CHANGES IN NASAL MORPHOLOGY DURING PRIMARY PALATE FORMATION IN THE C57BL/6J MOUSE EMBRYO AS REVEALED BY TRANSMISSION ELECTRON MICROSCOPY AND HISTOCHEMISTRY

By

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ABSTRACT

Primary palate formation in the C57BL/6J mouse embryo involves three facial prominences. These are the medial nasal prominence (MNP), lateral nasal prominence (LNP), and the maxillary prominence (MxP). The MNP and LNP come together and fuse forming a nasal fin which is a transient structure that persists for a time and then regresses. Mesenchymal cells invade the nasal fin and form a mesenchymal bridge.

In this study, I look at the morphological changes in nasal fin epithelium and its basal lamina as the fin regresses. I also describe the spatial and temporal distribution of filamentous actin (F-actin) at the time of primary palate formation. Morphological changes in the developing nasal cavity are illustrated by performing 3-Dimensional reconstructions from serial sections. This information is incorporated into a model describing cellular events that take place during primary palate formation.

C57BL/6J female mice were time mated with detection of a vaginal plug being called Day 0 of pregnancy. Females were sacrificed at times ranging from Day 10 to 13, and embryos were removed from the uteri and placed in saline buffer. The embryos were staged developmentally according to tail somite (T.S.) number and then prepared for either fluorescence or transmission electron microscopy. A total of 25 embryos were collected ranging from 7 to 27 T.S. Specimens used in the F-actin study were fixed and then snap frozen in liquid nitrogen in preparation for cryostat sectioning. Sections were stained with a
chemical probe for F-actin, NBD-phallacidin, which is a fluorescent phallotoxin that stains specifically only actin of the filamentous type.

There appear to be different zones or domains of epithelium within the nasal cavity, nasal fin, and facial prominences as the primary palate is forming with respect to F-actin staining. This reflects different states of epithelial differentiation in these various regions. F-actin appears to be distributed uniformly all around the nasal cavity with higher concentrations in apical epithelium facing the lumen. Also at regions where the nasal cavity bends or evaginates, as detected by the 3-D reconstructions, F-actin content in epithelium at these locations increases dramatically. The facial prominence epithelium stains with reduced intensity especially in regions of presumptive fusion. Nasal fin epithelium of the fused LNP and MNP stains weakly with a disorganized appearance compared to epithelium in other areas.

In the TEM study I found that prior to nasal fin formation the basal lamina of the MNP and LNP starts to break down before the prominences come into contact. Once the nasal fin forms, the zone of basal lamina destruction becomes larger. At the point of basal lamina destruction mesenchymal cell processes as well as epithelial cell processes touch and penetrate the lamina. Once the mesenchymal bridge has formed, new basal lamina beneath the base of the nasal cavity and oral cavity epithelia is formed.

Comparisons were made of changes in the basal lamina during primary palate formation and in other developmental systems such as Mullerian duct regression, thyroid formation and secondary palate formation. Similarities and
differences between these systems and the primary palate were observed. Also the actin distribution in the developing nasal cavity was compared to that seen in the salivary gland.

The primary palate may be another example of a system where an epithelial component is transformed to a mesenchymal component with timing of basal lamina changes being important.
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1. INTRODUCTION

1.1 General Introduction

The human primary palate forms during stage 17 (O'Rahilly and Müller, 1987) at about 41 days postfertilization (Moore, 1989). The mouse model has been used in studies involving primary palate formation to investigate both normal and abnormal development of facial structures (Reed, 1933; Trasler, 1968; Gaare and Langman, 1977a, b). It has been shown that different strains of mice have different susceptibilities toward developing certain facial clefts (Trasler, 1968). Specifically, A/J mice develop lateral cleft lips and C57BL mice develop medial clefts. The C57BL strain does not develop lateral cleft lips so it is a convenient strain in which to study normal primary palate formation. Failure of the primary palate to form may result in the facial abnormality known as cleft lip (Warbrick, 1960). In humans, cleft lip occurs with an incidence of 1 in 600 births in Caucasian populations, and as high as 1 in 363 in North American Indian populations (Lowry and Renwick, 1969). It is thought that embryonic face shape may be one factor contributing to cleft formation (Fraser and Pashayan, 1970; Trasler, 1968). It is also thought that a genetic component (Juriloff, 1986) as well as a number of environmental factors (Bornstein et al., 1969; Davidson et al., 1969) may contribute to producing this facial abnormality. The exact mechanism behind cleft lip formation is still not known. It is hoped that by understanding how the upper lip develops normally one could then better speculate what goes wrong during abnormal development.
1.2 Primary Palate Formation in the Mouse

A portion of the migrating neural crest population comes to rest in an area that will soon become the embryonic face. In fact, these neural crest cells constitute almost the entire facial mesenchymal cell population above the developing oral cavity (Johnston and Sulik, 1980). The mid-face develops first as thickenings, called the nasal placodes, of ectoderm on either side of the face. The mesenchyme at the sides of the placodes then condenses and proliferates as do the epithelial cells of the placodes themselves (Pourtois, 1972). This results in an apparent sinking inward of the placode and a rising of the medial and lateral rims to form a primitive nasal pit or groove. This olfactory pit becomes deeper as the lateral and medial nasal prominences (LNP and MNP) grow. The nasal pit is exposed to the maxillary prominence (MxP) at its floor. The maxillary prominence is formed by the proximal half of the mandibular arch that then grows forward at its tip to eventually meet up with the LNP and MNP (Johnston and Sulik, 1980). Johnston speaks of the maxillary prominence as being "overwhelmed" by the MNP and LNP. These prominences are extremely well developed in the mouse probably due to the mouse having a highly evolved sense of smell. The actual formation of the primary palate in the mouse starts at the bottom of the nasal pit, in the isthmus which is the region between the MNP and LNP. The MNP and the LNP converge superior to the isthmus with the medial surface of the LNP meeting the lateral surface of the MNP. Fusion of the LNP and MNP occurs around the
opening of the nasal pit as well as inside the pit. Fusion then proceeds in an antero-inferior direction. These two epithelial surfaces form a plate called the epithelial plate of Hochstetter or the nasal fin (Patten, 1968). The nasal fin is a transient structure that persists during the developmental period corresponding to 11 and 12 tail somites in the C57 mouse. There is question as to what actually happens to the epithelial cells of the nasal fin. Lejour (1969) states that these cells undergo necrosis while others feel that the epithelial cells may migrate away and become a part of the surrounding mesenchymal cell population (Patten, 1968). It is even speculated that the epithelial cells may transform into mesenchymal cells such as is proposed to occur during secondary palate formation (Fitchett and Hay, 1989). Then at about 13 T.S., the nasal fin starts to regress (Gaare and Langman, 1977b) and the epithelial seam is disrupted. Mesenchymal cells from the medial and lateral side break through the nasal fin and form what is called a mesenchymal bridge (MB).

Little is known, at the ultrastructural level, about the mechanism of nasal fin regression. It is not known whether contact is necessary to initiate nasal fin breakdown or whether it depends on a pre-existing genetic program inherent to the cells. Like all epithelial cells, the cells of the nasal fin have an underlying basement membrane. It also is not known what role the basement membrane may play in nasal fin regression; that is, whether or not it must disappear first before the mesenchymal bridge can form, or does it merely play a passive role. Once formed, the mesenchymal bridge grows and enlarges becoming more robust and it is here that the primary palate is considered to have formed. Failure
or incomplete fusion of the MNP and LNP and subsequent insufficient MB formation may result in a cleft lip. As the face and especially the brain grows and develops the resulting forces generated may tear the facial prominences apart. There are a number of theories, some of which will be discussed later, as to how a cleft lip may form.

1.3 Cellular Events Associated With Upper Lip and Nasal Cavity Formation.

Fusion between the epithelial linings of the MNP and LNP transforms the nasal pit into a primitive nasal cavity and forms an epithelial seam called the nasal fin. Only certain regions of the epithelium come into contact however, and remaining regions become part of the nasal and oral cavities.

Gaare and Langman (1980) investigated the process of epithelial fusion and studied DNA synthesis at stages prior to contact of the MNP and LNP. They found that the level of DNA synthesis in presumptive fusing epithelia and non-fusing epithelia decreased as the facial prominences came into contact. They concluded that since both types of epithelia, and not just the pre-fusion epithelia, showed decreased DNA synthesis, it was unlikely that the reduced level of DNA synthetic activity was an indication that the nasal fin was undergoing cell death. They felt that cells of the nasal fin were probably being incorporated into neighboring epithelial linings of the growing nasal and oral cavities. They had observed epithelial cell death in some cells of the nasal fin in an earlier study (Gaare and Langman, 1977a) and said that direct contact was not necessary to
trigger cell death. Using an acid phosphatase procedure that tests for lysosomal activity they found that adjacent epithelial cells transform to macrophages whose lysosomes digest the degenerating epithelial cells.

Gaare and Langman (1977b), also studied the epithelial cell surfaces of the approaching LNP and MNP using ruthenium red which is a positive dye that binds to negatively charged glycoproteins comprising proteoglycan moieties within the outer surface of the plasma membrane. They found that prior to contact, the pre-fusing superficial epithelia extend cell projections that are coated with glycoproteins, which they believed were necessary to mediate initial adhesion between the MNP and LNP.

The distribution of surface coat material has also been studied using lectins (Burk et al., 1979). Concanavalin A was used to bind surface coat material on pre-fusing and fused facial prominences. It was found that there was increased synthesis of surface coat material as the MNP and LNP were fusing and still a further increase once fusion had occurred. They concluded that this glycoprotein surface coat may aid adhesion and fusion of the facial prominences.

In a scanning electron microscope study (Yee and Abbott, 1978) looking at primary palate formation in the chick embryo, it was observed that prior to fusion, epithelium from the MNP, LNP and MxP started to exhibit long, slender filaments that extended towards the point of fusion. They believe that these "prefusion filaments" may function in alignment or adhesion of the facial prominences.

More recent studies have demonstrated that within the population of prefusion epithelium in the MNP and LNP a unique cell type may exist (Kosaka et
al., 1985; Kosaka and Eto, 1986). These cells have been called superficial cells and they are the ones that are thought to bridge the initial gap between the MNP and LNP. They possess well developed junctional complexes with associated intermediate filaments and microfilaments subjacent to the junctions. These superficial cells are located at the fusion site at a region of transition between squamous surface ectoderm of the primitive oral cavity and the pseudo-stratified columnar epithelium in the nasal pit. Kosaka states that superficial cells resemble the neural crest cells that make initial contact during neural tube closure. These cells like neural crest cells make initial contact first between lamellapodia, then between interdigitating cell projections. Fusing epithelia showed some cell death but so did epithelial cells at much earlier stages and at other locations such as at the nasal placode and early nasal pit. Kosaka proposes that epithelial cell death during primary palate formation is not only related to the fusion process between LNP and MNP but also to morphogenetic events in the growing face such as invagination of the nasal placode and growth of the prominences.

Recently, investigators have developed in vitro models to study the process of fusion between LNP and MNP (Gibson et al., 1989; Forbes and Steffek, 1989; Forbes et al., 1989). Epithelial cells in organ culture were found to extend filopodia across the fusion site that attach to the opposite facial prominence forming extensive bridges. These processes are thought to help during early fusion. This same group went on to compare the fusion process in a strain of mice susceptible to cleft lip. They used A/WySn mice, a strain with a 20-25% cleft lip
frequency (Kalter, 1979.) and found that the epithelial cells of the MNP and LNP in these mice had reduced epithelial bridging and fewer cell projections at the points of fusion compared to a non-cleft lip strain. This reduction in epithelial surface activity was proposed to account for the observed differences in facial clefting in the two strains.

1.4 Epithelial-Mesenchymal Interactions in Development: The basement membrane.

During embryogenesis there is constant interaction between adjacent tissues while a particular organ system is forming. These tissues are separated by substance known as the extracellular matrix (ECM). Grobstein in 1954 proposed that the ECM mediated these tissue interactions and was responsible for changes in tissue morphology as various organs developed.

In the early vertebrate embryo there exists a basic dichotomy of tissue organization. Cells can be grouped into one of two classes. The first is epithelia which consists of cells closely attached and linked by tight junctions, adherens junctions, and desmosomes (Bernfield et al., 1984). They form a sheet or covering that sits on top of an ECM. The cells facing the lumen usually have a specialized surface containing microvilli and a distinct glycoprotein surface coat. The basal surface of an epithelial cell faces a specialized type of ECM produced by the epithelial cell called the basement membrane.

The other class of cell is called mesenchyme and they migrate through ECM.
Mesenchymal cells can form clusters of cells grouped together but they are not always attached to their neighbors. The early embryo is made up mostly of epithelia. Mesenchymal cells are derived from these epithelia from sheets of cells that break away from the original cell population and migrate to new locations within the developing embryo (Noden, 1984; Moore, 1989). Primary mesenchyme forms from the primitive streak. Most of the mesenchyme of the developing face comes from the neural crest. This is a cell population that breaks away from neural ectoderm cells of the closing neural tube. As a few cells change shape in an epithelial sheet the form of the sheet will change possibly invaginating forming a tube or evaginating forming a bud. Cell shape changes starting with just a few cells can become morphologic changes at the tissue level. Epithelial cells that fold into buds such as the lung, liver, prostate and the kidney collecting tubules will go on to branch (Farquar et al, 1981). The budding and branching is due to specific epithelial-mesenchymal interactions that occur at precise times over short distances while an organ is developing. The interaction that takes place effects both tissues involved (Gurdon, 1987).

It is a common belief that the ECM is capable of controlling cell behavior since the ECM is closely associated with the cell surface. Molecules that link the cells to the ECM are closely associated with the cell membrane. They are integral glycoproteins that span the cell membrane and are thought to be able to interact with the cells cytoskeletal elements (Uitto and Larjava, 1991). These molecules can be in the form of protein receptors or proteoglycan receptors. An example is the recently discovered heparan sulfate proteoglycan named syndecan. This
molecule can bind a number of ECM components such as different collagens, fibronectin and tenascin, a molecule thought to be important in development (Halfer, et al., 1989). Syndecan is able to bind to cytoskeletal actin microfilaments and is thought to possibly be involved in cell-matrix and cell-cell interactions. It is thought that the arrangement of the cytoskeleton and the regulation of cell shape depends a great deal on the surrounding ECM. Cell shape changes on a multi-cell level could lead to morphological shape changes at the tissue level (Bernfield, M. 1984). Different interacting tissues can reciprocally modify the others ECM which may act as a signal for a group of cells to undergo a specific event such as mitosis or protein synthesis.

Epithelial cells and mesenchymal cells have different ECMs. The matrix is different in composition and in how it associates with adjacent cells. Both epithelial cells and mesenchymal cells are capable of making their own ECM. Epithelial cells synthesize a specialized type of ECM called a basal lamina and mesenchymal cells embed themselves in an interstitial matrix. The interstitial matrix of embryonic tissues is a highly hydrophilic gel, rich in interstitial fluid and the glycosaminoglycan hyaluronic acid. Fibronectin is often present as are collagen types I, III, and V. Chondroitin and heparan sulfate are also present. These molecules and others, are thought possibly to be involved in certain induction mechanisms that take place during morphogenesis. The basal lamina is specific for epithelial cells and is often called the basement membrane or basement lamina. Prior to the use of the electron microscope in biology the term basement membrane referred to the PAS-positive layer around epithelia and
muscle fibers visible with the light microscope. With the electron microscope it became apparent that the basement membrane was made up of component parts. The term basal lamina is an electron microscopic term that refers to the moderately electron dense layer found around epithelia, nerve, muscle and adipose tissue (Farquhar et al., 1981). In most locations the basal lamina consists of a lamina densa that is 20-50 nm thick, a lamina lucida which is less electron dense and is 10nm thick. The basal lamina consists of functional elements that regulate cell activities, and structural elements that impart a physical stability to the tissue. In every basal lamina there are certain components that may differ in relative amounts but are always present. These are: type IV collagen laminin, heparan sulfate proteoglycan, nidogen and bullous pemphigoid antigen (Charonis and Tsilibary, 1990; Kleinman et al., 1987). It is thought that these molecules in different proportions may impart to each basal lamina a certain specificity compared to others (Dziadek and Timpl, 1985; Wan et al., 1984). All basement membrane molecules are able to interact with each other. These associations, and others, with surrounding tissue and ECM may play a role in promoting growth and differentiation during development (Ferguson, 1984).

Recently, investigators looked at the spatial and temporal distribution of basement membrane components during maxillary process formation in the chick embryo (Xu, et al., 1990). They mapped the spatial and temporal distribution of laminin and type IV collagen in the face and found that regions that were undergoing rapid growth and expansion, such as the lateral surface of the maxillary process, showed less intense staining for type IV collagen. Regions that
were not undergoing rapid growth such as the roof of the stomodeum, stained with high intensity for type IV collagen. Laminin staining was uniformly high in both locations. They proposed that type IV collagen may confer structural stability to slow growing regions.

Epithelial-mesenchymal interactions are thought to be involved in the development of many organ systems in the body. Tooth, thyroid, lung, bone, kidney, mammary gland, salivary gland, gut, and pancreas are all thought to form as a result of interactions between epithelial and mesenchymal components (Mina and Kollar, 1987; Slavkin et al., 1984; Van Exan and Hall, 1983; Aufderheide and Ekblom, 1988). By performing tissue recombinations between different tissue types and even between different species it has been found that the mesenchymal component instructs the epithelium to follow a specific differentiation pathway. For example, Slavkin et al. (1984) used dental mesenchyme to induce the expression of enamel proteins in dental epithelium. He also stimulated undifferentiated lung mesenchyme to induce buds of epithelia to differentiate into alveolar type II cells. Hall in 1982 performed recombinations between mandibular arch epithelium and mesenchyme and induced chondrogenesis and osteogenesis in mandibular mesenchyme. He observed that different regions of mandibular epithelium induced a greater inductive response in the mesenchyme. He and others (Mina and Kollar, 1982; Van Exan and Hall, 1984; Lesot; et al., 1986) have hypothesized that inductive signals passing between the epithelia and mesenchyme may reside in the basal lamina. Direct cell-cell contact is not thought to be necessary for induction to occur although
some investigators believe cell contact may be important (Saber, 1989). This study performed recombinations between maxillary prominence epithelium and mesenchyme in the developing face. They performed homotypic (maxillary epithelium and maxillary mesenchyme), heterotypic (limb epithelium and maxillary mesenchyme), and heterochronic (epithelium and mesenchyme from different stages of development) recombinations and found that the mesenchyme would only remain viable if it was placed in contact with the epithelium being tested.

The morphogenesis of the submandibular salivary gland has often been cited as a model system for tissue interactions. The gland develops initially as a sheet of endodermally derived epithelial cells that starts to bud into mesenchymal cells that are derived from the neural crest (Bernfield et al., 1984). The bud elongates and forms a stalk so the structure now looks like a lollipop. Then notches start to form at the distal end of the bulb that then deepen and form clefts. This branching process repeats itself over and over. Eventually the epithelium becomes the secretory apparatus of the gland and the mesenchyme gives rise to the vascular supply and the supportive stroma. If the mesenchyme is removed from this system, branching ceases and the epithelial cells flatten and eventually die. Thus the mesenchyme is thought to be responsible for epithelial cell proliferation and epithelial integrity. Cell proliferation occurs most rapidly at the distal ends of the lobules. In order for notches and new clefts to form at the lobules distal ends the epithelial cells must first change shape. This is thought to be an actin mediated event. Basally arranged actin microfilaments contract giving
the epithelial cells a wedge shaped appearance (Spooner, 1973). These cells at
the cleft have a much lower rate of mitosis than do the epithelial cells at the
rowing tip. It was found by histochemistry that the clefts contain abundant
glycosaminoglycan and collagens type I and IV that are thought to aid
stabilization. The basal lamina is thick in the clefts but it is thinned and
discontinuous at the growing distal tips. There are direct contacts between
epithelial and mesenchymal cells at the growing tips that penetrate the basal
lamina (Cutler and Chaudry, 1973) and these may be locations for cell-cell
communication of some sort. The basal lamina is thought to help maintain the
lobular epithelial morphology of the gland. Banerjee et al. (1977) and Bernfield
(1981) propose that the basal lamina is influenced by the mesenchyme to
become discontinuous or become stabilized at different places at different times
as the salivary gland develops. Presumably the mesenchyme turns on and off
synthesis of basal lamina components by the epithelium.

Recently, the branching phenomenon of the developing salivary gland was
studied in organ-culture to determine what effect the mesenchyme had on
epithelial ability to branch (Takahashi and Nogawa, 1991). Epithelium was
separated from mesenchyme by a membrane filter. Normal branching
phenomenon was only observed when the epithelium was exposed to Matrigel, a
reconstituted matrix of basement membrane components. They used a
membrane filter with a pore size small enough to prevent direct cell-cell contact
and still observed normal branching if the Matrigel was present. It was concluded
that some sort of diffusible factor produced by the mesenchyme was necessary to
induce epithelial branching.

1.4 Some Examples of Epithelial to Mesenchymal Transformations in Development Associated With Basal Lamina Destruction.

In a number of developmental systems where an epithelial component is known to disappear and be replaced by a mesenchymal component there is a shared phenomenon that takes place. This is that the epithelial basal lamina must first break down before the epithelial cells are removed. The exact fate of the epithelial cells is as yet controversial and it still is not completely clear whether or not they 1) die as a result of a programmed cell death phenomenon, 2) migrate away into the mesenchymal cell population, 3) transform into mesenchymal cells, or 4) become incorporated into nasal and oral epithelium. In some systems it is thought that epithelial cells might become phagocytic like macrophages and degrade neighboring epithelial tissue. The developing organ systems to which this phenomenon might apply are: thyroid, lens, Mullerian duct and secondary palate formation.

Greenburg and Hay in 1988, studied thyroid follicular epithelium in vitro and found that isolated thyroid follicles that had their basal laminas enzymatically removed could transform into mesenchyme-like cells when grown on type I collagen gels. The epithelial cells switch from making type IV collagen to type I characteristic of mesenchymal cells. They also develop mesenchymal cell polarity, become elongate and develop pseudopodia and filopodia. In addition
they cease to make thyroglobulin. The newly formed mesenchymal cells elongate from the basal surface, detach from the follicle and invade the surrounding collagen matrix gel as single bipolar cells. Their cytoplasm becomes filamentous like mesenchymal cells and the intermediate filament production switches from keratin tonofilaments associated with desmosomes to vimentin characteristic of mesenchymal cells. There is no coexpression of keratin and vimentin. It is thought that a vimentin cytoskeleton is required for migration through a collagen matrix. It has been concluded that transformation from epithelium to mesenchyme is carefully controlled and only occurs in appropriate areas at predictable times.

Greenburg and Hay (1986) reported that anterior lens epithelium, when suspended in type I collagen matrices, gives rise to freely migrating mesenchymal-like cells. This transformation was also inhibited if the epithelial cells were grown on basal lamina substrate.

The regression of the Mullerian duct is another example of an epithelial to mesenchymal transformation where the basal lamina first disappears. The Mullerian duct is a structure that persists only if the phenotype is female. In the male this structure regresses. There is degeneration and phagocytosis of epithelial and mesenchymal cells, removal of the ductal basal lamina, and a loss of the distinction between the epithelium and the mesenchyme (Donahoe et al. 1984). This study reports that basal lamina integrity is lost prior to duct regression and this occurred only in areas where mesenchymal cell processes touch the basal lamina. Epithelial cell processes also extend into and through the basal lamina and come into contact with mesenchymal cells and their processes.
Disappearance of the basal lamina occurs only if the duct is destined to regress. They do report limited cell death in the regressing duct but they believe cell migration of epithelial cells into the mesenchymal cell compartment is a more important event. The study does not however report whether there was a switch from keratin to vimentin intermediate filament production in the transforming epithelial cells. Heparan sulfate proteoglycan synthesis did stop at the time of transformation and fibronectin lysis increased dramatically around the regressing duct. There is also increased hyaluronic acid production at the site where the epithelial cells are thought to be transforming which is thought to aid in motility of both epithelial and mesenchymal cells. The epithelial cells that apparently transform still express glucosamine residues even though they are now mesenchymal cells. Synthesis of laminin and type IV collagen also diminishes as the ductal basal lamina is degraded.

A recent example where there is evidence of an epithelial to mesenchymal transformation is during secondary palate formation (Fitchett and Hay, 1989). They investigated the fusion of palatine shelves in rat. These shelves rotate and approach each other in a medial direction and eventually fuse. The palatine shelves are lined with an epithelium and it is the medial edge epithelium (MEE) that comes into contact. The outer cells of the MEE initially slough off before contact is made. The two shelves then adhere and the epithelial seam thins and disappears leaving only islands of epithelial cells. Then these islands also disappear leaving mesenchymal cell confluence. This study reports that programmed cell death is probably not a major mechanism behind the epithelial
seams disappearance. There is cell death but only in the outermost peridermal
cells of the MEE. Again the epithelial cells that are presumptive mesenchymal
cells extend pseudopodia and filopodia before they migrate into the
mesenchymal cell compartment. The cells of the MEE seam also lose a keratin
type intermediate filament complement and gain a vimentin type cytoskeleton.
The basal lamina is continuous prior to fusion and is contacted by numerous
mesenchymal cell processes. The basal lamina then thins at the time contact is
made and soon becomes discontinuous and patchy and eventually disappears.
Hemidesmosomes are infrequently seen beneath the epithelial seam attaching
the epithelia to the basal lamina. In another study looking at secondary palate
formation, Ferguson (1988) reports that prior to contact of approaching palatal
shelves, the medial edge epithelium still possess an intact basal lamina. He used
type IV collagen as a marker. The epithelial cells stain intensely for desmoplakin,
a protein associated with desmosomes, until fusion occurs. After fusion
desmoplakin staining is reduced as the epithelial cells lose their desmosomal
attachments to their neighbors. The basal lamina becomes discontinuous and
fragmented after fusion occurs and TEM shows mesenchymal cell processes
extending through it.

1.5 Actin in Non Muscle Cells

During development there are many shape changes that occur in the
forming tissues. To understand the basis for morphogenesis in embryonic tissues
it is necessary to know where cells divide, migrate, die, or change shape. A number of investigators have proposed that components of the cytoskeleton could be involved in the coordinated shape changes often seen in developmental systems (Priess and Hirsh, 1986; Madreperla and Adler, 1989). These studies have attributed cell and tissue shape changes to the actin microfilament network and microtubules within the cytoplasm. A few of these examples will be discussed.

Actin is a major constituent protein of non muscle cells (Pollard, 1981). It is found in the periphery of most cells organized as filaments. It forms much of the so called "cytoplasmic matrix" and is a major component of the microfilament system. Globular monomeric forms of the actin molecule exist called G-actin. Filamentous forms also exist called F-actin. Many actin binding proteins regulate the size of actin filaments and the relationships of one filament to another (Pollard, 1981). Actin filaments can form either networks or bundles. Filament bundles can be of random arrangement and these usually have contractile properties, or they may be unipolar and tightly crosslinked with these having more structural properties (Vogl, 1989; Emerman and Vogl, 1986). Contractile rings located in the cleavage furrow of dividing cells, the zonula adherens junctions of most epithelial cells and fibroblast stress fibers are an example of contractile bundles. Actin filaments have been shown to be involved in numerous motility type events such as endocytosis, secretion, phagocytosis, cell translocation and intracellular vesicular movement. They also have more structural roles as in the cores of microvilli and stereocilia (Vogl, 1989). Many of
actin's functions involve an interaction between actin and the cell membrane. It is by this interaction that actin may provide the cell with a mechanism of: 1) establishing specific domains within a region of membrane, 2) internalizing regions of a membrane, 3) moving a membraneous organelle to a different location within the cell, 4) generating changes in the shape of the cell, and 5) moving the cell (Vogl, 1989). It is the last two mechanisms I am looking at during primary palate formation and changes in nasal morphology.

A good example illustrating the possible role of actin in morphogenesis is presented in a study by Preiss and Hirsh (1986). They investigated the growth pattern observed in the Caenorhabditis elegans embryo which undergoes rapid elongation of its body structure in the anterior-posterior axis. As the embryo elongates there is almost no division or migration of cells. Instead the cells throughout the embryo appear to change shape in a simultaneous and coordinated manner. It is thought that actin microfilaments constrict and microtubules distribute such that the embryo as a whole decreases in circumference and elongates. The cytoskeletal organization of certain cells within the embryo determines the embryo's shape during elongation while an extracellular cuticle maintains the body shape after elongation.

Retinal photoreceptors have also been studied to determine the role of microtubules and microfilaments in developing and maintaining the polarized shape of these cells (Madreperla and Adler, 1989). This study detected immunocytochemically longitudinally oriented actin microfilaments and microtubules in these photoreceptors and they postulated that within the retinal
photoreceptor cell there exists continuously active, oppositely directed, microtubule- and actin-dependent forces. These forces depending on how they balance, may be a determining factor in forming and keeping the shape and polarity of photoreceptor cells.

Other studies have taken a developing organ system and examined it in vitro under certain experimental conditions in order to test the idea of contractile proteins such as actin and myosin being involved in cell and tissue shape changes. Hilfer et al. (1977) examined thyroid placodes in the presence of a contractile medium consisting of Triton X-100 and ATP. Normally thyroid placodes take approximately 7 hours to evaginate in ovo, but in the presence of contractile medium, evagination was observed in minutes. They found that the cells involved in the shape changes within the placode were located at the periphery and the sharp bends that formed in contractile medium could not be explained by a simple pinching of cell apices at the point of folding. Instead they proposed that several forces may be acting at the site of evagination. The same contractile medium was used by Smuts (1981) in the study of nasal pit formation in the mouse. Previously it was thought that mesenchymal proliferation centers were responsible for the rise in the MNP and LNP on either side of the nasal placode (Lejour, 1969). Then it was thought that both epithelium and mesenchyme actively participate in the forming of the facial prominences (Portois, 1972). Minkoff and Kuntz (1977) found that the centers of mesenchyme did not undergo an increase in cell proliferation throughout the time of nasal placode invagination.
The morphological appearance of the forming nasal pits and facial prominences resembles the evagination of lens and thyroid (Hilfer et al., 1977). Both of these organs undergo early morphological movements in the absence of a contribution by mesenchymal cells. Development of a nasal pit normally takes about 8 hours from the placodal stage to an invaginated nasal pit. But in the presence of Triton X and ATP, this invagination occurred in 5 minutes (Hilfer, et al. 1977). The contraction medium stimulated the production of a small indentation in a newly formed placode region and a deep, pitlike invagination in a fully pseudostratified placodal epithelium. The heightened prominences were strictly epithelium and the mesenchyme had not changed position or appearance. They proposed that the rapid time for this precocious invagination does not allow the mesenchyme to add cells by either mitosis or cell migration to support the epithelium.

There has been much research done on the arrangement actin filaments take on in a number of different cell types. Most types of epithelial cells exhibit actin associated junctional complexes called zonula adherens junctions. These junctions have contractile bundles of actin filaments extending circumferentially along the cytoplasmic surface of the junctional membrane (Drenckhahn and Franz, 1986). Alpha actinin and vinculin are actin associated proteins that are located on the cytoplasmic side of the membrane. These proteins are thought to anchor actin filaments to the plasma membrane. Microfilaments can associate with the membrane laterally, where the filaments lie parallel to the cytoplasmic surface or end on, where the microfilaments seem to terminate at the surface.
(Rogalski and Singer, 1985). Cleavage furrows of dividing cells and stress fibers are examples of lateral associations while end on associations include the plaque like focal adhesion sites formed by fibroblasts to their substrata or with each other. Actin filament bundles have been shown to be present in specialized adherens junctions of Sertoli cells and are thought to play a functional role during spermatogenesis (Vogl and Soucy, 1985). F-actin is also found in myoepithelial cells of the mammary gland where these bundles are thought to control cell size and shape during lactation (Emerman and Vogl, 1986). During the development of the chicken eye, retinal pigmented epithelial cells extend apical projections containing abundant F-actin. These projections contain actin arranged in paracrystalline bundles that are thought to be more structural than contractile in nature (Owaribe and Eguchi, 1985). Cultured epithelial cells show different distributions of F-actin as they make a transition from a stationary to a motile state (Takeuchi, 1987). In a stationary state the cells are polygonal and F-actin is deposited along cell borders. The cells then become hemispherical and actin distributes along the inner cell surface. Then as the cells become motile they extend lamellae that contains an amorphous mass of F-actin at its distal end. Tucker et al. (1985) have studied neural crest (NC) migration and hypothesized that since cultured NC cells have actin microfilaments distributed at the cell cortex and not as localized dense focal contacts, the NC cells move through embryonic ECM making only weak adhesions with their substratum. They postulate that other embryonic cell types may generate stronger forces on the delicate ECM that would restrict their migration and thus set up morphogenetic events. Tomasek
and Hay (1984) cultured avian embryonic corneal fibroblasts in ECM and found that locomotory behavior was accompanied by adhesion of the cells to collagen fibrils in the ECM. The fibroblasts may then move by an interaction between the myosin rich cytosol and the F-actin rich cell cortex. By injecting phallotoxins into live cultured 3T3 fibroblasts, Wang (1987) showed that some actin filaments undergo continuous movement and reorganization in living cells.

1.6 Thesis Project

In this study I will describe the spatial and temporal distribution of F-actin in epithelium of the developing nasal cavity, nasal fin, and the facial prominences during the time period corresponding to primary palate formation. I will go on to describe changes in the basal lamina during nasal fin formation and regression using transmission electron microscopy. In the discussion, I will compare my observations to literature on other developmental systems.
2. MATERIALS AND METHODS

2.1 MOUSE COLLECTION PROCEDURES

C57Bl/6J mice were used for this study. Females were mated with males between 5 p.m. and 8 a.m., at which time the females were checked for the presence of a vaginal plug. Detection of a plug was considered to be day 0 of pregnancy. 12 midnight was considered as the time of conception. The mice were fed Mouse Chow and water ad libitum. The animal quarters were illuminated for 12 hours each day from 6 am to 6 pm. Pregnant females were sacrificed at specific times on the 10th, 11th, 12th, and 13th days of pregnancy. Ages of embryos were assumed, for example, to be 10 Days 8h (10/8) at 8 a.m. on the 11th day. Embryos were dissected from the uterus and placed in phosphate-buffered saline (PBS) at 4 °C. Before fixation the embryos were staged under a binocular dissecting microscope according to the number of somites from the caudal edge of the hind limb to the end of the tail (tail somites: T.S.). After counting the number of T.S. the embryos were prepared for either staining with NBD-phallacidin or observation using transmission electron microscopy. For this study 14 embryos were used for the F-actin experiments and 15 embryos were used for the TEM study with mice ranging from 7 T.S. to 27 T.S.

2.2 Tissue Preparation for Staining with Hematoxylin and Eosin (H/E)

Embryos were dissected from the uterus and placed in Bouins fixative. After a tail somite count was made, the heads were removed and placed in an Autotechnicon Model 2A for tissue processing. The tissue was dehydrated in a graded ethanol series: 1x75%, 2x95%, and 3x100%, and then cleared in
Chloroform. Each step in the procedure was for 20 minutes. The tissues were embedded in Paraplast Plus Tissue Embedding Medium containing dimethyl sulphoxide for rapid tissue infiltration. The melting point of the wax was 56-57 °C. The heads were then placed in a mould and orientated such that frontal sections could be taken of the faces. Sections were cut at a thickness of 7 micrometers on a microtome, mounted on glass slides, and dried in a 50 °C oven.

For staining with H/E sections were first dewaxed in xylene and then rehydrated in ethanol: 100%, 95%, 70% and washed in water. They were then stained in hematoxylin for 3 minutes and rinsed in tap water. This was followed by a dip in saturated lithium carbonate and the sections were washed again in water. Slides were then stained in eosin for 1 minute and then dehydrated through 3 baths of absolute alcohol and then cleared in xylene. The sections were then mounted in Entellan and photographed.

2.3 NBD-phallacidin as a Probe for Filamentous Actin

Phallacidin is one of the phallotoxins isolated from the American variety of the deadly Amanita phalloides mushroom. This compound has a low molecular weight (847 daltons) and thus is able to readily penetrate tissue. Phallacidin is a bicyclic peptide with a free carboxyl group to which the fluorophore Nitrobenzoxadiazole (NBD) is attached. Phallotoxins bind to both large and small F-actin but are unable to bind monomeric G-actin. Phalloidin is another phallotoxin with molecular weight 789 daltons. This molecule has a higher affinity for F-actin than does phallacidin and in this study it was used as a control. NBD is a small fluorescent molecule of 165 dalton molecular weight that is excited by
visible light and produces a yellow fluorescence when coupled to primary amines. The phallotoxins are highly specific (stain F-actin at nanomolar concentrations), are water soluble, and remain stable for a number of days. These characteristics make them very convenient probes for labeling and identifying F-actin in tissue sections.

2.4 NBD-phallacidin Protocol for F-actin Study

C57BL/6J mouse embryos were dissected from the uterus at different chronological ages and placed in 0.2 M phosphate-buffered saline (PBS). The embryos were then staged developmentally by counting the number of tail somites (T.S.). After T.S. counting the heads were removed. Fixation was performed in 3.7% paraformaldehyde for 30 minutes. A cryoprotection step was used to prevent tissue damage due to ice crystal formation upon freezing. This was performed using 40% sucrose for 12 hours. The specimens were then snap-frozen in hexane cooled to -70 °C. in liquid nitrogen. Embryos were embedded in OCT embedding media and oriented such that true frontal sections of the heads could be taken. The heads were sectioned on a cryostat at 8 to 10 micrometers thickness. Sections were placed on poly-lysine coated slides to prevent them from floating off the slide at later steps in the procedure. They were then post-fixed in acetone for 5 minutes and then allowed to air dry for 30 minutes. The tissue was rehydrated in PBS for 10 minutes and then incubated in one of the following treatment solutions: 1) PBS (a control for autofluorescence of the tissue); 2) PBS+ 1.65 x 10^-6 M NBD-phallacidin (fluorescent probe for filamentous
actin); 3) PBS + 1.65 x 10^{-6} M NBD-phallacidin + 1.04 x 10^{-4} M phallloidin
(competitive specificity control); and 4) PBS + 1.04 x 10^{-4} M phallloidin (control for
phallloidin treatment). Samples were incubated in the above solutions for 30
minutes and then washed 3 times in 100 microliters of PBS. This was followed by
dehydration in a graded series of alcohols; 70%, 95%, 2x100%, and then into
xylene. Sections were then mounted in Flo-Texx. Samples were viewed under a
Zeiss D-7082 Oberkochen standard ultraviolet microscope and photographed
using Fuji P-1600 film at 800 ASA.

2.5 Transmission Electron Microscopy Protocol for Basal Lamina Study.

C57BL/6J mouse embryos were dissected from the uterus and placed in
0.1M PBS for somite counting. The primary fixation was in 2.5% glutaraldehyde at
4 °C for 1 hour. This was followed by 3 washes, 5 minutes each in 0.1 M PBS.
After a 1 minute rinse in distilled water the tissue was fixed again this time in 1%
osmium tetroxide for 1 hour. Osmium tetroxide was made as a 4% stock solution
which was diluted to 2% in distilled water and then to 1% in 0.2 M PBS. This
secondary fixation was followed by two 5 minute washes in 0.1 M PBS and a 1
minute rinse in distilled water. Dehydration was performed in a graded series of
ethanols starting with 30% at 4 °C for 10 minutes, 50% at 4 °C for 10 minutes and
then 2% uranyl acetate at 4 °C for 30 minutes. Next the tissue was placed in 70%
at 4 °C for 10 minutes, then in 90% at room temperature for 15 minutes followed
by two 15 minute steps in 100% alcohol. Substitution was carried out in 1 part
propylene oxide: 1 part 100% ethanol first for 15 minutes and then in straight propylene oxide for 15 minutes. Embedding was first in 1 part epon: 1 part propylene oxide for 1 hour and then 1 part propylene oxide: 3 parts epon left overnight. Next the tissue was placed in straight epon plus catalyst for 1 hour. The samples were then placed in an incubator at 37 °C for 24 hours and then 60 °C for 48 hours. The blocks were trimmed and thin sections were taken using a diamond knife. Silver/grey sections were floated and collected on carbon/collodin coated 200 mesh Copper grids, counterstained with 2% uranyl acetate for 30 minutes and with lead citrate for 5 minutes. Five to ten sections were analyzed at each of the stages collected. The samples were then observed on a Phillips 300 transmission electron microscope and photographed using Kodak 4489 electron microscopic film. The film was developed for 2 minutes with Kodak D19 full strength, rinsed in tap water and fixed for 4 minutes in Kodak rapid fixer.

2.6 3-Dimensional Reconstruction of the Nasal Cavity

Five C57 mouse embryos (11,12,14,16, and 18 T.S.) were serially sectioned after being embedded in paraffin. The sections were taken at 7 micrometers thickness. A series of sections was chosen that illustrated the nasal cavity shape change anteriorly to posteriorly. Every second section (14 micrometers apart) was photographed using Fuji 100 film and made into 35 mm slides. These slides were then viewed through a Caramate slide projector and the faces were traced on
acetate transparencies. The three dimensional reconstruction program was implemented on a Hewlett-Packard 1000 Series E, minicomputer. It consists of two programs, one for data acquisition and the other for data display. Data acquisition is through the program BCDIG and data display is through BC VIEW. Each tissue section is plotted as a contour outlining the section. Contours are then plotted sequentially and observed at 40 degrees to the left and 0 degrees on the vertical plane.
**TABLE 1**  
**SAMPLE SIZES**

For the F-actin study 12 embryos were used:

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For the basal lamina study 13 embryos were collected:

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3. RESULTS

3.1 Nasal Fin Formation and Regression

Figures 1 and 2 show a series of C57 mouse embryos from 11 to 18 T.S sectioned in the frontal plane and stained with Hematoxylin and Eosin. These are intended to illustrate the various stages of primary palate formation. The LNP and MNP can be seen to approach each other and then fuse forming an epithelial seam known as the nasal fin (Fig. 1d). One can observe different stages of primary palate formation in a single embryo depending on the level of section. The nasal fin persists anteriorly before being replaced by a mesenchymal bridge. That is why Figure 1e shows MNP and LNP still prior to fusion and Figure 2e shows the same specimen in a more posterior region where a mesenchymal bridge has already formed.

3.2 F-actin Distribution at specific tail somite number

13 T.S

Various stages of prominence fusion were observed in the 13 T.S. embryos examined. Figure 3 shows LNP and MNP just prior to contact. Epithelium of the future nasal cavity is already stratified and possibly pseudo-stratified characteristic of fully differentiated olfactory epithelia. The roof of the nasal cavity is characterized by having epithelium that is several layers thick. This continues towards the nasal cavity base where there is a gradual transition to an epithelial cell layer of only a few cells. The epithelia that lay in the presumptive fusion area
of the two facial prominences varied in thickness but generally consists of fewer cell layers than nasal cavity epithelium.

In all embryos examined at 13 T.S., F-actin was found to concentrate in epithelia toward the apex of nasal cavity epithelia and it then gradually became more diffuse toward basal epithelia (Figs. 3c,d and 5c,d). This apical-basal differential was observed around the nasal cavity until the nasal cavity base where epithelium that lay in the pre-fusion region of the facial prominences seemed to lose its apical actin (Fig. 5c). In the 13 T.S. embryo examined that already had its MNP and LNP fused, the apical actin was re-established in regions facing the closed nasal cavity (Fig. 4d). Within the nasal fin however apical epithelial cells from the MNP and LNP that were now in contact had lost their apical actin (Fig. 4d). This point of transition can be seen in Figure 3e where the apical epithelial cells of the nasal fin appear to be disorganized and only spot densities of actin remain. These may be zonulae adherens junctions. Mesenchymal cells stained around the periphery of each cell and within cell processes (Fig. 6c). Mesenchymal cells take on a stellate appearance and are separated from their neighbours by extracellular matrix. At the junction separating epithelium from mesenchyme in nasal cavity epithelium there were observed concentrations of F-actin at the most basal aspect of the epithelium just above the basement membrane(Fig. 3d,e). This appeared to be arranged parallel to the basement membrane although it was difficult to discern the type of filament arrangement.
At 15 T.S. one of the embryos examined was sectioned more anteriorly than the other. Figure 6 shows a frontal section through the face where the MNP and LNP have not yet fused. The facial prominences are further apart in Figure 6a because this section was taken in a more anterior position relative to the sections stained with NBD-phallacidin. Prominent blood vessels can be seen in the MNP and the LNP. The epithelium of the nasal cavity is multi-layered and appears to be of the pseudostratified type. Again, looking at Figure 6b, it can be seen that staining for F-actin is most intense in epithelia toward the top of the nasal cavity. This intensity diminishes toward the base of the nasal cavity and toward the facial prominences. Along the LNP it can be seen that there is a transition zone roughly at the point where nasal epithelium starts to become oral epithelium (Fig. 6c). There is a marked decrease in the amount of F-actin present at this region. The MNP does not show a transition region as seen in the LNP epithelium. Actin staining was more intense in MNP epithelia. In apical epithelium certain cells showed spot densities of actin that were not seen in basal epithelium (Fig. 6d). There were however basally distributed actin filaments in epithelium just above the basement membrane that seemed to run parallel to the membrane. In the more posterior section (Fig. 7), the nasal fin has already broken down and has been replaced with a mesenchymal bridge. The thickening of epithelium seen medial to the nasal cavity is the naso-vomer organ which is a specialized epithelium designed for olfaction. This structure is quite pronounced in rodents but it is a vestigial organ in humans. Actin staining is again quite pronounced in
the most apical epithelium of the nasal cavity which can be more than 10 cells thick in some locations. In epithelium heading basally, actin appears to distribute around the cell periphery in the cortex of these epithelial cells. Intensity varies but again it appears to diminish in the basal direction. There seems to be increased actin content in apical epithelial cells located in regions of the nasal cavity where there is a bend and the epithelium changes its orientation. This is especially pronounced at the top of the nasal cavity but is also seen medially (Fig. 7b and c). Figure 8 is the same specimen as Figure 7 and it shows the nasal cavity more ventrally toward its base. It can be seen how the medial and lateral sides of the cavity appear to stain differently for actin when actually the epithelium has just been sectioned along a different plane. The more lateral aspect shows epithelial cells cut *en face* with the plane of section going through a layer of cells such that it just catches a sheet of circumferential bundles of actin filaments coursing around individual epithelial cells. Actin staining is quite intense in this region as well as through the epithelial layers heading basally. Toward the more lateral aspect, the actin staining pattern more closely resembles nasal cavity epithelium encountered previously with higher actin content in the apical epithelia compared to the basal epithelia. It is interesting that even though there is a bend in the epithelium at the base of the nasal cavity one does not observe the increase in actin content of apical epithelium in this region as seen at the top of the cavity. This epithelium instead appears to have very little actin. Perhaps this epithelium is somehow different than other regions of nasal epithelium in that it is at a different level of differentiation. One does see the occasional spot density of F-
actin that may be due to the presence of adherens junctions (Fig. 8). The most basal layer of epithelial cells that contacts the underlying mesenchyme seems to have re-established its characteristic pattern of actin filament arrangement where the filaments appear to align parallel to the basement membrane. The nasal fin has disappeared at this point and has been replaced by a mesenchymal bridge. This would imply that the organization of F-actin in epithelium of this region would have been temporarily disrupted while the mesenchymal bridge was forming and that it would then have to re-assemble once the nasal fin epithelium had disappeared. Mesenchymal cells also stain for actin, some more intensely than others. Mesenchymal cell processes containing F-actin can be seen to extend and come in contact with the epithelial cell layers (Fig. 6c and d). These processes also contact other mesenchymal cells. Mesenchymal cells beneath the nasal cavity comprising the mesenchymal bridge in Figure 8 stain quite intensely for F-actin.

16 T.S

The embryo examined at 16 T.S. shows a frontal section through the face of an embryo where the nasal fin has broken down and been replaced with a mesenchymal bridge. The MNP and LNP can be seen in Figure 9 and a portion of the maxillary prominence is also included. This nasal cavity provides a good example of the F-actin distribution seen at areas where there is a bend in the nasal cavity. At the top one observes high concentrations of actin in apical epithelia that radiates out in all directions proceeding basally. There is an abrupt
transition at the junction between epithelium and mesenchyme. Actin filaments take on an arrangement parallel to the basement membrane but it is difficult to tell whether the epithelial cells or the mesenchymal cells contain the actin arranged like this (Fig. 9). As one proceeds toward the base of the nasal cavity, the high concentration of actin in apical regions of the epithelium continues and appears to be relatively equal on both medial and lateral sides. This high actin density diminishes however about midway down the cavity. The apical epithelia on the lateral side shows this most apparently. The epithelia toward the more medial side continues to exhibit high concentrations of actin. The nasal cavity bulges medially and the epithelium increases the number of layers in this region. Where the epithelium bends and reorients to progress laterally there is a high concentration of actin at the apex of epithelia at the bend that seems to radiate out in finger-like projections towards basal epithelia. These fingers extend to only about halfway through the stratified epithelia. Then, as was the case at 15 T.S., there is a marked decrease in the amount of actin seen in apical regions of epithelia located at the base of the nasal cavity.

17.T.S.

At 17 T.S. the tissue has been torn beneath the brain cavity; however the nasal cavity remained completely intact. Figure 10 shows a face again in the frontal plane where the MNP, LNP, and MxP are clearly visible. One can also see a thickening of epithelium on the medial side of the nasal cavity that is the naso-vomer organ. The heart can also be seen although it is damaged in this
preparation. At higher power, the nasal cavity can be seen to stain abundantly with F-actin. At this later stage of development it can be seen that the shape of the nasal cavity has become more complex. The epithelium takes on numerous folds and evaginations as development proceeds. The top of the nasal cavity contains the characteristic high concentration of apical actin in epithelium closest to the cavity lumen. The finger-like projections of F-actin only extend to about one third the thickness of the nasal epithelium and they disappear. Progressing laterally there is a gradual bend in the nasal cavity that has a high content of actin in its epithelium. The medial side at this same level contains only a thin but intense band of actin at the most apical aspect of the epithelium next to the cavity lumen. These may actually be adherens junctions connecting epithelial cells together. The medial and lateral layers of epithelium at the base of the nasal cavity in this specimen approximate and continue along side one another. It appears that these epithelial layers are still a component of the nasal fin which has just started to break down (see Fig. 11). The actin staining appears more intense in this region of contact because there are now two layers of apical epithelium contributing to the fluorescence. On the lateral side of the face at the base of the nasal cavity lumen, the epithelium becomes quite disorganized. It is difficult to see where the epithelium ends and the mesenchyme begins in this region. Possibly the epithelium is undergoing some morphogenetic shape changes. The epithelium here seems to thin and not every epithelial cell stains for actin with the same intensity. At the extreme base of the nasal cavity, where the mesenchymal bridge is just one or two cells wide, the actin staining is quite faint and perhaps
the epithelium is still reorganizing and new actin filaments that will comprise the basal epithelium have not yet been synthesized. Across the mesenchymal bridge one can see future oral epithelium. Part of the nasal fin still persists in this epithelium as a bud or thickening (Fig. 11). There appears to be a concentration of actin filaments in the basal layers of this oral epithelium similar to that seen in nasal epithelium previously. Mesenchymal cells toward the medial side between the nasal cavity and the naso-vomer organ stain quite intensely for F-actin (Fig. 11) as do the mesenchymal cells on the medial side of the mesenchymal bridge. On the lateral side of the mesenchymal bridge the mesenchymal cells still stain for actin but do so with less intensity.

18 T.S.

This specimen (Fig. 12) although at a higher level of development than the previous 17 T.S. embryo shows almost the same stage of nasal fin regression and mesenchymal bridge formation. This embryo at 18 T.S. has a mesenchymal bridge that is slightly larger and more robust than the 17 T.S. specimen. All three facial prominences can be identified easily and it is interesting to note that the MxP has appeared prominently in specimens where the mesenchymal bridge has started to form. In this example there is differential distribution of actin staining in different regions of the nasal cavity. At the top, there is the characteristic high concentration of F-actin where there is a bend in the epithelium. The more apical epithelium stains more intensely than does the basal epithelium again. The finger-like projections of F-actin radiate out in the basal
direction. Midway down the nasal cavity on the lateral side there appears to be abundant spot densities of actin possibly due to adherens junctions. On the medial side of the nasal cavity the plane of section has exposed epithelial cells cut en face as seen previously in Figure 10. It is notable that even when there is only a small bend or directional change in the epithelium lining the nasal cavity as the epithelium evaginates, that one still observes an increase in actin staining intensity (Fig. 12 and 13). The epithelium beneath the cells cut en face shows actin filaments arranged differently than that observed previously. Towards the base of the nasal cavity, the apical regions of epithelium continue to show strong fluorescence. In Figure 12 on the lateral side, the epithelial-mesenchymal junction is very prominent. One can see numerous focal densities of F-actin in the basal epithelium that demarcates the transition to mesenchyme. The mesenchymal bridge in Figure 13 appears to be three or four cells wide at this point. The basal epithelium of the nasal epithelium appears disorganized and the epithelial-mesenchymal junction is not apparent. These cells may be undergoing rearrangement and the final organization of F-actin has not yet been established. The same could be said about the oral epithelium at the base of the mesenchymal bridge. Its epithelial cells also appear to be somewhat disorganized (Fig.13).

19 T.S.

Figure 14a illustrates that the nasal fin persists for a certain time before it regresses. The section was taken in the anterior of the face where the nasal fin
epithelium was still present. In any one embryo it may be possible to observe all stages of primary palate formation. In this case we are still observing the nasal fin comprising epithelium from the MNP and LNP. The nasal cavity shows prominent actin staining in apical epithelium which becomes more diffuse basally. Towards the base of the nasal cavity the apical actin concentrations continue but to a lesser extent (Fig. 15). The epithelial-mesenchymal junction is quite apparent around the base of the nasal cavity. It does however seem to disappear on both lateral and medial sides of the nasal fin. The basal actin then reappears at the oral side of the nasal fin (Fig. 15). This may indicate a different type of epithelium may be present along different regions of the nasal fin. In these regions that may be just about to disperse and break up there are no spot densities of F-actin and possibly therefore no adherens junctions. This region of no actin filament assembly in basal epithelium corresponds to the area of initial mesenchymal bridge formation seen in Figures 11 and 13.

27 T.S.

The primary palate in this embryo (Fig. 16) has formed completely at this stage of development. The mesenchymal bridge is quite robust and there is no nasal fin present. The shape of the nasal cavity has changed to that of a more differentiated state. Figure 16a shows how at points where the nasal cavity bends one still finds increased apical actin concentration. Nasal cavity epithelium still shows an increased actin distribution in apical epithelium with a network appearance of intermediate epithelial layers and then a prominent parallel
arrangement at the epithelial-mesenchymal junction (Fig. 16a,b,c).

Actin is generally more uniformly distributed all around the nasal cavity; however there are higher concentrations at bends. Actin at the apex of epithelium is more uniform around the nasal cavity as well.

3.3 Basal Lamina Changes During Primary Palate Formation- TEM

Results.

7 T.S.

At 7 T.S. the LNP and MNP have not yet fused and what is shown in Figure 17 is a high magnification view of the epithelium comprising the MNP. The most basal layer of epithelial cells is shown and a quite intact basal lamina can easily be distinguished. The epithelial cells themselves contain abundant polyribosomes as well as numerous cisternae of rough endoplasmic reticulum (RER). The epithelial cells can be seen to extend cell processes toward the basal lamina, but at no point do these processes extend through it. Stacks of the Golgi apparatus can also frequently be seen in the epithelial cells. These cells would seem to be highly metabolically active due to the large number of mitochondria present (Fig. 17b). Also apparent are a number of endocytotic vesicles that may in fact be clathrin coated pits located just above the basal lamina (Fig. 17b,c). Mesenchymal cells that underlie the epithelial cell layer also contain abundant clusters of free polyribosomes, mitochondria, but relatively less RER. The mesenchymal cells appear attached to neighbouring cells through junctional complexes (Fig. 17b,c). Endocytotic vesicles can also be seen in the occasional
mesenchymal cell as well (Fig. 17b).

10 T.S.

The MNP and LNP have not yet made contact at this stage of development. Basal epithelial cells that are in the presumptive fusion area of the MNP and LNP have a basal lamina that is still intact (Fig. 18a,b). The epithelial cells look active and contain many free polyribosomes and mitochondria. In the LNP basal epithelia there are what appear to be endocytotic vesicles within the cell cytoplasm as well as fused to the plasma membrane on the side of the cell opposite to the basal lamina (Fig. 18b). At this point the epithelial cells and the mesenchymal cells do not extend cell processes into the basal lamina, but on the MNP further down in the direction of the oral cavity one does see cell processes from both epithelial and mesenchymal cells (Fig. 18c). In Fig 18c, a cell process from the epithelial cell seems to extend into the basal lamina and even break through it. The opposing mesenchymal cell also extends a cell process that makes a direct cell-cell contact with the epithelial cell. There may even be a junctional complex forming between these cells. One can also observe debris possibly basal lamina remnants and other extracellular matrix material.

11 T.S.

At this stage the MNP and LNP have fused and formed a nasal fin. The epithelial cells on the side of the MNP have become fragmented and their basal lamina has become patchy and discontinuous (Fig. 19a). It appears as though the
epithelial cell processes have penetrated the basal lamina. The epithelial cells themselves appear alive with no signs of necrosis at this level of the nasal fin. Endocytotic vesicles also appear in some of the epithelial cells. Mesenchymal cells also extend cell processes that approach the basal lamina. On the side of the LNP the basal lamina is still intact and no damage is apparent. The epithelial cells have started to extend small cell processes into the basal lamina but these have not yet broken through it (Fig. 19b). Mesenchymal cells also extend cell processes but they do not come in close contact with the basal lamina. The nuclei of the epithelial and mesenchymal cells in Figure 19b is very euchromatic indicative of a high level of cell activity. Mitochondria are also quite abundant in both these cell types.

Figure 20 shows an embryo at the same number of tail somites where a nasal fin has formed and there does seem to be some indicators of necrosis in some of the epithelial cells comprising the nasal fin. The basal lamina is fragmented in some areas although the epithelial cells do not possess an elaborate cell process network yet. The basal lamina appears patchy on both the LNP and MNP sides of the nasal fin (Fig. 20a,b). Figure 20c shows an epithelial cell within the nasal fin that is about six cells thick at this point that looks necrotic. The cell appears vesiculated and the nucleus is condensed. Multivesicular bodies are also present.
12 T.S.

The embryo shown at 12 T.S. has a nasal fin that has not yet been broken down and there is an increase in the amount of necrotic activity in these epithelial cells compared to that seen in the 11 T.S. example. Figure 21a, and b shows a number of epithelial cells comprising the nasal fin on the MNP side. One can observe that some of the cells have condensed nuclei, tertiary lysosomes and electron dense multivesicular bodies. The cells appear to be fragmented and there are also large intercellular spaces some of which could be lumen of blood vessels. Figure 21b is a portion of the nasal fin below 21a in a more ventral position. The mesenchymal cells show no signs of necrosis. These cells have large euchromatic nuclei many free polyribosomes and abundant mitochondria.

13 T.S.

The nasal fin epithelia shown in Figure 22 illustrates that although the epithelia should be starting to regress by 13 T.S. certain regions exist completely intact. Nasal fin breakdown does not occur simultaneously in all epithelial cells comprising the fin. Depending on where the section was taken one could observe different stages of primary palate formation. The nasal fin may be regressing in this embryonic face in a more posterior position, but this section was taken anteriorly. The epithelial cells of the MNP appear quite active with many polyribosomes in the cell cytoplasm and abundant mitochondria and RER. In contrast to the cells shown in Fig. 21, this portion of the nasal fin shows no areas of necrosis. Some of the epithelial cells of the fin more toward the oral cavity have
started to extend cell processes into the intact basal lamina but these cell extensions have not penetrated through it yet (Fig. 22b). On the lateral side these epithelial cells lie flat against their basal lamina still and no cell processes are evident. The mesenchymal cells at this stage are very fragmented and their cell processes extend and approach the basal lamina but do not penetrate it. The mesenchymal cells also contain abundant mitochondria indicative of their being highly active metabolically.

15 T.S.

The nasal fin shown in Figure 23 is one that is likely to regress quite soon. The lateral side of the fin is illustrated. Epithelial cells appear fragmented and have developed numerous cell processes that come in close contact with but do not pass through the basal lamina. The basal lamina is still quite intact although portions of it may have started to breakdown (Fig. 23b). The mesenchymal cell processes appear to contain microfilaments as shown in Fig. 23a. These processes may extend and touch the basal lamina and possibly even the epithelial cell on the other side. Figure 23b shows a higher magnification view of a mesenchymal cell process in contact with the epithelial basal lamina. There is an electron dense region just at the point of contact possibly the site of a cell-matrix interaction of some kind.
By 19 T.S. the nasal fin has broken down and mesenchymal cells have invaded the region. The fin may still persist in anterior locations however. Figure 24 and 25 show the same nasal fin just at a location where it is breaking down. The fin is perhaps six cells thick and is composed of epithelial cells from both medial and lateral facial prominences. The surface cells in contact with epithelia of the opposing prominence are somewhat squamous in appearance whereas the more basal cells are more cuboidal in shape (Fig. 24a). The cells appear quite healthy and no necrotic activity is seen. Figure 24b shows a high magnification view of an epithelial cell on the lateral side of the nasal fin illustrating an intact basal lamina with one possible endocytotic vesicle. Figure 25 illustrates how the basal lamina appears just at the top of the nasal fin that has regressed and is exposed to mesenchymal cells that are beginning to form a mesenchymal bridge. The lamina is quite fragmented and patchy at the tip of the nasal fin (Fig. 25b). It is unclear whether it is still being degraded at this point or whether it is being resynthesized. It is also difficult to tell whether the epithelial cell at the uppermost tip of the nasal fin is necrotic or whether it is preparing to migrate and become a constituent cell of the surrounding mesenchymal cell population. Figure 25a illustrates how at the tip of the regressing nasal fin the cells are very fragmented and it is not clear whether these cells are epithelial or mesenchymal in nature. Figure 26 shows the same nasal fin as Figs. 24 and 25 but this figure shows epithelial cells of the MNP side of the nasal fin more in the ventral direction toward the future oral cavity. One can see mesenchymal cell
processes making direct contacts with the basal lamina and possibly the epithelial cells as well (Fig. 26a,b). Figure 26c shows a mesenchymal cell that is about to divide. Its chromatin is condensed and it remains attached to neighbouring mesenchymal cells via tight junctions.

21 T.S.

Figure 27 illustrates an anterior region of the face where a nasal fin still temporarily persists but is most likely about to regress. A section of the basal layer of epithelial cells on the lateral side of the fin is shown. The epithelial cell in Fig. 27a has extended a cell process through the basal lamina. The lamina is fragmented and discontinuous in regions where epithelial cell processes are located. The epithelial cells show no necrotic activity and appear to be quite metabolically active. The cell processes of both the epithelial and mesenchymal cells contain microfilaments that can not be seen in other parts of the cells.

27 T.S.

By 27 T.S. the mesenchymal bridge has formed and the primary palate is considered to be completely formed. Figure 28 shows two basal epithelial cells that comprise the base of the nasal cavity and the top of the mesenchymal bridge. This surface is no longer denuded and it appears as though the basal lamina underlying the epithelial cells in the region has been repaired and laid down again. Figure 28a shows an area where basal lamina material appears to be thicker than normal and may be a site where resynthesis is occurring. The
epithelial cells here now will go on to differentiate and become components of the nasal cavity epithelia. It is interesting that the mesenchymal cell processes are still present in the resynthesizing stages of primary palate formation and one could speculate that they may be involved in cell-cell communication or signalling between epithelia and mesenchyme.

3-Dimensional Reconstructions

Figure 29 shows a series of developing nasal cavities during the time of primary palate formation. The reconstructions make it easier to interpret the 2-D sectioned material and the changing nasal morphology can be better visualized. Early in primary palate formation, at 11 and 12 T.S., the nasal cavity has a simple shape resembling a tube. The nasal fin is about to form as the MNP and LNP approach and fuse. Later, its shape becomes more complex as the region grows and expands (Figure 29c-e). The epithelium bends at a number of locations and if one compares this to Figures 9 and 10, the actin staining in apical regions of epithelium at these bends can be seen.
FIGURE 1. a) An 11 T.S. C57BL/6J mouse embryo frontal section taken anteriorly. These specimens were embedded in paraffin and stained with hematoxylin and eosin. Facial prominences not yet fused. MNP, medial nasal prominence; LNP, lateral nasal prominence. b) 12 T.S. Prominences not yet fused. c) 14 T.S. Prefusion stage. d) 16 T.S. Nasal fin has formed (NF). e) 18 T.S. Prefusion stage. NC, nasal cavity; OC, future oral cavity.
FIGURE 2. The same embryos as Figure 1 sectioned in a more posterior position. 

a) 11 T.S. A nasal fin has formed (NF). MNP, medial nasal prominence; LNP, lateral nasal prominence; Mxp, maxillary prominence; NC, nasal cavity. 

b) 12 T.S. Nasal fin has formed. NV, nasovomer organ. 

c) 14 T.S. Nasal fin has regressed and a mesenchymal bridge has formed (MB). 

d) 16 T.S. A nasal fin still persists. 

e) 18 T.S. A mesenchymal bridge has formed and will now continue to enlarge as the primary palate.
FIGURE 3. A 13 T.S. face. a) Prefusion stage stained with hematoxylin and eosin (H&E) showing MNP and LNP just prior to contact. b) Same specimen stained with NBD-phallacidin. c) Top of the nasal cavity. Note the intense fluorescence of the apical epithelial cells. E, epithelium; M, mesenchyme. d) Wall of the nasal cavity on the medial side. e) Nasal fin region. Note the spot densities of F-actin (arrows).
FIGURE 4. a) 13 T.S. embryo showing a nasal fin stained with H&E. b) Section stained with NBD-phallacidin. MxP, maxillary prominence. c) Nasal cavity toward the lateral side. Note the mesenchymal cell processes (arrows) extending to neighbouring cells and how the level of fluorescence varies. d) A nasal fin. Note how the apical epithelial cells of the nasal fin do not fluoresce.
FIGURE 5. a) T.S. section stained with H&E showing MNP and LNP just prior to fusion. b) Section stained with NBD-Phallacidin. c) View of the nasal cavity just approaching the nasal fin. Note the disappearance of fluorescent activity in apical epithelial cells (arrow). d) Nasal fin. Note the minimal fluorescent activity in nasal fin epithelia.
FIGURE 6. a) A 15 T.S. frontal section during the pre-fusion stage stained with H&E. b) A section further posteriorly stained with NBD-phallacidin. c) Closer view of the LNP. Note the transition zone where epithelium stops fluorescing (arrow). d) The MNP with a blood vessel.
FIGURE 7. a) A 15 T.S. embryo stained with NBD-phallacidin. NV, nasovomer organ; H, heart. b) Nasal cavity on the medial side. Note the intense staining apically (arrow). c) Top of the nasal cavity. Note the bend in the epithelium and the intense fluorescence in this region (arrows).
FIGURE 9. a) A 16 T.S. embryo stained with NBD-phallacidin. Note the increased level of fluorescence at points in the nasal cavity where there is a bend or evagination. b) Higher power view of the nasal cavity. Observe the differential distribution of F-actin at different locations around the cavity. Note the finger-like extensions of actin at the evagination points (arrows). Note also the absence of apical actin staining at the base of the nasal cavity. Dotted line, the epithelial mesenchymal junction.
FIGURE 10. a) A 17 T.S. embryo stained with NBD-phallacidin. b) Top of the nasal cavity. Note the intense actin staining in mesenchymal cells on the medial side between the naso-vomer organ. Also see the increased fluorescent activity at bends in the nasal cavity.
FIGURE 11. Same 17 T.S. embryo as Figure 10 but at the base of the nasal cavity. Note the disorganized appearance of these epithelial cells at the base. Note also the mesenchymal bridge that is one cell wide (large arrow). OE, oral epithelium.
FIGURE 12. a) An 18 T.S. embryo stained with NBD-Phallacidin. Note the mesenchymal bridge. b) The top of the nasal cavity. Note the epithelial cells on the medial side of the cavity have been sectioned en face. See how even at small bends or evaginations (E) one still sees an increase in the amount of F-actin. Observe the epithelial-mesenchymal junction (dotted line).
FIGURE 13. The base of the nasal cavity of the same 18 T.S. embryo shown in the previous figure. See also the mesenchymal bridge (MB) here perhaps three cells wide. BV, blood vessel.
FIGURE 15. A close up view of the nasal fin of the previous 19 T.S. embryo. Note the spot densities of F-actin in epithelium at the base of the nasal cavity. Also notice how the actin fluorescence at the epithelial-mesenchymal junction disappears at areas where the mesenchymal bridge is about to form (between the arrows).
FIGURE 16. a) A 27 T.S. embryo stained with NBD-phallacidin. Note the site of possible ingrowth of olfactory nerves (N). The primary palate has now formed completely. b) This is a region of the nasal cavity on the left side of the face. Note the differential actin distribution in apical versus basal epithelium. c) This is a section of the left nasal cavity at its base. This is also the top of the mesenchymal bridge (MB). d) A section of the left nasal cavity indicating the slightly reduced level of fluorescence in this region of apical epithelium.
FIGURE 17. a) A transmission electron micrograph (TEM) of a 7 T.S embryo showing the basal layer of epithelial cells of the MNP. At this stage of development the LNP and MNP have not yet fused. Note the epithelial cell process extending into the basal lamina (arrows). b) A view of these basal epithelia in a more ventral position. Note the endocytic vesicle (V). c) A view further ventrally showing how the basal lamina is continuous at this stage of development. (Bar=1 micrometer)
FIGURE 18. a) A TEM of a 10 T.S. embryo where the facial prominences have not yet fused. The basal lamina is continuous and intact. M-medial nasal prominence. b) This micrograph was taken at about the same level as (a) but on the lateral side. L, lateral nasal prominence. Note the endocytotic vesicles. c) A region on the MNP in a more ventral position showing an epithelial cell process extending into the basal lamina. (Bar=1 micrometer). Arrows show the basal lamina.
FIGURE 19. a) A TEM of an 11 T.S. embryo where the LNP and MNP have formed a nasal fin. The micrograph was taken on the MNP showing regions of basal lamina destruction. Note the epithelial cell processes penetrating the basal lamina and the debris of degraded extracellular matrix remaining. b) A view from a similar level on the side of the LNP showing an intact and still continuous basal lamina. c) A necrotic epithelial cell within the nasal fin. (Bar=1 micrometer).
FIGURE 20.  a) A TEM of an 11 T.S. embryo that has formed a nasal fin. The micrograph was taken on the MNP side. Note that the basal lamina is continuous but patchy in regions. b) This is a micrograph of the basal epithelia at about the same level as (a) but on the LNP side. See how the basal lamina is patchy and starting to fragment. c) This is an epithelial cell on the MNP side that appears to be necrotic. (Bar=1 micrometer)
FIGURE 21. a) A TEM of a 12 T.S. embryo where a nasal fin has formed and has not yet broken down. Note the necrotic activity in some of the epithelial cells. b) This micrograph is of a more ventral position showing necrotic epithelial cells containing tertiary lysosomes and multivesicular bodies. (Bar=1 micrometer)
FIGURE 22. a) A TEM of a 13 T.S. embryo where the MNP and LNP have formed a nasal fin. This is a view from the MNP showing a continuous and intact basal lamina. b) This is a more ventral position on the MNP showing a continuous basal lamina and epithelial cell processes extending into but not through it. c) Shown here is a corresponding position on the LNP side of the nasal fin. Basal lamina is intact and continuous. This continues ventrally in d. (Bar=1 micrometer)
FIGURE 23. a) A TEM of a 15 T.S. embryo where a nasal fin has formed and is likely to soon regress. Shown is a view from the LNP. Note the fragmented epithelial cells and the mesenchymal cell process. b) A higher magnification view of the point of mesenchymal cell contact with the basal lamina. Note the electron dense region. Also see the fragmented basal lamina material (arrow). (Bar=1 micrometer)
FIGURE 24. a) A TEM of a 19 T.S. embryo sectioned anteriorly where a nasal fin still persists. Above this in a dorsal direction the nasal fin has regressed. Observe that the fin consists of about six cell layers. b) This is an epithelial cell on the LNP side of the nasal fin showing an intact basal lamina and possibly an endocytotic vesicle (V). (Bar=1 micrometer)
FIGURE 25. a) A TEM showing the tip of a nasal fin in the same 19 T.S. embryo as Figure 25 and (b) is the same view at a higher magnification. Notice the fragmented appearance of the cells close to the tip of the nasal fin as well as the basal lamina debris (arrows). A, artifact (Bar= 1 micrometer)
FIGURE 26.  a) This is a TEM of the residual nasal fin as seen from the MNP of the 19 T.S. embryo shown in Figs. 25 and 26. The mesenchymal cell processes make direct contact with the epithelial basal lamina and possibly with the epithelial cell itself. Possibly the site of a cell-cell interaction. b) Mesenchymal cell processes contact the basal lamina in a more ventral location as well. c) A micrograph showing a mesenchymal cell about to divide. This cell is located on the LNP side of the nasal fin near the tip. (Bar=1 micrometer)
FIGURE 27. a) A TEM of a 21 T.S. embryo where a nasal fin still persists since this section was taken anteriorly. These micrographs are from the LNP. An epithelial cell process has penetrated the basal lamina which is patchy and discontinuous. The epithelial and mesenchymal cell processes appear to contain microfilaments (MF). Progressing ventrally along the nasal fin (b,c, and d) one can see patchy basal lamina and epithelial cell processes extending into it. One also sees a number of endocytotic vesicles (V). (bar=1 micrometer)
FIGURE 28. a) A TEM of a 27 T.S. embryo where the primary palate has completely formed. This is a view from the most dorsal aspect of the mesenchymal bridge at the base of the nasal cavity. Observe the newly synthesized basal lamina (arrows in a). b) An epithelial cell just lateral to the one in a). Note that the basal lamina is completely intact. E, epithelial cell, MB, mesenchymal bridge. (Bar=1 micrometer)
FIGURE 29. A series of three dimensional reconstructions of the developing nasal cavity during the time of primary palate formation. Nasal epithelium, medial nasal prominence epithelium, and lateral nasal prominence epithelium can be seen. a) An 11 T.S. embryo. b) 12 T.S. c) 14 T.S. d) 16 T.S. e) 18 T.S.
4. DISCUSSION

4.1 Possible Role of F-actin in Regulating Nasal Morphology in Light of the 3-Dimensional Reconstructions

The nasal cavity was shown to undergo dramatic changes in shape anteriorly to posteriorly. This shape change became more pronounced as the nasal cavity continued to differentiate and develop. At 11 and 12 T.S., the cavity resembled a hollow tube that formed a ridge on its most dorsal aspect and an evagination towards its medial aspect. The cavity by 14 T.S. started to bend in its mid region and by 16 T.S. this bending became more pronounced.

After visualizing the shape of the nasal cavity from the 3-D reconstructions, it was easier to analyze the actin staining pattern from 2-D sections. Actin distribution was uniform all the way around the nasal cavity, however staining intensity was increased at points where there was a bend. Particularly, the apical layers of epithelium were more intense than basal layers. The epithelium lining the primitive nasal cavity showed a constant pattern of actin distribution at all stages of development. Occasionally, spot densities of actin staining were seen in epithelium facing the lumen that could represent actin associated adherens junctions. Possibly, actin in these regions could provide structural support to the nasal cavity. This was similar to thyroid evagination studied by Hilfer et al. (1977) where the apical epithelial cells contained more microfilaments in their cytoplasm than did basal epithelial cells. My results in the nasal cavity contradicted the actin
arrangement found in salivary gland morphogenesis (Spooner, 1973) where the basal cells constricted due to F-actin contraction causing the cells to assume a wedge shape. The system in the salivary gland is an invagination rather than an evagination as in the nasal cavity and could explain the basal and apical differences in staining.

I observed different patterns of actin distribution in the facial prominences at different stages of primary palate formation. Prior to fusion, the actin staining seen in the epithelium that lined the prominences was similar to the nasal cavity just described with apical regions staining more intensely. As the MNP and LNP approached each other about to fuse, the epithelium of the presumptive fusion site of each process showed a marked decrease in staining intensity. Once the prominences had fused forming a nasal fin, this epithelium showed more diffuse concentrations of actin. Also, in epithelium comprising the nasal fin, spot densities were no longer observed. Possibly, these cells were in the process of losing their attachments to neighbouring cells and were preparing to migrate. They may have lost junctional contact.

The differences in actin distribution observed in the nasal cavity versus the facial processes may have reflected epithelium that was at a different level of differentiation. Nasal epithelium may have represented a different epithelial domain than facial prominence epithelium. Croucher and Tickle (1989) studied nasal placode epithelium in chick embryos and found that different cell surface molecules and extracellular matrix molecules existed in different regions of the olfactory epithelia. They found that olfactory epithelium expressed different cell
surface molecules than respiratory epithelium of the nasal passages. My observations suggest that different epithelial domains in mouse nasal epithelium show differential actin staining.

4.2 Timing of Basal Lamina Disappearance During Primary Palate Formation

During primary palate formation there were a series of observable morphological changes that took place within the environment of the LNP and MNP epithelium. This epithelium was always in close proximity to its basal lamina which in turn was closely apposed to mesenchymal cells underneath. It would be expected that epithelial and mesenchymal components interact such that the events of nasal fin formation and regression, as well as mesenchymal bridge formation, took place in a carefully timed and coordinated manner. With the electron microscope, these interactions could only be visualized on a morphological and descriptive level, but there were observable changes in the shape of the epithelial cells and mesenchymal cells that could be described. Specifically, prior to fusion of the facial prominences, the epithelial cells were seen to rest against their basal lamina with the point of contact being quite flat. Mesenchymal cells had an elongated appearance and portions of these cells extended outwards as cellular projections. These projections were observed to be closely associated or in close proximity to the basal lamina. At all developmental stages looked at from 7 to 27 T.S. mesenchymal cell processes were observed approaching the basal lamina and often in contact with it.
Epithelial cells also extended their own cell processes toward their basal lamina as the MNP and LNP came together. As early as 10 T.S. which was still in the prefusion stage, epithelial cell processes extended into and through the basal lamina. There appeared to be basal lamina fragmentation of the basal lamina occurring in regions where there were mesenchymal cell processes, but in other regions the basal lamina was still completely intact. This observation of basal lamina destruction before MNP and LNP epithelial cells make contact was different from the situation described by some authors during secondary palate formation (Fitchett and Hay, 1989; Ferguson 1988). There, the basal lamina was intact until the palatal shelves had fused. Then it became patchy, fragmented, and disappeared. In the primary palate these events occurred earlier prior to fusion. Once the nasal fin had formed there was rapid basal lamina destruction as epithelial and mesenchymal cell processes penetrated through it. This destruction was localized to specific regions of the nasal fin however. Often toward the nasal cavity the basal lamina remained intact and sometimes destruction was only seen on the side of one facial prominence. One of the embryos at 11 T.S. had a nasal fin where on the MNP side, the basal lamina was breaking down while on the LNP side it remained completely intact but generally this process appeared to be simultaneous on both MNP and LNP sides. The basal lamina became patchy and discontinuous beneath presumptive fusion epithelium only. Other portions of the facial prominences had an intact basal lamina during primary palate formation. At sites where the basal lamina was fragmenting one could often see vesicles in epithelial cells that may have been
endocytotic possibly taking in components of the lamina aiding in its dissolution.

Regions of necrosis were observed in the nasal fins of a few embryos but this was rare. Cells contained tertiary lysosomes and multivesicular bodies but surrounding epithelial cells remained metabolically active and vital.

During primary palate formation there were no residual epithelial cell rests as seen in secondary palate as the epithelial seam starts to thin (Fitchett and Hay, 1989). In primary palate once the nasal fin started to regress the epithelial cells quickly disappeared and mesenchymal cells invaded the area. The epithelial rests were still bound by basal lamina in secondary palate formation but no analogous structures were seen in this study. Once the basal lamina disappeared, it did not leave any remnants behind. At the denuded edge of a regressing nasal fin, the epithelium adjacent to the mesenchymal bridge had deposits of basal lamina material beneath it. This material appeared quite thick on the MNP side and it was impossible to tell whether this represented degraded basal lamina or lamina that was being resynthesized as the mesenchymal bridge was enlarging. Presumably there would be some epithelial "shuffling" and rearrangement taking place as the tissue was taking on a new level of organization.

In a number of the nasal fins studied, there were numerous points of contact between the cell processes of mesenchymal cells and the epithelial basal lamina. A 15 T.S. nasal fin showed a mesenchymal cell process in contact with the basal lamina but not through to the epithelial cell. There appeared to be an electron dense layer at the point where the mesenchymal cell touched the basal
lamina. This could be some sort of communication point. Sometimes mesenchymal cells contacted the basal lamina and the epithelial cell underneath. This would imply a direct cell-cell contact as is thought to occur in facial prominence epithelium and mesenchyme where mesenchymal cell contact was necessary to maintain maxillary epithelium viability (Saber et al., 1989). The epithelial and mesenchymal cell processes observed in this study often appeared to contain microfilaments. Possibly these filaments served to maintain the structural integrity of the process.

The primary palate is considered to have formed once the nasal fin has disappeared and the mesenchymal bridge has enlarged. The timing involved in nasal fin formation and regression is likely important. The basal lamina must break down before the epithelial surfaces make contact. I observed that once the nasal fin was formed, the areas of basal lamina destruction were already quite extensive, and the epithelial cells became displaced presumably allowing mesenchymal cells to move in.

4.3 Description of Hays "Fixed Cortex" Model applied to Epithelial to Mesenchymal Transformation.

Recently a new model has been published that attempts to describe a mechanism by which epithelial cells that transform to mesenchyme can move away from the epithelial cell population and become a part of the surrounding mesenchymal cell population (Hay, 1989; Bilozur and Hay, 1989). This theory would explain how epithelial cells in a number of developmental systems that are
thought to involve epithelial to mesenchymal transformation are able to leave their original epithelial arrangement. Systems like thyroid, lens, Mullerian duct regression and secondary palate development are all potential candidates where an epithelial to mesenchymal transformation is thought to occur. They are also possible systems that this model could serve to explain.

The fixed cortex theory was originally applied to fibroblasts and it stated that the fibroblast cell surface consisting of an actin rich cortex and plasmalemma is left behind as the myosin and intermediate filament rich endoplasm slides continuously forward along the actin cortex into a new front end of the cell. Essentially, the front end of a moving fibroblast becomes the rear end and is left behind as a membrane bound actin cortex attached to the ECM. This theory is applied by Hay to explain the movement out of epithelium of newly forming mesenchymal cells during epithelial to mesenchymal transformation in the embryo. She puts forward the idea that myosin in the endoplasm is attached to microtubules and intermediate filaments such that a force is produced that could drive the whole inner part of the cell into the F-actin rich front end of the cell. The intermediate filaments of the epithelial cells have switched from a keratin type characteristic of epithelial cells to vimentin which characterizes mesenchymal cells. Vimentin-actin interactions with ECM may be a major factor in the ability of a cell to become elongated and form an actin rich new front end. Bilozur and Hay (1989) propose that a master gene is turned on during epithelial-mesenchymal transformations that turns on a mesenchymal cell phenotype. The epithelial cell could now produce collagen type I, fibronectin, and the cytoskeletal system of a
mesenchymal cell. As the epithelial cell becomes a newly forming mesenchymal cell it extends actin rich filopodia into the ECM. The leading end of the cell is being rebuilt with new actin, new membrane, and new ECM receptors. The trailing end of the cell that still has epithelial cell characteristics is left behind at sites of the zonula adherens junctions and cell adhesion proteins. The epithelium leaves the old fixed surface behind and moves the endoplasm containing the nucleus out of this shell.
4.4 Applying the Fixed Cortex Model to the Primary Palate Model

It is interesting to speculate whether an epithelial to mesenchymal transformation takes place during primary palate formation as the nasal fin epithelium regresses. If such a system were operating would it follow the principles of the fixed cortex theory? This would imply that the epithelial cells of the nasal fin that were destined to regress must lose their basal lamina first before they can transform into a mesenchymal phenotype. That would mean that nasal fin regression was similar to thyroid placode evagination, Mullerian duct regression, and secondary palate formation which are all systems where this model could be applied. Once the basal lamina had disappeared, only certain epithelial cells, that no longer had their basal lamina intact, could then migrate into the surrounding mesenchymal cell population. As the mesenchymal bridge increased in size, more and more epithelial cells would be transforming into mesenchyme. The TEM observations in this study do support the idea that only certain regions of the nasal fin break down at any one point in time; these regions being places where mesenchymal cell processes contacted the basal lamina. Also, regions of epithelium at various stages of differentiation existed that stained differentially for actin. Around the nasal cavity would be one type of epithelium. This was a pseudostratified primitive olfactory epithelium that contained abundant F-actin in its apical epithelial layers facing the lumen. The epithelium of the MNP and LNP that was in the region of presumptive fusion was another epithelial
domain. This epithelium would go on to fuse and form the nasal fin. It did not exhibit apical actin staining. Only the occasional spot or plaque of F-actin, possibly zonula adherens junctions existed within nasal fin epithelium. This would correspond to the part of the epithelial cell left behind as the mesenchymal component was migrating away in the Hay model. There appeared to be more adherens junctions in nasal epithelium compared to nasal fin epithelium. This could be due to the nasal fin being transitory and it would not be practical to have these epithelial cells as firmly attached to each other as in olfactory epithelium. The other epithelial domain was the future oral epithelium where there was not intense actin staining in apical epithelium like that seen in nasal epithelium.

My results were consistent with the Hay model in that primary palate formation could be a system where an epithelial to mesenchymal transformation takes place. There is a transitory epithelial cell population that eventually disappears and is replaced with mesenchymal cells. Also the basal lamina breaks down and can no longer act as a barrier.

Further studies need to be done to look at nasal fin regression in order to understand whether a transformation to mesenchyme takes place in this epithelium. Immunohistochemistry would be useful to detect whether there was a switch to a vimentin type intermediate filament production in cells of the nasal fin, or were they still producing keratin.

The basal lamina phenomenon should be studied using immunological tags to basement membrane components. This should be done at the light and electron microscopic levels to better understand its breakdown as the epithelium
of the prominences fuse. My results indicated that the basal lamina breaks down prior to fusion of the MNP and LNP, but it would be interesting to know whether all of its component molecules disappeared simultaneously or sequentially during primary palate formation.

5. CONCLUSIONS

- Differential staining for F-actin was observed in epithelium of the nasal cavity, facial processes, and nasal fin.

- In nasal cavity epithelium staining was uniform with higher concentrations of actin in apical regions of epithelium towards the lumen and at locations where there was bending.

- In the LNP and MNP epithelium, staining was uniform with occasional spot densities of actin seen mostly in epithelium that was not in the presumptive fusion zone.

- After fusion, LNP and MNP epithelium formed the nasal fin, and actin staining was greatly reduced and disorganized. Spot densities were less frequent in nasal fin epithelium.

- Results of this study were consistent with the Hay Fixed Cortex Theory.
- The basal lamina regresses prior to contact of the LNP and MNP but only in regions of presumptive fusion. The lamina remained intact beneath epithelium of other regions of the facial prominence.

- My results indicate that the timing of basal lamina regression is different in primary palate formation than during secondary palate where the basal lamina is disrupted after the medial edge epithelia have made contact.

- Regions of basal lamina regression had mesenchymal cell processes extending into and through the lamina. This observation was similar to Mullerian duct regression where basal lamina integrity was lost prior to duct regression and only in regions where mesenchymal cell processes touch the lamina.

- Little necrosis was observed in nasal fin epithelium suggesting that programmed cell death in this area was unlikely.
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