters cardiac myocyte survival. Development 122:419-428.

Shima, I., Sasaguri, Y., Kusukawa, J., Nakano, R., Yamana, H., Fujita, H., Kakegawa, T., and Morimatsu, M. (1993): Production of matrix metalloproteinase 9 (92-kDa gelatinase) by human oesophageal squamous cell carcinoma in response to epidermal growth factor. Br. J. Cancer 67:721-727.

Shigeta, H., Taga, M., Katoh, A., and Minaguchi, H. (1993): Ontogenesis and distribution of epidermal growth factor immunoreactivity and binding activity in the mouse fetal and neonatal tissues. Endocrine J. 40:641-647.

Shimizu, S., Malik, K., Sejima, H., Kishi, J., Hayakawa, T., and Koiwai, O. (1992): Cloning and sequencing of the cDNA encoding a mouse tissue inhibitor of metalloproteinases-2. Gene 114:291-292.

Shuler, C.F., Halpern, D.E., Guo, Y., and Sank, A.C. (1992): Medial edge epithelium fate traced by cell lineage analysis during epithelial-mesenchymal transformation *in vivo*. Dev. Biol. 154:318-330.

Shum, L., Sakakura, Y., Bringas, P. Jr., Luo, W., Snead, M.L., Mayo, M., Crohin, C., Millar, S., Werb, Z., Buckley, S., Hall, F.L., Warburton, D., and Slavkin, H.C. (1993): EGF abrogation-induced fusilli-form dysmorphogenesis of Meckel's cartilage during embryonic mouse mandibular morphogenesis *in vitro*. Development 118:903-917.

Sibilia, M., and Wagner, E.F. (1995): Strain-dependent epithelial defects in mice lacking the EGF receptor. Science 269:234-237.

Silver, J., and Hughes, A.F.W. (1973): The role of cell death during morphogenesis of the mammalain eye. J. Morphol. 140:159-170.

Sissman, N. (1970): Developmental landmarks in cardiac morphogenesis: comparative chronology. Am. J. Cardiol. 25:141-148.

Smith, M.M., and Hall, B.K. (1990): Developmental and evolutionary origins of vertebrate skeletogenic and odontogenic tissues. Biol. Rev. 65:277-373.

Smith, J.M., Sporn, M.B., Roberts, A.B., Derynck, R., Winkler, M.E., and Gregory, H. (1985): Human transforming growth factor-α causes precocious eyelid opening in newborn mice. Nature 315:515-518.

Snead, M.L., Luo, W., Oliver, P., Nakamura, M., Don-Wheeler, G., Bessem, C., Bell, G.I., Rall, L.B., and Slavkin, H.C. (1989): Localization of growth factor precursor in tooth and lung during embryonic mouse development. Dev. Biol. 134:420-429.

Snell, G.D., Fekete, E., Hummel, K.P., and Law, L.W. (1940): The relation of mating, ovulation and the estrus smear in the house mouse to time of day. Anat. Rec. 76:39-54.

Soini, Y., Hurskainen, T., Hoyhtya, M, Oikarinen, A., and Autio-Harmainen H. (1994): 72-kDa and 92-kDa type IV collagenase, type IV collagen, and laminin mRNAs in breast cancer: a study by *in situ* hybridization. J. Histochem. Cytochem. 42:945-951.

Solursh, M., Fisher, M., Meier, S., and Singley, C.T. (1979): The role of the extracellular matrix in the formation of the sclerotome. J. Embryol. Exp. Morphol. 54:75-98.

Solursh, M., and Jensen, K.L. (1988): Accumulation of basement membrane components during the onset of chondrogenesis and myogenesis in the chick wing bud. Development 104:41-49.

Song, K., Wang, Y., and Sassoon, D. (1992): Expression of Hox-7.1 in myoblasts inhibits terminal differentiation and induces cell transformation. Nature 360:477-481.

Spemann, H. (1901): Über Correlationen in der Entwickelung des Auges. Verh. Anat. Ges. 15:61-79.

Stahlman, M.T., Orth, D.N., and Gray, M.E. (1989): Immunocytochemical localization of epidermal growth factor in the developing human respiratory system and in acute and chronic lung disease in the neonate. Lab. Invest. 60:539-547.

Stark, R.B. (1954): The pathogenesis of harelip and cleft palate. Plast. Reconstruct. Surg. 13:20-32.

Steffensen, B., Wallon, U.M., and Overall, C.M. (1995): Extracellular matrix binding properties of recombinant fibronectin type II-like modules of human 72-kDa gelatinase/type IV collagenase. High affinity binding to native type I collagen but not native type IV collagen. J. Biol. Chem. 270:11555-11566.

Stenman, S., and Vaheri, A. (1978): Distribution of a major connective tissue protein, fibronectin, in normal human tissue. J. Exp. Med. 147:1054-1064.

Stern, C.D. (1993): Immunocytochemistry of embryonic material. In: Stern, C.D., and Holland, P.W.H. (eds.) "Essential Developmental Biology." Oxford University Press, Oxford. pp.193-212.

Stetler-Stevenson, W.G., Brown, P.D., Onisto, M., Levy, T. and Liotta, L.A. (1990): Tissue inhibitor of metalloproteinases-2 (TIMP-2) mRNA expression in tumor cell lines and human tumor tissues. J. Biol. Chem. 265:13933-13938.

Stetler-Stevenson, W.G., Krutzsch, H.C., and Liotta. L.A. (1989): Tissue inhibitor of metalloproteinase (TIMP-2). A new member of the metalloproteinase inhibitor family. J. Biol. Chem. 264:17374-17378.

Stetler-Stevenson, W.G., Liotta, L.A., and Kleiner, D.E. Jr (1993): Extracellular matrix 6: Role of matrix metalloproteinases in tumor invasion and metastasis. FASEB J. 7:1434-1441.

Stoker, M., Gherardi, E., Perryman, M., and Gray, J. (1987): Scatter factor is a fibroblast-derived modulator of epithelial cell motility. Nature, (London) 327:239-242.

Streeter, G.L. (1948): Developmental horizons in human embryos. Description of age groups XV, XVI, XVII, and XVIII, being the third issue of a survey of the Carnegie collection. Contrib. Embryol. Carnegie Inst. 32:133-203.

Strongin, A.Y., Collier, I., Bannikov, G., Marmer, B.L., Grants, G.A., and Goldberg, G.I. (1995): Mechanism of cell surface activation of 72-kDa type IV collagenase. J. Biol. Chem. 270:5331-5338.

Strongin, A.Y., Marmer, B.L., Grant, G.A., and Goldberg, G.I. (1993): Plasma membrane-dependent activation of the 72-kDa type IV collagenase is prevented by complex formation with TIMP-2. J. Biol. Chem. 268:14033-14039.

Sugi, Y., Sasse,J., Barron, M., and Lough, J. (1995): Developmental expression of fibroblast growth factor receptor-1 (*cek-1; flg*) during heart development. Dev. Dynamics 202:115-125.

Sulik, K.K., Johnston, M.C., Ambrose, L.J.H., and Dorgad, D. (1979): Phenytoin (Dilantin)-induced cleft lip and palate in A/J mice: a scanning and transmission electron microscopic study. Anat. Rec. 195:243-256.

Suzuki, H.R., Solursh, M., and Baldwin, H.S. (1995): Relationship between fibronectin expression during gastrulation and heart formation in the rat embryo. Dev. Dynam. 204:259-277.

Swiderski, R.E., Daniels, K.J., Jensen, K.L., and Solursh, M. (1994): Type II collagen is transient expressed during avian cardiac valve morphogenesis. Dev. Dynamics 200:294-304.

Sympson, C.J., Talhouk, R.S., Alexander, C.M., Chin, J.R., Clift, S.M., Bissell, M.J., and Werb, Z. (1994): Targeted expression of stromelysin-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene expression. J. Cell. Biol. 125:681-693.

Takahashi, S., Barry, A.C., and Factor, S.M. (1990): Collagen degradation in ischemic rat hearts. Biochem. J. 265:233-241.

Takino, T., Sato, H., Shinagawa, A., and Seiki, M. (1995): Identification of the second membrane-type matrix metalloproteinase (MT-MMP-2) gene from a human placenta cDNA library. MT-MMPs form a unique membrane-type subclass in the MMP family. J. Biol. Chem. 270:23013-23020.

Talhouk, R., Chin, J.R., Unemori, E.N., Werb, Z., and Bissell, M.J. (1991): Proteinases of the mammary gland: development regulation *in vivo* and vectorial secretion in culture. Development 112:439-449.

Talhouk, R., Werb, Z., and Bissell, M.J. (1992): Functional interplay between extracellular matrix and extracellular matrix-degrading proteinases in the mammary gland: a coordinate system for regulating mammary epithelial function. In: Fleming, T.P. (ed.) "Epithelial Organization and Development." Chapman and Hall, London. pp.329-351.

Tam, J.P. (1985): Physiological effects of transforming growth factor in the new born mouse. Science 229:673-675.

Tamarin, A. (1982): The formation of the primitive choanae and the junction of the primary and secondary palates in the mouse. Am. J. Anat. 165:319-337.

Thesleff, I., and Hurmerinta, K. (1981): Tissue interactions in tooth development. Differentiation 18:75-88.

Thesleff, I., Partanen, A., and Vainio, S. (1991): Epithelial-mesenchymal interactions in tooth morphogenesis: the roles of extracellular matrix, growth factors, and cell surface receptors. J. Craniofac. Genet. Dev. Biol. 11:229-237.

Thiery, J.-P., Duband, J.-L., Dufour, S., Savagner, P., and Imhof, B.A. (1989): Roles of fibronectin in embryogenesis. In: Mosher, D.F. (ed.) "Fibronectin." Academic Press, New York. pp.181-212.

Thompson, M.W., McInnes, R.R., and Willard, H.F. (1991): Genetics in medicine. Fifth edition, W.B. Saunders, Toronto.

Thomson, B.M., Atkinson, S.J., McGarrity, A.M., Hembry, R.M., Reynolds, J.J., and Meikle, M.C. (1989): Type I collagen degradation by mouse calvarial osteoblasts stimulated with 1,25-dihydroxyvitamin D-3: evidence for a plaminogen-plasmin-metalloproteinase activation cascade. Biochem. et Biophy. Acta 1014:125-132.

Threadgill, D.W., Dlugosz, A.A., Hansen, L.A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., Harris, R.C., Barnard, J.A., Yuspa, S.H., Coffey, R.J., and Magnuson, T. (1995): Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. Science 269:230-234.

Tidball, J.G. (1992): Distribution of collagens and fibronectin in the subepicardium during avian cardiac development. Anat. Embryol. 185:155-162.

Timpl, R. (1989): Structure and biological activity of basement membrane proteins. Eur. J. Biochem. 180:487-502.

Timpl, R., and Brown, J.C. (1994): The laminins. Matrix Biol. 14:275-281.

Timpl, R., and Dziadek, M. (1986): Structure, development, and molecular pathology of basement membranes. Int. Rev. Exp. Pathol. 29:1-112.

Timpl, R., and Martin, G.R. (1982): Components of basement membranes. In Furthmayr, H. (ed.) "Immunohistochemistry of the Extracellular Matrix." CRC Press, Boca Raton, Florida. Vol II, pp.119-150.

Todaro, G.J., Fryling, C., and DeLarco, J.E. (1980): Transforming growth factors produced by certain human tumor cells: polypeptides that interact with epidermal growth factor receptors. Proc. Natl. Acad. Sci. U.S.A. 77:5258-5262.

Trainor, P.A., and Tam, P.P. (1995): Cranial paraxial mesoderm and neural crest cells of the mouse embryos: co-distribution in the craniofacial mesenchyme but distinct segregation in branchial arches. Development 121:2569-2582.

Trasler, D.G. (1968): Pathogenesis of cleft lip and its relation to embryonic face shape in A/J and C57BL mice. Teratology 1:33-50.

Trasler, D.G., and Leong, S. (1982): Mitotic index in mouse embryos with 6-aminonicotinamide-induced and inherited cleft lip. Teratology 25:259-265.

Trelstad, R.L., Hayashi, A., Hayashi, K., and Donahoe, P.K. (1982): The epithelial-mesenchymal interface of the male rat Mullerian duct: Loss of basement membrane integrity and ductal regression. Dev. Biol. 92:27-40.

Tuckett, F., and Morriss-Kay, G.M. (1986): The distribution of fibronectin, laminin and entactin in the neurulating rat embryo studied by indirect immunofluorescence. J. Embryol. Exp. Morphol. 94:95-112.

Turksen, K., Choi, Y., and Fuchs, E. (1991): Transforming growth factor alpha induces collagen degradation and cell migration in differentiating human epidermal raft cultures. Cell Regulation 2:613-625.

Tyagi, S.C., Kumar, S.G., Banks, J., and Fortson, W. (1995a): Co-expression of tissue inhibitor and matrix metalloproteinase in myocardium. J. Mol. Cell Cardiol. 27:2177-2189.

Tyagi, S.C., Kumar, S.G., and Glover, G. (1995b): Induction of tissue inhibitor and matrix metalloproteinase by serum in human heart-derived fibroblast and endomyocardial endothelial cells. J. Cell. Biochem. 58:360-371.

Tyagi, S.C., Ratajska, A., and Weber, K.T. (1993): Myocardial matrix metalloproteinase(s): localization and activation. Mol. Cell Biochem. 126:49-59.

Van Helden, W.C.H., Kok-Verspuy, A., Harff, G.A., and Van Kamp, G.J. (1985): Rate-nephrolometric determination of fibronectin in plasma. Clin. Chem. 31:1182-1184.

Van Mierop, L.H.S., Alley, R.D., Kausel, H.W., and Stranahan, A. (1962): The anatomy and embryology of endocardial cushion defects. J. Thorac. Cardiovasc. Surg. 43:71-83.

Vassar, R., and Fuchs, E. (1991): Transgenic mice provide new insights into the role of TGF-α during epidermal development and differentiation. Genes Dev. 5: 714-727.

Vermeij-Keers, C. (1972): Transformation in the facial region of the human embryo. Adv. Anat. Embryol. Cell Biol. 46:7-28.

Wallon U.M., and Overall C.M. (1996): The C-terminal hemopexin-like domain of human 72-kDa gelatinase requires calcium for structural integrity and for binding to fibronectin and heparin: Analysis of the binding properties of recombinant 72-kDa gelatinase C-domain to extracellular matrix and basement membrane components. J. Biol. Chem. (submitted).

Wang, K.-Y. (1992): Morphometric studies of normal and abnormal primary palate formation in noncleft and cleft lip strains of mice. Ph.D. Thesis. Vancouver: University of British Columbia Library.

Wang, K.-Y., Chen, K.C., Chiang, C.P., and Kuo, M.Y.P. (1995): Distribution of $p21^{ras}$ during primary palate formation of non-cleft and cleft strains of mice. J. Oral. Pathol. Med. 24:103-108.

Wang, K.-Y., Juriloff, D.M., and Diewert, V.M. (1995): Deficient and delayed primary palatal fusion and mesenchymal bridge formation in cleft lip-liable strains of mice. J. Craniofac. Genet. Dev. Biol. 15:99-116.

Warbrick, J.C. (1960): The early development of the nasal cavity and upper lip in the human embryo. J. Anat. 94:351-362.

Warburton, M.J., Mitchell, D., Ormerod, E.J., and Rudland, P. (1982): Distribution of myoepithelial cells and basement membrane proteins in the resting, pregnant, lactating, and involuting rat mammary gland. J. Histochem. Cytochem. 30:667-676.

Warburton, D., Seth, R., Shum, L., Horcher, P.G., Hall, F.L., Werb, Z., Slavkin, H.C. (1992): Epigenetic role of epidermal growth factor expression and signalling in embryonic mouse lung morphogenesis. Dev. Biol. 149:123-133.

Ward, R.V., Atkinson, S.J., Reynolds, J.J., and Murphy, G. (1994): Cell surface-mediated activation of progelatinase A: demonstration of the involvement of the C-terminal domain of progelatinase A in cell surface binding and activation of progelatinase A by primary fibroblasts. Biochem. J. 304:263-269.

Wartiovaara, J., Leivi, I., and Vaheri, A. (1979): Expression of the cell surface-associated glycoprotein, fibronectin, in the early mouse embryo. Dev. Biol. 69:247-257.

Wartiovaara, J., Nordling, S., Lehtonen, E., and Saxen, L. (1974): Transfilter induction of kidney tubules: Correlation with cytoplasmic penetration into Nucleopore filters. J. Embryol. Exp. Morphol. 31:667-682.

Waseem, N.H., and Lane, D.P. (1990): Monoclonal antibody analysis of the proliferating cell nuclear antigen (PCNA). J. Cell Sci. 96:121-129.

Weber, B.H.F., Vogt, G., Pruett, R.C., Strohr, H., and Felbor, U. (1994): Mutations in the tissue inhibitor of metalloproteinases-3 (TIMP3) in patients with Sorsby's fundus dystrophy. Nature Genet. 8:352-356.

Wedden, S.E. (1987): Epithelial-mesenchymal interactions in the development of chick facial primordia and the target of retinoid action. Development 99:341-351.

Welgus, H.G., Fliszar, C.J., Seltzer, J.L., Schmid, T.M., and Jeffrey, J.J. (1990): Differential susceptibility of type X collagen to cleavage by two mammarian interstitial collagenases and 72-kDa type IV collagenase. J. Biol. Chem. 265:13521-13527.

Werb, Z. (1990): Expression of EGF and TGF-α genes in early mammalian development. Molec. Reprod. Dev. 27:10-15.

Werb, Z., Alexander, C.M., and Adler, R.R. (1992): Expression and function of matrix metalloproteinases in development. In: Birkedal-Hansen, H., Werb, Z., Welgus, H.G., and Van Wart, H.E. (eds.). "Matrix Metalloproteinases and Inhibitors." Matrix. Spec. Suppl. No.1. Gustav Fischer, Stuttgart. pp.337-343.

Wessells, N.K. (1977): In: Wessells N.K. (ed.) "Tissue Interactions and Development." W.A. Benjamin, Inc., California. pp.3-5.

Wilcox, J.N., and Derynck, R. (1988): Developmental expression of transforming growth factor-alpha and beta in mouse fetus. Mol. Cell Biol. 8:3415-3422.

Wiley, L.M., Adamson, E.D., and Tsark, E.C. (1995): Epidermal growth factor receptor function in early mammalian development. BioEssays 17:839-846.

Wilhelm, S.M., Collier, I.E., Marmer, B.L., Eisen, A.Z., Grant, G.A., and Goldberg, G.I. (1989): SV40-transformed human lung fibroblasts secrete a 92-kDa type IV collagenase which is identical to that secreted by normal human macrophages. J. Biol. Chem. 264:17213-17221.

Wilkinson, D.G. (1993): In situ hybridization. In: Stern, C.D., and Holland, P.W.H. (eds.) "Essential Developmental Biology." Oxford University Press, Oxford. pp.257-274.

Wilson, G.D., Soranson, J.A., and Lewis, A.A. (1987): Cell kinetics of mouse kidney using bromodeoxyuridine incorporation and flow cytometry: preparation and staining. Cell Tissue Kinet. 20:125-133.

Winkler, M.E., O'Connor, L., Winger, M., and Fendly, B. (1989): Epidermal growth factor and transforming growth factor α bind differently to the epidermal growth factor receptor. Biochem. 28:6373-6378.

Woessner, J.F. (1991): Matrix metalloproteinases and their inhibitors in connective tissue remodelling. F.A.S.E.B. J. 5:2145-2154.

Wong, S.T., Winchell, L.F., McCune, B.K., Earp, H.S., Teixido, J., Massague, J., Herman, B., and Lee, D.C. (1989): The TGF-a precursor expressed on the cell surface binds to the EGF receptor on adjacent cells, leading to signal transduction. Cell 56:495-506.

Wrenn, J.T., and Wessells, N.K. (1969): An ultrastructural study of lens invagination in the mouse. J. Exp. Zool. 171:359-367.

Xu, Z., Parker, S.B., and Minkoff, R. (1990): Distribution of type IV collagen, laminin and fibronectin during maxillary process formation in the chick embryo. Am. J. Anat. 187:232-246.

Yamada, K.M. (1991): Fibronectin and other cell interactive glycoproteins. In Hay, E.D. (ed.) "Cell Biology of Extracellular Matrix." Plenum Press, New York. pp.111-146.

Yurchenco, P.D. (1994): Assembly of laminin and type IV collagen into basement membrane networks. In: Yurchenco, P.D., Birk, D.E., and Mecham, R.P. (eds.) "Extracellular Matrix Assembly and Structure." Academic Press, Inc., San Diego. pp.351-388.

Zempo, N., Kenagy, R.D., Au, Y.P.T., Bendeck, M., Clowes, M.M., Reidy, M.A., and Clowes, A.W. (1994): Matrix metalloproteinases of vascular wall cells are increased in balloon-injured rat carotid artery. J. Vascular Surgery 20:209-217.

Zetter, B.R., and Martin, G.R. (1978): Expression of a high molecular weight cell surface glycoprotein (LETS protein) by preimplantation mouse embryos and teratocarcinoma stem cells. Proc. Natl. Acad. Sci. U.S.A. 75:2324-2328.

Zhang, J., Hagopian-Donaldson, S., Serbedzija, G., Elsemore, J., Plehn-Dujowich, D., McMahon, A.P., Flavell, R.A., and Williams, T. Neural tube, skeletal and body wall defects in mice lacking transcriptional factor AP-2. Nature 381:238-241.

Zwaan, J., and Hendrix, R.W. (1973): Changes in cell and organ shape during early development of the ocular lens. Am. J. Zool. 13:1039-1049.

APPENDIX 1: The primary antibodies and their concentrations and sources

primary antibody	host	concentration	source
1. polyclonal anti-mouse type IV collagen	rabbit	1:50	Chemicon, Temecula, CA (AB756)
2. polyclonal anti-human type IV collagen	rabbit	1:25	Chemicon, Temecula, CA (AB748)
3. polyclonal anti-EHS mouse sarcoma laminin	rabbit	1:100	Sigma, St. Louis, MO (L-9393)
4. polyclonal anti-human fibronectin	rabbit	1:100	Dako, Glostrup, Denmark (A 245)
5. polyclonal anti-mouse EGF	rabbit	1:50	Sigma, St. Louis, MO (E-2635)
6. polyclonal anti-human recombinant TGF-α	sheep	1:50	Chemicon, Temecula, CA (AB1412)
7. monoclonal anti-human EGF-R	mouse	1:20	Sigma, St. Louis, MO (E-3138)

8. monoclonal anti-human PCNA (PC 10)	mouse	1:20	Dimension, Mississauga, ONT, Canada
9. monoclonal anti-human BrdU	mouse	1:10	Amersham, Oakville, ONT, Canada (RPN202)
10. polyclonal anti-human MMP-2 (non-affinity-purified)	rabbit	1:200	created & provided by U.M. Wallon and C.M. Overall, UBC
11. polyclonal anti-human MMP-2 (affinity-purified)	rabbit	1:10	created & provided by U.M. Wallon and C.M. Overall, UBC

APPENDIX 2: The secondary antibodies and their concentrations and sources

secondary antibody	host	concentration	source
1. anti-rabbit TRITC conjugate	goat	1:200	Sigma, St. Louis, MO (T-6778)
2. anti-sheep FITC conjugate	donkey	1:200	Sigma, St. Louis, MO (F-7634)
3. anti-mouse biotin conjugate (Vectastain ABC Kit)	goat	1:100	Vector, Burlingame,CA (PK-4002)
4. anti-mouse FITC conjugate	goat	1:200	Sigma, St. Louis, MO (F-9006)
5. anti-rabbit FITC conjugate	sheep	1:200	Sigma, St. louis, MO (F-7512)
6. anti-rabbit biotin conjugate	goat	1:100	Sigma, St. Louis, MO (B-8895)

APPENDIX 3: Positive control tissues

Antibodies	Tissues	References
anti-EGF	embryonic mouse lung	Warburton *et al.*, 1992
anti-TGF-α	human oral squamous cell carcinoma	Christensen *et al.*, 1993
anti-MMP-2	embryonic mouse mandibular bone	Reponen *et al.*,1992
anti-laminin	basement membranes	Timpl, 1989
anti-fibronectin	mesenchymal tissue, basement membranes	Timpl, 1989
anti-type IV collagen	basement membranes	Timpl, 1989
anti-BrdU	mouse intestine	Dolbeare, 1995
anti-PCNA	mouse intestine	Dolbeare, 1995
	human tonsil	Hall *et al.*, 1990

APPENDIX 4: Samples of mouse embryos used for immunohistochemistry.

Laminin (Primary Palate)

7-11 TS	12-17 TS	18-20 TS
CD1 10.21.10 (9 TS) 4 slides, 4 sections/slide =16 sections	CD1 8.7 (13 TS) 11 slides, 4 sections/slide = 44 sections	CD1 B-4.8 (19 TS) 3 slides, 4 sections/slide =12 sections
CD1 10.21.2 (9 TS) 2 slides, 4 sections/slide =8 sections	CD1 6.1 (13 TS) 7 slides, 4 sections/slide =28 sections	CD1 B-4.10 (20 TS) 1 slide, 4 sections/slide =4 sections
CD1 1.3 (11 TS) 1 slide, 30 sections/slide =30 sections	CD1 10.21.6 (14 TS) 9 slides, 4 sections/slide =36 sections	CLFR 7.8s (19 TS) 1 slide, 4 sections/slide =4 sections
CLFR 1.7 (11 TS) 2 slides, 4 sections/slide = 8 sections	CD1 1.10 (15 TS) 8 slides, 4 sections/slide =32 sections	CLFR 7.4s (19 TS) 2 slides, 4 sections/slide =8 sections
	CD1 1.4 (15 TS) 1 slide, 30 sections/slide =30 sections	
	CD1 6.6 (17 TS) 14 slides, 4 sections/slide =56 sections	
	CD1 B-1.5 (16 TS) 2 slides, 4 sections/slide =8 sections	
	CLFR 10.22 (13 TS) 11 slides, 4 sections/slide =44 sections	
	CLFR 4.8 (14 TS) 1 slide, 30 sections/slide =30 sections	
	CLFR 4.7 (14 TS) 1 slide, 30 sections/slide =30 sections	
Total 3 CD1 embryos (50 sections) 1 CLFR embryo (8 sections)	**Total** 7 CD1 embryos (234 sections) 3 CLFR embryos (104 sections)	**Total** 2 CD1 embryos (20 sections) 2 CLFR embryos (12 sections)

Type IV Collagen (Primary Palate)

7-11 TS	12-17 TS	18-20 TS
CD1 10.21 (9 TS) 6 slides, 4 sections/slide =24 sections	CD1 3.2 (12 TS) 6 slides, 4 sections/slide =24 sections	CD1 B-4.8 (19 TS) 2 slides, 4 sections/slide =8 sections
CD1 10.18 (11 TS) 8 slides, 4 sections/slide =32 sections	CD1 10.8.7 (13 TS) 7 slides, 4 sections/slide =28 sections	CD1 7.1 (19 TS) 10 slides, 4 sections/slide =40 sections
CD1 10.19.9 (9 TS) 1 slide, 3 sections/slide =3 sections	CD1 10.23.6 (13 TS) 1 0 slides, 4 sections/slides =40 sections	CD1 4.3 (20 TS) 1 slide, 4 sections/slide =4 sections
	CD1 10.21 (14 TS) 12 slides, 4 sections/slide =48 sections	CD1 2.6 (20 TS) 2 slides, 4 sections/slide =8 sections
	CD1 3.1 (17 TS) 1 slide, 4 sections/slide =4 sections	CLFR 2.1 (19 TS) 2 slides, 4 sections/slide =8 sections
	CLFR 10.22 (13 TS) 1 1 slides, 4 sections/slide =44 sections	
Total 3 CD1 embryos (59 sections)	**Total** 5 CD1 embryos (144 sections) 1 CLFR embryo (44 sections)	**Total** 4 CD1 embryos (60 sections) 1 CLFR embryo (8 sections)

Fibronectin (Primary Palate)

7-11 TS	12-17 TS	18-20 TS
CD1 D-4.7 (7 TS) 1 slide, 20 sections/slide =20 sections	CD1 B-1.8 (15 TS) 10 slides, 4 sections/slide =40 sections	CD1 2.7 (18 TS) 2 slides, 4 sections/slide =8 sections
CD1 10.21.2 (9 TS) 13 slides, 4 sections/slide =52 sections	CD1 1.5 (15 TS) 1 slide, 30 sections/slide =30 sections	CD1 B-4.8 (19 TS) 8 slides, 4 sections/slide =32 sections
CD1 1.11 (11 TS) 1 slide, 30 sections/slide =30 sections	CD1 18.1 (17 TS) 2 slides, 5 sections/slide =10 sections	CD1 18.12 (19 TS) 1 slide, 4 sections/slide =4 sections
	CD1 10.22 (15 TS) 15 slides, 4 sections/slide =60 sections	CD1 18.9 (19 TS) 1 slide, 4 sections/slide =4sections
Total 3 CD1 embryos (102 sections)	**Total** 4 CD1 embryos (140 sections)	**Total** 4 CD1 embryos (48 sections)

Epidermal Growth Factor (EGF)

<7 TS	7-11 TS	12-17 TS	18-20 TS	day12
CD1 25.8 (3 TS) 2 slides 6 sections/slide =12 sections	CD1 A-9.5 (9 TS) 2 slides 30 sections/slide =60 sections	CD1 3.2 (12 TS) 1 slide 30 sections/slide = 30 sections	CD1 1.8 (18 TS) 1 slide 4 sections/slide =4 sections	CD1 19.4 1 slide 20 sections/slide =20 sections
CD1 25.5 (4 TS) 1 slides 5 sections/slide =5 sections	CD1 10.21.6 (10 TS) 2 slides 4 sections/slide =8 sections	CD1 D-5.5 (15 TS) 6 slides 4 sections/slide =24 sections	CD1 18.8(18 TS) 1 slide 4 sections/slide =4 sections	CD1 19.9 1 slide 9 sections/slide =9 sections
CD1 25.6 (5 TS) 2 slide 5 sections/slide =10 sections	CD1 10.19.7 (11 TS) 3 slides 4 sections/slide =12 sections	CD1 18.1 (17 TS) 3 slides 5 sections/slide =15 sections	CD1 18.9 (19 TS) 1 slide 4 sections/slide =4 sections	CD1 28.6 1 slide 10 sections/slide =10 sections
		CD1 1.5 (17 TS) 3 slides 4 sections/slide =12 sections	CD1 18.12(19 TS) 1 slide 3 sections/slide =3 sections	CD1 19.2 1 slide 10 sections/slide =11 sections
		CLFR A-1.3 (14 TS) 7 slides 4 sections/slide =28 sections		
		CLFR A-1.1 (15 TS) 9 slides 4 sections/slide =36 sections		
Total 3 CD1 embryos (32 sections)	**Total** 3 CD1 embryos (38 sections)	**Total** 4 CD1 embryos (81 sections) 2 CLFR embryos (64 sections)	**Total** 4 CD1 embryos (15 sections)	**Total** 4 CD1 embryos (50 sections)

Transforming Growth Factor-alpha (TGF-alpha)

<7 TS	7-11 TS	12-17 TS	18-20 TS	day12
CD1 25.8 (3 TS) 2 slides 6 sections/slide =12 sections	CD1 D-7.1 (8 TS) 2 slides 30 sections/slide =60 sections	CD1 D-5.6 (13 TS) 2 slides 30 sections/slide =60 sections	CD1 1.8 (18 TS) 3 slides 4 sections/slide =12 sections	CD1 19.4 1 slide 20 sections/slide =20 sections
CD1 25.5 (4 TS) 1 slide 5 sections/slide =5 sections	CD1 B-1.9 (9 TS) 5 slides 4 sections/slide =20 sections	CD1 10.23.8 (15 TS) 2 slides 4 sections/slide =8 sections	CD1 1.11 (18 TS) 1 slide 4 sections/slide =4 sections	CD1 19.9 1 slide 9 sections/slid e=9 sections
CD1 25.6 (5 TS) 1 slide 5 sections/slide =5 sections	CD1 D-7.4 (10 TS) 1 slide 30 sections/slide =30 sections	CD1 10.18.1 (15 TS) 1 slide 4 sections/slide =4 sections	CD1 11.18.5 (18 TS) 2 slides 4 sections/slide =8 sections	CD1 19.2 1 slide 13 sections/slide =13 sections
		CD1 18.1 (17 TS) 1 slide 5 sections/slide =5 sections	CD1 11.11 (19 TS) 2 slides 4 sections/slide =8 sections	
		CLFR 4.1 (12 TS) 5 slides 4 sections/slide =20 sections	CLFR 6.7 (18 TS) 1 slide 4 sections/slide =4 sections	
		CLFR 10.22.8 (14 TS) 4 slides 4 sections/slide =16 sections	CLFR 2.5 (18 TS) 2 slides 30 sections/slide =60 sections	
		CLFR 3.7 (15 TS) 5 slides 4 sections/slide =20 sections		
Total 3 CD1 embryos (22 sections)	**Total** 3 CD1 embryos (110 sections)	**Total** 4 CD1 embryos (77 sections) 3 CLFR embryos (56 sections)	**Total** 4 CD1 embryos (32 sections) 2 CLFR embryos (64 sections)	**Total** 3 CD1 embryos (42 sections)

Epidermal Growth Factor Receptor (EGF-R)

<7 TS	7-11 TS	12-17 TS	18-20 TS	day12
CD1 25.8 (3 TS) 2 slides 6 sections/slide =12 sections	CD1 10.21.5 (8 TS) 1 slide 4 sections/slide =4 sections	CD1 D-7.3 (12 TS) 2 slides 4 sections/slide =8 sections	CD1 11.18.5 (18 TS) 2 slides 4 sections/slide =8 sections	CD1 19.4 1 slide 6 sections/slide =6 sections
CD1 25.5 (4 TS) 1 slide 5 sections/slide =5 sections	CD1 B-1.9 (9 TS) 1 slide 4 section/slide =4 sections	CD1 10.19.5 (15 TS) 2 slides 4 sections/slide =8 sections	CD1 1.8 (18 TS) 1 slide 4 sections/slide =4 sections	CD1 19.9 1 slide 5 sections/slide =5 sections
CD1 25.6 (5 TS) 1 slide 5 sections/slide =5 sections	CD1 A-8.5 (11 TS) 1 slide 30 sections/slide =30 sections	CD1 10.21.1 (15 TS) 2 slides 4 sections/slide =8 sections	CD1 18.8 (18 TS) 1 slide 3 sections/slide =3 sections	CD1 19.2 1 slide 6 sections/slide =6 sections
		CD1 10.21.5 (15 TS) 2 slides 4 sections/slide =8 sections	CD1 18.9 (19 TS) 1 slide 4 sections/slide =4 sections	
		CD1 10.23.8 (15 TS) 3 slides 4 sections/slide =12 sections	CD1 18.12 (19 TS) 1 slide 4 sections/slide =4 sections	
		CD1 18.1 (17 TS) 1 slide 5 sections/slide =5 sections		
		CD1 1.6 (17 TS) 3 slides4 sections/slide =12 sections		
		CD1 1.5 (17 TS) 5 slides 4 sections/slide =20 sections		
		CLFR 4.1 (12 TS) 5 slides 4 sections/slide =20 sections		
Total 3 CD1embryo (22 sections)	**Total** 3 CD1 embryos (38 sections)	**Total** 5 CD1 embryos (52 sections) 1 CLFR embryo (20 sections)	**Total** 5 CD1 embryos (23 sections)	**Total** 3 CD1 embryos (17 sections)

5-Bromodeoxyuridine (BrdU) Incorporation

7-11 TS	12-17 TS	18-22 TS
CD1 u18.8 (10 TS) 2 slides (30 sections/slide) =60 sections	CD1 u19.4 (12 TS) 1 slide (30 sections/slide) =30 sections	CD1 5.7 (20 TS) 2 slides (30 sections/slide) =60 sections
CD1 u20.1 (10 TS) 2 slides (30 sections/slides) =60 sections	CD1 u4.3 (13 TS) 1 slide (30 sections/slide) =30 sections	CD1 u5.10 (22 TS) 1 slide (30 sections/slide) =30 sections
CD1 4.8 (10 TS) 1 slide (30 sections/slide) =30 sections	CD1 u3.5 (14 TS) 1 slide (30 sections/slide) =30 sections	CD1 6.3 (22 TS) 1 slide(30 sections/slide) =30 sections
CD1 u19.10 (11 TS) 1 slide (30 sections/slide) =30 sections	CD1 1.6 (15 TS) 1 slide (30 sections/slide) =30 sections	Balb/c u7.8 (18 TS) 1 slide (30 sections/slide) =30 sections
Balb/c 6.5 (9 TS) 1 slide (30 sections/slide) =30 sections	Balb/c u5.7 (14 TS) 1 slide (30 sections/slide) =30 sections	Balb/c u11.3 (20 TS) 1 slide (30 sections/slide) =30 sections
Balb/c u1.2 (10 TS) 1 slide (30 sections/slide) =30 sections	Balb/c 1.6 (15 TS) 1 slide (30 sections/slide) =30 sections	
	Balb/c u2.4 (16 TS) 1 slide (30 sections/slide) =30 sections	
Total 4 CD1 embryos (180 sections) 2 Balb/c embryos (60 sections)	**Total** 4 CD1 embryos (120 sections) 3 Balb/c embryos (90 sections)	**Total** 3 CD1 embryos (90 sections) 2 Balb/c embryos (60 sections)

Proliferating Cell Nuclear Antigen (PCNA)

7-11 TS	12-17 TS	18-22 TS
CD1 1.1 (10 TS) 2 slides (30 sections/slide) =60 sections	CD1 B-1.7 (12 TS) 2 slides (4 sections/slide) =8 sections	CD1 4.2 (18 TS) 2 slides (30 sections/slide) =60 sections
CD1 1.11 (11 TS) 2 slides (30 sections/slide) =60 sections	CD1 B-1.6 (13 TS) 2 slides (4 sections/slide) = 8 sections	CD1 u7.7 (18 TS) 1 slide (30 sections/slide) =30 sections
	CD1 u17.4 (15 TS) 1 slide (30 sections/slide) =30 sections	CD1 B-4.8 (19 TS) 1 slide (4 sections/slide) =4 sections
	CD1 14 (16 TS) 1 slide (30 sections/slide) =30 sections	CD1 u5.2 (20 TS) 1 slide (30 sections/slide) =30 sections
	CD1 B-1.5 (16 TS) 3 slides (4 sections/slide) =12 sections	CD1 u11.7 (21 TS) 2 slides (30 sections/slide) = 60 sections
	CD1 6.9 (17 TS) 1 slide (4 sections/slide) =4 sections	
Total 2 CD1 embryos (120 sections)	**Total** 6 CD1 embryos (92 sections)	**Total** 7 CD1 embryos (188 sections)

72-kDa Gelatinase (Primary Palate and Mandible)

<7 TS	7-11 TS	12-17 TS	18-20 TS	day12
CD1 5 (day 9) 1 slide 4 sections/slide =4 sections	CD1 D-7.2 (7 TS) 2 slides 30 sections/slide =60 sections	CD1 D-5.10 (12 TS) 2 slides 30 sections/slide =60 sections	CD1 1.8 (18 TS) 2 slides 4 sections/slide =8 sections	CD1 28.6 2 slides 10 sections/slide =20 sections
CD1 5.2.95 (day 9) 1 slide 6 sections/slide =6 sections	CD1 B-1.9 (9 TS) 5 slides 4 sections/slide =20 sections	CD1 10.19.3 (13 TS) 2 slides 4 sections/slide =8 sections	CD1 11.11 (19 TS) 2 slides 4 sections/slide =8 sections	CD1 19.4 1 slide 5 sections/slide =5 sections
CD1 25.3 (4 TS) 1 slide 6 sections/slide =6 sections	CD1 10.21.2 (9TS) 8 slides 4 sections/slide =32 sections	CD1 D-5.7 (13 TS) 2 slides 30 sections/slide =60 sections	CD1 1.1 (20 TS) 2 slides 30 sections/slide =60 sections	CD1 19.9 1 slide 9 sections/slide =9 sections
CD1 25.5(4 TS) 2 slides6 sections/slide 12 sections	CD1 10.21.10 (9 TS) 4 slides 4 sections/slide =16 sections	CD1 10.18.3 (13 TS) 1 slide 4 sections/slide =4 sections	CD1 3.2 (20 TS) 1 slide 30 sections/slide =30 sections	CLFR 6.2s 3 slides 5 sections/slide =15 sections
CD1 25.6 (5 TS) 2 slides 5 sections/slide =10 sections	CLFR 14 3 slide 2 sections/slide =6 sections	CD1 18.2 (13 TS) 1 slide 4 sections/slide =4 section	CD1 18.12 (19 TS) 1 slide 5 sections/slide =5 sections	CLFR 6.3 s 1 slide 10 sections/slide =10 sections
CLFR 1 (day 9) 2 slides 10 sections/slide =20 sections		CD1 10.23.3 (15 TS) 2 slides 4 sections/slide =8 sections	CLFR 1.4 (18 TS) 3 slides 3 sections/slide =9 sections	CLFR 14 3 slides 4 sections/slide =12 sections
		CD1 18.1 4 slides 5 sections/slide =20 sections		
		CLFR 10.22.8 (14 TS) 3 slides 4 sections/slide =12 sections		
		CLFR 3.7 (15 TS) 5 slides 4 sections/slide =20 sections		
		CLFR 10.21.3 (16 TS) 2 slides 4 sections/slide =8 sections		
Total 5 CD1 embryos (38 sections) 1 CLFR embryo (20 sections)	**Total** 4 CD1 embryos (138 sections) 1 CLFR embryo (6 sections)	**Total** 7 CD1 embryos (148 sections) 2 CLFR embryos (36 sections)	**Total** 5 CD1 embryos (111 sections) 1 CLFR embryo (9 sections)	**Total** 3 CD1 embryo (34 sections) 3 CLFR embryos 37 sections)

72-kDa Gelatinase (Eye)

day10	day11	day12	day13	day14
CD1 D-7.2 1 slide 30 sections/slide =30 sections	CD1 D-5.6 1 slide 30 sections/slide =30 sections	CD1 28.6 2 slides 5 sections/slide =10 sections	CD1 22.3 2 slides 5 sections/slide =10 sections	CD1 16.8s 3 slides 6 sections/slides =18 sections
CD1 D-7.5 1 slide 30 sections/slide =30 sections	CD1 10.23 2 slides 4 sections/slide =8 sections	CD1 19.4 2 slide 20 sections/slide =40 sections	CD1 22.2 2 slides 5 sections/slide =10 sections	CD1 16.7s 3 slides 15 sections/slide =45 sections
CD1 A-9.5 1 slide 30 sections/slide =30 sections	CD1 4.2 1 slide 25 sections/slide =25 sections	CD1 19.2 1 slide 17 sections/slide =17 sections	A/J 13 1 slide 4 sections/slide =4 sections	CD1 14.8s 1 slide 4 sections/slide =4 sections
CD1 25.8 2 slides 6 sections/slide =12 sections		CLFR 6.3s 2 slides 10 sections/slide =20 sections	A/J c 2 slides 3 sections/slide =6 sections	CLFR 12.7s 1 slide 4 sections/slide =4 sections
Total 4 CD1 embryos (102 sections)	**Total** 3 CD1 embryos (63 sections)	**Total** 3 CD1 embryos (67 sections) 1 CLFR embryo (20 sections)	**Total** 2 CD1 embryos (20 sections) 2 A/J embryos (10 sections)	**Total** 3 CD1 embryos (67 sections) 1 CLFR embryo (4 sections)

Co-distribution of 72-kDa Gelatinase, EGF and TGF-alpha (Primary Palate)

7-11 TS	12-17 TS	18-20 TS
CD1 10.19.9 (9 TS) 1 slide, 3 sections/slide =3 sections	CD1 A-8.6 (12 TS) 1 slide, 3 sections/slide =3 sections	CD1 18.8 (1 TS) 1 slide, 3 sections/slide =3 sections
CD1 10.19.6 (10 TS) 1 slides, 3 sections/slide =3 sections	CD1 10.8.8 (14 TS) 1 slide, 3 sections/slide =3 sections	CD1 18.9 (19 TS) 1 slide, 3 sections/slide =3 sections
CD1 10.19.10 (9 TS) 1 slide, 3 sections/slide =3 sections	CD1 A-8.7 (15 TS) 1 slide, 3 sections/slides =3 sections	CD1 18.12 (19 TS) 1 slide, 3 sections/slide =3 sections
Total 3 CD1 embryos (9 sections)	**Total** 3 CD1 embryos (9 sections)	**Total** 3 CD1 embryos (9 sections)

72-kDa Gelatinase (Heart)

day9	day10	day11	day12	day13
CD1 5 1 slide 4 sections/slide =4 sections	CD1 25.3 1 slide 6 sections/slide =6 sections	CD1 18.1 4 slides 5 sections/slide =20 sections	CD1 28.6 2 slides 10 sections/slide =20 sections	CD1 22.3 1 slide 4 sections/slide =4 sections
CD1 5.2.95 1 slide 6 sections/slide =6 sections	CD1 25.5 2 slides 6 sections/slide =12 sections	CD1 18.12 2 slides 5 sections/slide =10 sections	CD1 19.4 2 slides 20 sections/slide =40 sections	CD1 22.5 1 slide 4 sections/slide =4 sections
CD1 5.3.95 1 slide 6 sections/slide =6 sections	CD1 25.6 2 slides 5 sections/slide =10 sections	CLFR 1.1 1 slide 10 sections/slide =10 sections	CD1 19.2 1 slide 17 sections/slide =17 sections	CD1 22.7 1 slide 4 sections/slide = 4 sections
CLFR 1 2 slides 10sections/slide =20 sections	CD1 25.4 2 slides 10 sections/slide =10 sections	CLFR 4.3 1 slide 5 sections/slide =5 sections	CLFR 6.2s 3 slides 5 sections/slide =15 sections	CD1 22.2 1 slide 4 sections/slide =4 sections
	CLFR 13.2 3 slides 2 sections/slide =6 sections	CLFR 7.4s 1 slide 5 sections/slide =5 sections	CLFR 6.3s 1 slide 10 sections/slide = 10 sections	
		A/W 5.2 2 slides 10 sections/slide =20 sections	CLFR 17.5 3 slides 4 sections/slide =12 sections	
Total 3 CD1 embryos (16 sections) 1 CLFR embryo (20 sections)	Total 4 CD1 embryos (38 sections) 1 CLFR embryo (6 sections)	Total 2 CD1 embryos (30 sections) 3 CLFR embryos (20 sections) 1 A/W embryo (20 sections)	Total 3 CD1 embryos (77 sections) 3 CLFR embryos (37 sections)	Total 4 CD1 embryos (16 sections)

Laminin (Heart)

day9	day10	day11	day12	day13
CD1 5.2.95 1 slide 6 sections/slide =6 sections	CD1 25.3 1 slide 6 sections/slide =6 sections	CD1 18.13 1 slides 5 sections/slide =5 sections	CD1 19.9 1 slide 4 sections/slide =4 sections	CD1 22.3 1 slide 4 sections/slide =4 sections
CD1 5.3.95 1 slide 6 sections/slide =6 sections	CD1 25.4 1 slides 6 sections/slide =6 sections	CD1 18.9 1 slide 4 sections/slide =10 sections	CD1 19.4 1 slides 20 sections/slide =20 sections	CD1 22.5 1 slide 4 sections/slide =4 sections
CLFR 1 1 slides 10 sections/slide =10 sections	CD1 25.10 1 slides 5 sections/slide =5 sections	A/W 5.2 1 slides 10 sections/slid e=10 sections	CD1 19.2 1 slide 17 sections/slide =17 sections	CD1 22.2 1 slide 4 sections/slide =4 sections
Total 2 CD1 embryos (12 sections) 1 CLFR embryo (10 sections)	**Total** 3 CD1 embryos (17 sections)	**Total** 2 CD1 embryos (15 sections) 1 A/W embryo (10 sections)	**Total** 3 CD1 embryos (41 sections)	**Total** 3 CD1 embryos (12 sections)

Type IV Collagen (Heart)

day9	day10	day11	day12	day13
CD1 5.2.95 1 slide 6 sections/slide =6 sections	CD1 25.3 1 slide 6 sections/slide =6 sections	CD1 18.13 1 slide 5 sections/slide =5 sections	CD1 19.7 1 slide 4 sections/slide =4 sections	CD1 22.7 1 slide 4 sections/slide =4 sections
CD1 5.3.95 1 slide 6 sections/slide =6 sections	CD1 25.5 1 slide 6 sections/slide =6 sections	CD1 18.9 1 slide 4 sections/slide =10 sections	CD1 19.2 1 slide 20 sections/slide =20 sections	CD1 22.5 1 slide 4 sections/slide =4 sections
CLFR 1 1 slide 10 sections/slide =10 sections	CD1 25.7 1 slide 5 sections/slide =5 sections	CD1 18.12 1 slide 5 sections/slide =5 sections	CLFR 6.3s 1 slide 4 sections/slide =4 sections	CD1 22.2 1 slide 4 sections/slide =4 sections
Total 2 CD1 embryos (12 sections) 1 CLFR embryo (10 sections)	**Total** 3 CD1 embryos (17 sections)	**Total** 3 CD1 embryos (15 sections)	**Total** 2 CD1 embryos (24 sections) 1 CLFR embryo (4 sections)	**Total** 3 CD1 embryos (12 sections)

Fibronectin (Heart)

day9	day10	day11	day12	day13
CD1 5.2.95 1 slide 6 sections/slide =6 sections	CD1 25.6 1 slide 6 sections/slide =6 sections	CD1 18.13 1 slide 5 sections/slide =5 sections	CD1 19.7 1 slide 4 sections/slide =4 sections	CD1 22.7 1 slide 4 sections/slide =4 sections
CD1 5.3.95 1 slide 6 sections/slide =6 sections	CD1 25.10 1 slides 6 sections/slide =6 sections	CD1 18.9 1 slide 4 sections/slide =10 sections	CD1 19.2 1 slides 20 sections/slide =20 sections	CD1 22.5 1 slide 4 sections/slide =4 sections
CLFR 1 1 slides10 sections/slide =10 sections	CD1 25.7 1 slides 5 sections/slide =5 sections	CD1 18.12 1 slides 5 sections/slide =5 sections	CD1 19.9 1 slide 4 sections/slide =4 sections	CD1 22.2 1 slide 4 sections/slide =4 sections
Total 2 CD1 embryos (12 sections) 1 CLFR embryo (10 sections)	**Total** 3 CD1 embryos (17 sections)	**Total** 3 CD1 embryos (15 sections)	**Total** 3 CD1 embryos (28 sections)	**Total** 3 CD1 embryos (12 sections)