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Tooth Induction and Establishment of Temporal Patterns in Palatal Epithelium of Mice

Joy Marion Richman

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TOOTH INDUCTION AND
ESTABLISHMENT OF TEMPORAL PATTERNS IN
PALATAL EPITHELIIUM OF RATS

Joy Marion Richman
D.M.D., University of Manitoba, 1979

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TOOTH INDUCTION AND
ESTABLISHMENT OF TEMPORAL PATTERNS IN
PALATAL EPITHELium OF MICE

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1984
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- Recombination; In Vitro Culture
- Intraocular Grafting; In Vivo Culture
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INTRODUCTION

The process of gastrulation involves transformation of a homogeneous cell population into the three germ layers, ectoderm, mesoderm and endoderm. Although all cells are identical genetically, there are epigenetic influences which ultimately make various populations of cells different from one another. With the significant event of gastrulation, mesodermal cells become associated with ectoderm or endoderm. The juxtaposition of these different tissues initiates organogenesis and the formation of ectodermal and endodermal derivatives.

Induction is the process by which stable, permanent changes in one population of cells result from signals sent by adjacent, different cell populations. The inducer can play one of two roles. The first is merely supportive in which favourable conditions are provided that permit expression of genetic potential. The genes selected for expression are already present from biases formed during previous stages of development. This is termed permissive induction. In the second, variation, an inducer actively directs the responding tissue to behave in a specific way. This type of interaction is called instructive induction. These two types of cellular interactions, permissive and instructive, are quite different. Consequently, it is important to design experiments in such a way that one can distinguish between them.
General Principles of Induction

Instructive Tissue Interactions

The lens was one of the first embryonic structures to be examined for inductive interactions. Spemann (‘01), showed that by removing the optic cup from a frog, the lens did not form. Subsequently, it was shown that the retinal anlage is not the only lens inducer; rather, it is the last in a series of tissues that exert inductive influences on head ectoderm. There is a sequence of steps by which one tissue acts upon the next until finally the optic cup alone has the capacity to induce the lens. All the intermediary tissues eventually lose this ability. However, early in embryonic development there are many tissues which permit the lens to form despite the absence of the optic cup (Jacobson, ’66). Although this is a clear example of a permissive interaction, Karkinen-Jaasklainin (’78), has shown there are also instructive influences operating later in development. The final inducing structure, the optic cup, can force trunk ectoderm to form a lens that is identifiable not only morphologically, but also contains lens-specific proteins. Thus, temporal sequences are important in tissue interactions and can significantly alter the instructive or permissive quality of inductive message transmitted.

Some of the clearest examples of instructive interactions are provided during the development of integumental derivatives. The highly organized, distinctive morphology of structures such as feathers, scales, or teeth, make the identification of positive inductive events easy. It is possible to distinguish between instructive and permissive interactions based on the type of structure induced. If a different cutaneous appendage is formed instead of the
normally developing ectodermal derivative, the induction is instructive. However, if the original, programmed structure continues to develop despite alterations in mesenchyme, this event is permissive.

The technique of tissue recombination after trypsin separation at cold temperatures was introduced by Rawles ('63). Tissues are incubated in trypsin at 4°C which digests the basement membrane between the epithelium and mesenchyme while preserving cellular viability and tissue integrity. Rather than dissociating tissues into individual cells, the tissues can be separated cleanly and as intact sheets. In her pioneering work, Rawles studied feather and scale development in chick embryos. She found that the dermis determined the kind of cutaneous appendage produced by the epithelium. Her culture technique involved grafting of the combination to the chorioallantoic membrane of embryonic eggs. With a blood supply established, the grafts survived up to 9 days. She demonstrated, using various combinations of tissues of different ages, that the stage of development of the dermis and epidermis was critical during the process of induction. The dermis in scale bearing areas of avian embryos gains strength as an inducer with increasing age. At day 12 the dermis causes feathered scales to develop in epithelium taken from a feathered region, whereas at day 13 only scales develop. There is an optimal age at which the anterior shank dermis exerts its instructive influence. Before and after day thirteen, this potential for scale induction is diminished. Whatever form the inductive signal takes, timing is critical both in the strength of its message and in the way it is interpreted by the responding tissue. From Rawles' work we
conclude that the morphology of the cutaneous appendage is dermis dependent, and stage dependent.

McLoughlin ('61b) studied combinations of heterotypic mesenchyme with chick epidermis. She observed that limb bud and esophagus mesenchyme did support normal differentiation and keratinization of the epidermis but that gizzard mesenchyme resulted in differentiation of mucous secreting epithelial cells. Heart myoblasts caused the epidermis to spread out into a thin layer much like cardiac endothelium. However, when the epidermal cells came in contact with fibroblasts they did not spread; instead, they keratinized. Thus she concluded that the mesenchyme does affect such growth qualities as spreading and keratinization. Although these observations were not as dramatic as those instructive tissue interactions of Rawles, the mesoderm was shown to control the differentiation of the epithelium.

Some of the more interesting experiments probing the mechanism of induction have involved combinations of tissues from different species. Coulombre and Coulombre ('71) placed mouse mesenchyme taken from a haired region, directly under embryonic chick cornea and induced feathers from the epithelium. The mesenchyme instructed the epithelium in a general way to make an appendage. However, the cornea could only respond in a way characteristic of its species origin; that is, to make feathers. Instructive induction is not a unidirectional flow of information. Rather, it is a bi-directional interaction, with both tissues contributing to the final outcome.

**Experimental Manipulation of Tooth Germs**

The tooth germ has provided investigators with an experimental model system which clearly demonstrates instructive interactions. When
a positive result is seen, enamel protein is deposited by the epithelium. Enamel protein is made only by specialized epithelial cells derived from the inner dental epithelium. As the majority of experiments utilize murine tooth germs, a synopsis of incisor (Hay, '61) and molar (Cohn, '57) development in the mouse may be useful.

Mice have one incisor, a diastema, and then three molars in each quadrant in their oral cavity. The incisor begins its development earlier than the molar, at twelve days of gestation. An epithelial downgrowth gives rise to the incisor tooth germ and also produces the lip furrow (vestibular lamina). At fourteen to fifteen days the enamel organ has begun to differentiate into the inner and outer enamel epithelium and the less organized intermediate layers, stratum intermedium and the stellate reticulum. Between days fifteen and sixteen the inner enamel epithelium differentiates into preameloblasts over the future, enamel bearing labial surface of the incisor. On the seventeenth day odontoblasts in the dental papilla become functional and secrete dentin, triggering the differentiation of polarized secreting ameloblasts. With continued apical development, the incisor comes to lie inferior to the molar tooth buds. As the incisor is a continually erupting tooth in the rodent, there is always a population of differentiating cells at the apical end even in the adult animal.

The first molar begins forming at fourteen days of gestation as an epithelial downgrowth of the dental lamina similar to the incisor. The invagination forms a cup-shaped enamel organ enclosing an area of condensed mesenchymal cells, the dental papilla. As the shape of the crown develops, a layer of polarized mesenchymal cells become
odontoblasts. The odontoblasts are first seen at day fifteen at the cusp tips. By day seventeen dentin secretion has begun and a bell-stage enamel organ can be identified. The enamel organ includes polarized ameloblasts which begin to produce enamel matrix after the first layer of dentin is laid down. Cell differentiation in molar teeth is sequential with more mature cells found at the cusp tips and progressively less mature cells encountered as one approaches the cervical margin. At seventeen days, the second molar begins its development just posterior to the first molar. The third molar begins its development between four and six days after birth. Unlike the incisor, the molars do not grow continually and complete their root development once the tooth has erupted.

Huggins et al. (’34), were the first investigators to use the developing tooth as a model for studying epithelio-mesenchymal interactions. They did not use trypsin to separate their tissues, instead they used mechanical separation. Dog teeth at an advanced developmental stage contain some calcified dentin and enamel, therefore separation of the two tissues could be accomplished fairly easily with the assurance that there was no contamination by adhering ameloblasts. They explanted their tooth rudiments to the abdominal wall. Even with their less elaborate techniques they demonstrated an absolute requirement for both mesenchyme and epithelium to be present for continued tooth development. These investigators showed some incisive insight by realizing that only dental mesenchyme could support tooth development. Other connective tissue in contact with enamel organs produced only epithelial islands composed mostly of basal cells.
Several decades later Kollar and Baird ('69), found that when incisor mesenchyme and molar epithelium were combined, an incisor was formed. In reciprocal combinations molar mesenchyme and incisor epithelium produced molars. These experiments demonstrated that the enamel organ did not play a dominant role in determining tooth shape. Rather, the information for tooth morphology resides in the mesenchyme. To test further the instructive capabilities of the dental papilla, Kollar and Baird combined this tissue with other sorts of epithelium ('70a). Lip furrow (vestibular) epithelium develops from the oral mucosa as do the adjacent incisor teeth, yet, it is destined to become surface epithelium and not dental epithelium. In combination with tooth mesenchyme vestibular epithelium formed teeth, establishing there is no inherent quality within this epithelium which keeps it different. Perhaps this dentally related epithelium is biased in its response to a dental papilla because of its close origins to dental lamina. To confirm that tooth mesenchyme was capable of instructive induction, other more distant epithelia would have to be tested.

Kollar and Baird ('70b), next showed that even foot pad epithelium could be induced to form teeth. They also showed that enamel organs when combined with foreign, heterotypic mesenchyme do not form teeth, but exhibit ameloblastoma-like invasive characteristics. This finding fits well with the observed tendency of the dental lamina to migrate into the mesenchyme. In a subsequent paper, Kollar ('72), demonstrated that the diastema between the molars and incisors could also be induced to form tooth structures indicating that probably any epithelium from the oral cavity can be induced to synthesize enamel, de novo. Even more recently Thesleff ('77),
described tooth induction in the gingival epithelium of mice.

Perhaps the most unusual demonstration of the instructive potential of the dental papilla comes from an experiment in which the oral epithelium of embryonic chicks was combined with mouse molar papillae and grafted into the anterior chambers of the eyes of nude (nu/nu) mice (Kollar and Fisher, '80). In this nutritionally rich explantation site tooth morphogenesis occurred. They concluded that the genes for tooth formation are still contained within the chick genome, although they are not normally expressed.

Developing teeth also illustrate the notion that the stage of development affects instructive potential. Yoshikawa and Kollar ('80) showed that sixteen day dental sac mesenchyme would form teeth in combination with enamel organ just as molar papillae do. However, this capacity for instructive tooth induction diminished considerably with seventeen-, eighteen- and nineteen-day dental sacs. In contrast, dental papillae were inductive from sixteen to nineteen days of gestation. It was not possible based on the results to distinguish one age of papilla that was most inductive within the sixteen to nineteen day range. The authors concluded that the cells of the dental sac were beginning to be determined as early as sixteen days of gestation. This is especially important when one considers that both dental papilla and sac cells derive from the same progenitor population.

**Permissive Tissue Interactions; Organ Morphogenesis**

It is obvious that morphogenesis is an integral part of induction. In all of the examples cited above, complex tissue architecture and anlage morphology precedes cytodifferentiation. How can an unorganized mass of mesenchyme interact with epithelium to
form, for example, a salivary gland? This relationship was examined by Grobstein ('53) using salivary gland mesenchyme and epithelium cultured on a plasma clot. He found that only submandibular gland mesenchyme would permit typical glandular morphogenesis in the epithelium. Other types of mesenchyme would merely allow the epithelium to grow into large, round cysts. Even though this is not an instructive interaction, there seemed to be stringent requirements which the mesenchyme had to meet before the epithelium would undergo normal morphogenesis.

Kratochowil ('69) combined mammary gland epithelia with salivary gland mesenchyme in vitro. When the pattern of branching in mammary epithelium was examined, it was characteristic of the salivary gland. Thus, there seems to be a signal in the mesenchyme which directed the epithelium to branch and to branch in the manner specific to the source of the stroma. However when the reciprocal combination was made, Grobstein's findings ('53a) were supported in that mammary mesenchyme would not support branching of submandibular epithelium.

After further investigations, the absolute specificity of submandibular gland epithelium for its own mesenchyme was shown not to be true. Cunha ('72) showed that mesenchyme taken from a male secondary sex organ, for example the preputial gland, promoted branching of salivary gland epithelium. As submandibular gland mesenchyme in the male animal is androgen sensitive, the data suggests that in order to be adequately replaced by heterotypic tissue, the foreign mesenchyme must also be androgen sensitive.

Lawson ('74) demonstrated that mouse submandibular gland branching would continue in the presence of rat lung mesenchyme
whereas this was not as common with mouse lung mesenchyme. In this series of experiments the absolute specificity of submandibular epithelium was overcome provided there was an increased mass of heterotypic mesenchyme combined with it. The species difference in inductive potential was a reflection of the larger gland size of the rat versus the mouse. Since Grobstein ('53a) and Spooner and Wessels ('72) used glass–clot interface and millipore filter respectively both of which promote spreading of mesenchyme, it is likely their results were due to a reduction of the mesenchyme below the critical mass necessary for induction.

Interpreting the above data from the perspective of mesenchymal influences rather than responses of the epithelia suggests that lung mesenchyme has a permissive effect on development. In large quantities, it will permit salivary morphogenesis to occur. However, it will not instruct salivary gland epithelium to branch in a lung pattern. Thus, these combinations of submandibular epithelium and heterotypic mesenchyme are examples of permissive tissue interactions. The epithelium requires the interaction with mesenchyme from a branching organ to permit the initiation of branching, but the instructions to form the clefts and stalks of submandibular gland are inherent within the epithelial developmental repertoire.

A more recent example of permissive interactions in glandular development can be found in the work of Lawson ('83). She examined the reciprocal combination of lung epithelium and salivary gland mesenchyme. One would expect on the basis of Kratochwil's work ('69), that the mesenchyme would instruct the lung epithelium to take part in salivary morphogenesis. However, the salivary gland mesenchyme
supported bronchiolar morphogenesis in lung epithelium provided the initial bronchial buds had developed. Continued morphogenesis would not occur unless this phase of development had been passed. These findings are similar to those of Rawles ('63) in this regard. Therefore, just as temporal effects are important in instructive induction, so are they critical in permissive tissue interactions.

The Relationship of Organ Morphology to the Function of the Individual Cell

Does the change in morphology of an epithelial derivative in response to heterotypic mesenchyme, also change the cytodifferentiation of the individual epithelial cell? Thus far we have dealt with instructive induction at the organ level. If the message of induction is a complete one, it should instruct both morphologic changes and differences in gene expression during cytodifferentiation where organ specific protein are produced. Stated differently, protein synthesis should be consistent with the induced morphology. With the aid of histochemical and biochemical techniques, investigations of this hypothesis are possible.

There are two papers which support the premise that form does not dictate function. Sakakura et al. ('76), combined mammary epithelium with submandibular gland mesenchyme. The results of Kratochwil ('69) were confirmed; the epithelium looked like salivary gland. The second step was to graft the combinations under the kidney capsules of female mice. The females were mated, produced a litter, and eventually began lactation. The combinations were harvested and their composition analyzed biochemically. In one of four successfully recovered recombinants, significant quantities of an enzyme specific for milk
production was found. Thus, although the epithelium had the
dichotomous branching pattern typical of the submandibular gland,
there was evidence that the cells were behaving according to their
origin and were producing mammary gland specific proteins. An
incomplete message of induction was transmitted, in that instructions
to alter cytodifferentiation were not given to individual cells.

The second example also supporting the theory of partial
transmission of inductive messages comes from the work of Tyler and
Koch ('77). In vitro culture of palatal epithelium with submandibular
gland mesenchyme resulted in glandular morphogenesis in the
epithelium. Close examination of the cells lining the ducts showed
positive birefringence in the outer cell layers and the presence of
hematoxylin granules, both of which are suggestive of keratinization.
A re-examination of the figures in this paper leaves considerable
doubt as to the appropriateness of the term glandular in describing
these combinations. There may be lumen-like spaces, but the absence of
ducts, lobules and clefts is also apparent. The structures could just
as readily be described as keratin cysts. Perhaps cytodifferentiation
of the epithelium had occurred prior to the time of tissue
recombination, therefore it was unable to alter its histogenesis in
the presence of foreign mesenchyme.

On the other hand, there is much work which supports the premise
that induction does alter cell behavior. There are many instances in
which a complete instructive event can be demonstrated such that the
epithelium synthesizes new proteins in response to the inductive
signal from the mesenchyme. The tooth model gives us numerous examples
of enamel genes being switched on in non-dental epithelia.(Kollar and
Baird '72; Thesleff, '77; Ruch et al., '73; Kollar and Fisher, '80). As previously mentioned, Karkinen-Jaasklainin, ('78) demonstrated the presence of lens specific proteins in induced trunk ectoderm.

A recent example of altered gene expression that occurred in concert with changes in morphology, was seen in bladder epithelium. Cunha et al. ('83), combined adult mouse bladder epithelium with urogenital sinus mesenchyme of fetal mice and induced ascini which morphologically were identical to prostate gland. In addition, histochemical analysis revealed that the in epithelium secreted non-specific esterase, and glycosaminoglycans. The presence of both of these substances is characteristic of prostatic epithelium. Alkaline phosphatase which is a histochemical marker for bladder epithelium, could no longer be detected in the heterotypic recombinants. As added proof, hormone binding studies showed that bladder epithelial cells were able to bind androgens, a characteristic of prostate gland. In addition, stimulation of the combinations by adding androgens to the media caused an increase in DNA synthesis. Thus, not only were binding sites present but they were also active. What is particularly impressive about Cunha's work is that he was using adult epithelium with fetal mesenchyme. Perhaps the results are somewhat biased considering that bladder epithelium originates in close proximity to the inducing mesoderm of the urogenital sinus which gives rise to the prostate. Nonetheless, this series of experiments was the first to demonstrate considerable developmental plasticity in a mature adult mammalian epithelium.

All the aforementioned work has used the epithelium as the responding tissue. There is one combination that has resulted in
uncharacteristic gene products being produced by the mesenchyme. Hata and Slavkin ('78) combined apical ectodermal ridge (AER) epithelium from chick limb buds with cap-stage molar mesenchyme from mice. They observed a cartilagenous matrix was deposited by the mesenchymal cells. In addition, they were able to confirm the matrix was composed of type II collagen, a protein found in cartilage. This is unusual in that teeth are surrounded by membranous not endochondral bone, therefore a complete change in cytodifferentiation has occurred. The instructive abilities of limb bud epithelium suppressed the normally inductive molar mesenchyme. This may be a feature only associated with the AER because it has since been shown that avian mandibular epithelium will secrete enamel protein in combination with molar papillae (Kollar and Fisher, '80).

Theories of Induction

Developmental biologists have studied a variety of organs to gain an understanding of the factors which are involved in the process of induction. The questions asked are: which tissue provides the inductive signal, what form does the signal take, and what factors can modulate induction?

There are three popular hypotheses which attempt to explain embryonic induction. One proposes that the inductive signal is a diffusible ion or molecule which can travel long distances (Holtfreter, '55; Wolpert, '69). Another implicates cell to cell contact as a necessary event in the transmission of the signal (Lehtonen et al., '75; Slavkin and Bringas, '76). The third alternative is that the inductive message is encoded within the extracellular matrix (Grobstein and Dalton, '55; Kollar, '83).
Diffusible Ion Concept

The inducer as a diffusible ion was discussed most recently by Wolpert ('69) in describing how positional information may be transmitted between populations of cells. He hypothesized that there are ionic gradients which provide differences in ionic concentration within cell populations. This idea suggests that individual cells interpret their environment in a different way from their neighbour. The evidence to support this hypothesis rests principally on the presence of gap junctions. These structures contain pores which connect the cytoplasm of adjacent cells and allow passage of ions and certain small molecules between cells (Gilula et al., '72). Gap junctions have been demonstrated with electron microscopy in a variety of tissues and provide morphologic evidence that cells can communicate with each other directly. There is however, little experimental evidence to indicate the nature of the inducing ion or molecule. It has not been possible to isolate a substance, to add it to the culture medium and thus to simulate an inductive event. An analogy often cited for this proposal is the transient effect of hormones on cell differentiation in development and throughout the life of the organism. However, these fleeting influences are unlike the stable permanent changes caused by induction.

Cell to Cell Contact

Plasma membrane contacts between heterotypic cell populations, has been the object of many ultrastructural studies. Morphologic support for this theory is confounded by the presence of a trilaminar structure, the basement membrane, interposed between epithelium and mesenchyme. This layer is ubiquitous in any region where there is an
association between heterotypic cell populations. Ultrastructurally, the basement membrane consists of a lamina lucida under the epithelial cells supported by the lamina densa, beneath which is a fibrillar network of extracellular matrix molecules adjacent to the mesenchyme (Bloom and Fawcett, '75). As this structure presents a barrier of sorts, the tissues must communicate with each other through it.

The first ultrastructural observations of breaches in the basement membrane were made by Pannese ('62). He looked at the embryonic cat tooth and found that there were desmosomes between enamel epithelium cells and odontoblast processes. Reexamination of the photomicrographs in Pannese's paper by Kallenbach ('76) lead to a different interpretation. Instead of ameloblasts making contact with odontoblasts it was more likely they were forming junctions with cells of the stratum intermedium. The observation of heterotypic cell contacts has not been replicated despite numerous attempts. Cutler and Chaudhry ('73), found that in developing salivary gland there were regions where the basal lamina was breached. However, the presence of epithelio-mesenchymal contacts in the form of tight junctions, desmosomes, or hemidesmosomes were not seen in electron micrographs. Lehtonen ('75) also found gaps in the basal lamina of embryonic kidney tissue and Slavkin and Bringas ('76), showed evidence of very close association between preameloblasts and odontoblasts but once again no actual plasma membrane contact. More recently Riso ('83) and Goldberg and Hardy ('83), found very close heterotypic cell associations in developing lung and hair follicle respectively. In all cases, despite claims of cell to cell contact, no membrane contact is ever observed. The cells are always separated by at least 50 nm which is filled with
an electron dense substance, probably consisting of extracellular matrix. In fact, the only time during development when specialized contacts between cells of different origin can be conclusively demonstrated is during the establishment of the primary germ layers (Trelstad et al. '67). However the tight junctions are described as transitory and cease to exist with the secretion of extracellular matrix. Thus, the basement membrane appears to allow close association, but, as yet, no direct plasma membrane contact between heterotypic cell populations has been demonstrated.

Cell contact may not necessarily require plasma membrane junctions. The proximity of cell surface molecules may be more important (Lehtonen et al. '75). A method which provides insight into the role of cell proximity in tissue interactions, is the culturing of tissues across millipore or nucleopore filters. It was thought that the very small pores of the filters would prohibit cell to cell contact (Grobstein, '53b; Wessels, '62;). Thus, if induction could take place, the inducer would have to be small enough to pass through the pores. Increasing the thickness of the filter provided information about the limiting distance across which an inducing agent could act. Examining the filter itself permits biochemical characterization of the material trapped in the pores. Finally, by altering pore size, the molecular weight of the substance involved in the interaction could be estimated (Saxen, '76).

Earlier data was not interpreted in favour of cell to cell contact. Grobstein and Dalton ('57), combined spinal chord epithelium with metanephrogenic mesenchyme on either side of millipore filters. They found induction of kidney tubules in 3 out of 8 combinations
using filters with 0.1 μm pores. Since they were not able to demonstrate cytoplasmic penetration into the filters they concluded cell to cell contact was not necessary to transmit the inductive signal. Instead it was likely to be contained in an extracellular substance.

In 1972, Wartiovaara examined the ultrastructure of millipore filters using fixation superior to that of Grobstein and Dalton ('57) and found there was a great variation in pore size. In fact, he was able to demonstrate penetration of cytoplasmic processes a considerable distance into the filter with pore sizes as small as 0.2 μm. This was subsequently confirmed in kidney cultures by Wartiovaara et al. ('74) and in salivary gland cultures (Lehtonen et al., '75), who saw shallow cytoplasmic penetration in filters with 0.1 μm pores. Moreover, induction was not seen in cases where cytoplasm could not be demonstrated throughout the depth of the filter. Further demonstration of inhibition of induction in 0.1 μm nucleopore filters was provided by Thesleff et al. ('77). In experiments with tooth buds, they found that in order for the differentiation of ameloblasts from inner enamel organ to occur, significant ingrowth of odontoblast processes into the filter was necessary. The smaller the pores the longer it took to induce predentin and polarization of ameloblasts. Close examination of the material within the pores revealed it to be collagenous, hence originating from the mesenchyme. The authors concluded that it was essential for odontoblast processes to make contact with the extracellular material under the inner enamel epithelium, in order for them to secrete dentin. If the signal is long range, and diffusible, the thickness and pore size of the filter should have little effect on
inductive ability. This was not the case. The transfilter experiments suggest that intimate cell contact is necessary and, perhaps, a short-range communication system is involved. The presence of cytoplasmic extensions reaching the opposite side of the filter, may expedite the transmission of extracellular matrix through the pores. It is important to note that although cell processes were often very close to one another, the presence of specialized junctions within the filter, was not confirmed in any of the above cited work.

**Extracellular Matrix; The Role of the Basal Lamina in Induction**

If cells do not form actual plasma membrane junctions, perhaps the material filling the space between the membranes plays a role in induction. There may be short-range interactions with larger, less mobile molecules than the ions described in the first hypothesis (Wolpert, '69). This encompasses the third hypothesis which says the signal for induction is encoded within the extracellular matrix. This is by no means a new proposal. As early as 1955 Grobstein described this possibility. He stated;

"A labile intercellular continuum, locally alterable in penetrability and other properties by physiological shifts, endowed with a high degree of specificity, closely responsive to the genotype, able through polymerization to condense to higher levels of order thus providing boundaries and interfaces, seems to be exactly the missing piece required in many puzzles of development."

The basement membrane fits Grobstein's description very closely, and not surprisingly experimental evidence in support of the role of this structure in induction has accumulated. The basement membrane is composed of fibronectin (Courtoy et al., '80), laminin (Timple et al., '79), type IV collagen (Kefalides et al., '79), and proteoglycans (see review Toole, '81). Of these laminin and type IV collagen are thought
to reside only in basal lamina, which consists of lamina densa and the lamina rara adjacent to the epithelial cells (Bloom and Fawcett, '75). The others are associated with connective tissue in a more general way (see review, Hay, '81). Epithelia synthesize type IV collagen, laminin and hyaluronic acid as shown by culturing these tissues in vitro without mesenchyme (Kefalides, '73; Hay and Dodson, '73; Bernfield et al. '76; Timple et al., '79). Thus, the remaining component fibronectin, is likely to be produced by the mesenchyme (Yamada et al., '80).

All the molecules composing the basement membrane are associated with cell adhesion. Laminin enhances cell attachment and spreading of epithelial or endothelial cells. It has been shown to bind epithelial cells to type IV collagen and glycosaminoglycans (Yamada et al., '80). Fibronectin has also been established as a molecule promoting cell adhesion, spreading and motility (see review, Yamada et al., '80). Type IV collagen has also been claimed to possess specific adhesive properties for particular cell types. (see recent reviews, Sanders, '83; Hay, '81)

Keeping the aforementioned properties of basement membrane components in mind, one can propose several roles it may play in development. The basement membrane may act as a stabilizing scaffold against which morphogenesis occurs. In the submandibular gland it breaks down in areas where budding is about to occur and is reinforced in the cleft regions (Bernfield and Bannerjee, '78). Grobstein and Cohen ( '65), added collagenase to the medium and prevented glandular morphogenesis. This effect was reversible when collagenase was removed. However, when soluble tropocollagen was added to the media in
place of mesenchyme, it did not encourage morphogenesis. Thus, collagen as a part of the epithelio-mesenchymal interface was important and a soluble form would not have substituted for the intact molecular component of the extracellular matrix. A second function of the basement membrane may be a contact guidance system along which cells move. Fibronectin has been implicated in this role (see review, Yamada et al., '79). A third possibility is that it acts as a filter. The filtering capabilities of the basement membrane are best exemplified by glomerular basement membrane of the kidney. Perhaps by controlling ionic diffusion the basement membrane could influence differentiation (see review, Toole, '81).

Despite these plausible ideas, the exact role of the basement membrane is not clearly understood. When tissues are separated by trypsin that digests the basal lamina and are then recombined, the first sign of tissue interaction is the deposition of a new basement membrane (Karcher-Djuricic et al., '78, Slavkin et al., '82). In the developing odontoblast, differentiation cannot occur unless the basement membrane is redeposited. Perhaps the best example of the effect of the basement membrane on odontoblast histodifferentiation is seen in an experiment by Osman and Ruch ('81), in which they used EDTA separation to preserve the basement membrane contacting the dental papilla. They found that post-mitotic odontoblasts would continue to differentiate and secrete dentin if EDTA separation was used. However, if trypsin was employed, those same non-dividing odontoblasts would not differentiate further. We know that in tooth development the ameloblasts will not differentiate unless they are in contact with that first layer of predentin which of course will not be secreted
unless the odontoblasts polarize (Koch, '67). Thus, the basement membrane may determine in an indirect way whether or not induction of the ameloblasts occurs.

Lesot, et al. ('81), and Thesleff et al. ('81), showed with indirect immunofluorescence staining that there are changes in the distribution pattern of the various components of the basal lamina depending on the stage of development of the tooth. There was more fibronectin directly under the inner enamel epithelium at the time of odontoblast differentiation than elsewhere in the tooth germ. The other components of the basal lamina were more evenly distributed. Perhaps fibronectin is involved in polarization of the odontoblasts as it is synthesized by the mesenchyme. Once the first layer of predentin is secreted the basement membrane is degraded, fibronectin dissappears first and the rest of the constituents leave as a group.

Further indirect evidence of the need for an intact basal lamina during normal morphogenesis, comes from work with inhibitors of extracellular matrix synthesis. Kerley and Kollar ('78), cultured 15-day molars and incisors in the presence of tetracycline. This antibiotic chelates divalent ions such as Fe^{++} and Ca^{++}, which can interfere with mineralization of hard tissues and collagen synthesis. The effect on in vitro culture of tooth germs was to retard their development and suppress normal morphogenesis. It was possible for normal development to resume after transferring the teeth to control medium. The authors concluded that the effect of tetracycline on collagen synthesis was the reason for inhibited morphogenesis.

Thesleff and Pratt ('80) utilized a different antibiotic, tunicamycin, which interferes with glycoprotein biosynthesis.
Glycoproteins such as fibronectin, are constituents of the basal lamina. If the tooth germs are immersed in trypsin prior to culturing them in the presence of tunicamycin, severe retardation of development occurs. The odontoblasts do not polarize, no predentin is secreted, and consequently the ameloblasts do not polarize. The authors postulate that the basal lamina is necessary to trigger the alignment and polarization of preodontoblastic cells. If this does not happen, normal cell-matrix interaction cannot occur. However, this inhibitor study has an inherent weakness since the agent may have generalized effects. Thus the results should be interpreted with caution.

The mechanism of induction is still an unresolved question and may involve other concepts such as surface charge or compounds on the plasma membrane surface. (see review, Saxen, et al., '77) At the moment the strongest evidence is for participation of the extracellular matrix and in particular the basement membrane. Close association between epithelial and mesenchymal cells is essential for them to communicate with each other as shown by the transfilter work and recent descriptions of gaps in the basement membrane. Whether the basement membrane must be removed to permit the signal to be transmitted from one population of cells to another, or whether it contains encoded within its molecular constituents, the actual signal remains to be established.
Tissue Interactions of Oral Mucosa

In most experiments using adult tissues it is difficult to clearly demonstrate an instructive tissue interaction. The response of the epidermis to heterotypic dermis is modification of the keratinization pattern. There is no expression of new gene products, merely the rearrangement of proteins normally made by those cells into patterns specified by the dermis.

One of the first experiments utilizing oral mucosa was actually a small part of a larger body of work performed by Billingham and Silvers ('68). The majority of the experiments were devoted to the response of adult guinea pig epidermis to various types of dermis. They were able to alter the characteristics of adult epidermis according to the pattern dictated by the dermis. Foot dermis could modify the keratinization pattern of ear epidermis; however, mucosa taken from the esophagus, tongue or cheek pouch could not be altered. These investigators thought that there was some feature of oral mucosa that was radically different from that of sole of foot and ear and therefore, the dermal fibroblasts could not alter oral mucosal development.

The resistance of oral mucosa to inductive stimuli was re-examined by Karring et al. ('75). The question they asked was whether heterotypic connective tissue in combination with various oral epithelia could modulate epithelial expression. It is apparent that in the adult there are a variety of oral mucosal types: lining, masticatory and specialized tongue epithelium. Karring et al. ('71), as well as others more recently (MacKenzie and Hill, '81), had already shown that the specificity of the oral epithelium resides in the
underlying dermis. Karring et al. ('75) mechanically dissected monkey gingival connective tissue from the epithelium and placed it in subepithelial pouches in the alveolar mucosa. Control grafts of connective tissue were placed in similar pouches in the alveolar mucosa. After several weeks, the covering epithelium (alveolar mucosa) was stripped away from the connective tissue grafts. In the experimental grafts, re-epithelialization occurred with characteristic keratinization and rete pegs of attached gingiva. This change in the epithelium was not demonstrated unless the overlying mucosa was removed, suggesting that its own connective tissue stroma was posing a barrier to the inductive influence of the foreign connective tissue. Control grafts of alveolar connective tissue became indistinguishable from the surrounding tissue. This study provided support for the idea that the oral epithelium is labile and can be induced to change its characteristics even in the adult animal.

The previous experiments used mechanical dissection to separate the connective tissue from epithelium. More recent experiments used enzymatic separation of the tissue components. Heaney ('77), and Heaney and Jones ('78), used adult pigs as the source of tissue. Strips of alveolar mucosa and attached gingiva with lamina propria were removed and separated with trypsin at cold temperatures. The dorsum of the pig was the recipient site for recombination and control grafts which exposes the tissue to a dry atmosphere, unlike that of the oral cavity. Grafts of whole mucosa exposed to the external environment became keratinized where in their natural environment this would not have happened. However, there was no granular layer seen despite the orthokeratinization of the superficial layers. Gingival
grafts in contrast to those of alveolar mucosa became hyperkeratotic and maintained a stratum granulosum. Combinations of alveolar epithelium and gingival connective tissue produced gingival-like epithelium when compared with environmental controls of whole gingiva. The most impressive results were seen in grafts of pure connective tissue that re-epithelialized. These also assumed gingival features, including a stratum granulosum and hyperkeratosis (Heaney, '77). The reciprocal experiment (Heaney and Jones, '78) which combined alveolar connective tissue with gingival epithelium were difficult to interpret once again, because of the explantation site. However, there was a quantitative decrease in keratinization of gingival epithelium when combined with alveolar connective tissue as well as the absence of a granular layer.

A less ambiguous study done with human tissue, placed trypsin separated palatal connective tissue into a pouch in the alveolar mucosa. (Bernimoulin and Schroeder, '80) This is similar to the experiments of Karring et al. ('75), except for two features. First, the connective tissue was inverted to prevent any palatal epithelium from contributing to the new mucosal surface. Secondly, and perhaps more importantly, the connective tissue was not buried in the pouch but rather sutured to the border of alveolar mucosa. This permitted re-epithelialization to occur without the barrier of alveolar mucosal connective tissue. Bernimoulin and Schroeder ('80) found the regrowth of epithelium covering the graft to be indistinguishable from that of the palate. They also concluded that the message of keratinization was conveyed by all levels of connective tissue not only by the superficial lamina propria.
One other study that confirmed the keratinization pattern is determined by connective tissue, utilized biopsy material from human leukoplakic lesions (MacKenzie et al., '79). The control specimens were taken from non-keratinized alveolar mucosa. These investigators employed EDTA separation of tissues rather than trypsin. This method leaves the basement membrane in contact with the connective tissue. Tissue combinations were grafted to immunodeficient nude (nu/nu) mice. In two of three successfully recovered grafts, normal connective tissue in combination with leukoplakic epithelium caused a reversion of the neoplastic epithelium. There were less marked changes when neoplastic connective tissue was combined with normal epithelium, but in two of four specimens there was a suggestion of induced leukoplakia. These results are similar to those of Van Scott and Reinertson ('61) who used human basal cell carcinoma biopsy specimens. They found the basal cell epithelium would not survive unless its own connective tissue was carried with it. Thus, it appears that in the adult human, epithelial specificity is determined by the underlying connective tissue.

Although much has been written about gingival epithelium, alveolar epithelium, and palatal connective tissue (Karring, et al., '75; MacKenzie et al., '79; Heaney, '77; Heaney and Jones, '78; and Bernimoulin and Schroeder, '80), very little work has been done with palatal epithelium. The masticatory epithelium of the palate follows a similar course of development to that of the epidermis. At the time of fusion, 14 to 14 1/2 days of gestation in the mouse, the oral epithelium that is to become keratinized begins accumulating tonofilaments. The palatal epithelium is only a few cell layers thick.
and all are cuboidal in shape. Between the 15th and 17th day the epithelium becomes stratified and by day 18 completed histodifferentiation is present. The full complement of strata consists of: stratum basale, stratum spinosum, stratum granulosum and the flattened stratum corneum on the surface (Idoyaga-Vargas et al., '72).

The first recombination study using palatal epithelium was done by Tyler and Koch ('77). They combined 12-day epithelium from the pre-fusion palates of mice with incisor papillae of 16-day mice, tongue mesenchyme and submandibular gland mesenchyme. The tissues were cultured in direct contact with each other on top of a millipore filter for 5–8 days in a complex medium. Sections made for light microscopic examination revealed branching morphology in epithelium combined with submandibular gland mesenchyme. As mentioned earlier in this review there is some question of the aptness of the term glandular, especially considering the the cells appeared to deposit keratin rather than secreting salivary glycoproteins. The tongue mesenchyme allowed full differentiation of palatal epithelium similar to control combinations with palatal mesenchyme. Incisor mesenchyme was the least supportive of the palatal epithelium. They did not observe enamel organ induction and the mesenchyme did not support full histodifferentiation of the epithelial cells. They concluded that the cells of the secondary palate become determined prior to palatal fusion and hence, will not respond to an instructive message from heterotypic mesenchyme. Thus, palatal epithelium does not seem as labile as vestibular (Kollar and Baird, '70a), diastema (Kollar, '72), or gingival epithelium (Thesleff, '77). Whether or not this reflects a
regional variation in the determination of oral epithelium or the effects of in vitro versus in vivo culture techniques remains to be seen.

Effects of Experimental Method on Induction

Much work was done in the 1960's on what effect various alterations of tissue culture methods had on epidermal differentiation. Dodson ('67a), combined embryonic chick epidermis with whole living dermis, frozen-killed dermis, and heat-killed dermis. Isolated epidermis was grown on collagen gels, and millipore filters. A new ad-epidermal membrane (basement membrane) was formed in all combinations except those with heat-killed dermis. In fact, collagen gels replaced the dermis supporting not only stratification and keratinization but also secretion of an ad-epidermal membrane. He discovered that certain media, containing high levels of embryo extract would permit isolated epithelium to maintain its stratification after trypsin separation. Additional observations in Dodson's paper were; the epidermis would not grow unless the basal side was placed in contact with the dermis; epidermal spreading is controlled by the dermis and the dermis in turn reorganizes its fibers according to information provided by the epidermis; and, third, the factor that supports growth of the epidermis is heat labile and is probably contained within the extracellular matrix.

In contrast Briggman and Wheeler ('71) discovered that with adult tissues collagen gel would not substitute for living dermis. Perhaps adult epithelium is more restricted in developmental lability and, requires a substrate more closely resembling the homotypic connective tissue.
Wessels ('61) grew chick epidermis in a chemically defined medium and compared it closely to the growth seen in vivo. He found that omitting L-methionine from the medium resulted in necrosis, whereas adding cystine and cysteine promoted cornification. Thus there are certain nutritional requirements in the media that must be met.

Mcloughlin ('61a) studied isolated chick epidermis grown in culture with or without excess vitamin A. As just described above, epithelium does not do well on its own. McLoughlin observed that the epidermis does not spread on glass but instead it rounds up. Ultimately, even the basal cells keratinize. Adding vitamin A to the medium of cock's plasma and one part embryo extract prevented the epidermis from keratinizing and encouraged mucous secretion. Whether or not this fits into the category of an inductive event is debatable. Most of the literature tends to refer to this as a metaplastic event, (Dodson, '67b; Fell and Mellanby, '53) partly because it is reversible upon removal of the retinoic acid (Fell and Mellanby, '53). It may be more correct to say that the actions of vitamin A are similar to hormonal modulation of cellular physiology.

Certain criteria must be fulfilled in order to permit an organ to express its shape in culture. Gospodarowicz et al.('78) said that organ culture favours growth in three dimensions, while tissue culture promotes two dimensional growth. Even within various cell culture systems there are differences in cellular shape dictated by the substratum. For example, this same group of investigators found that collagen gels permit corneal cells to round up and become sensitive to epidermal growth factor while plastic substrates will make the cells flatten and become insensitive to EGF. Karasek ('71) also found post
embryonic mouse epidermal cells were modified by the surface of the culture substratum. Thus, if the objective is to produce a three dimensional structure the culture environment should not be one which promotes cell spreading. As Lawson pointed out (')74), critical mass necessary for induction is considerably reduced if cells are allowed to disperse on a millipore filter.

Several environments to which tissue rudiments have been grafted have proven to be suitable for supporting morphogenesis. Chorioallantoic membrane, subcapsular space of the kidney, anterior chamber of the eye and other subcutaneous sites on the homologous animal are frequently used. They all have in common a fluid rich substratum, humid atmosphere, and result in the establishment of a blood supply to the graft. Consequently, these techniques are better termed in vivo cultivation as opposed to in vitro methods on chemically defined media or various other serum mixtures. An interesting comparison of culture methods can be made between the work of Cummings et al. ('81) and Arechaga et al. ('83) on the one hand, and that of Kollar and Fisher ('80) on the other. The former investigators used chemically defined media to grow combinations of quail mandibular epithelium and mouse molar papillae. They did not see any differentiation of ameloblasts and secretion of enamel matrix. Although Cummings et al. ('81) state that chemically defined media supported growth for a 10 day period and that during that time the odontoblasts produced dentin matrix, the figures do not bear this out. There is a single light micrograph of a heterotypic combination which had been cultured for three days, and the dentin layer is very thin. The authors also acknowledge that the matrix was not mineralized.
Thesleff et al. ('77) and Koch ('67) in transfilter experiments showed that a definite layer of predentin must be secreted prior to ameloblast differentiation. Thus the failure to induce ameloblasts in the experiments of Cummings et al. may have been related to the lack of dentin. Even less successful results were obtained by Arechaga et al. ('83) as odontoblast polarization was not observed without concomitant homotypic enamel epithelium contamination. In contrast, the figures in Kollar and Fisher's ('80) paper show definite tooth morphology attained in similar combinations of chick mandibular epithelium and mouse molar papillae when grown in the anterior chamber of the eye for two to three weeks. Perhaps the difference in results was partly due to the intraocular culture method. Establishing a blood supply to the graft more closely simulates in vivo conditions and permits mineralized matrix deposition not possible in vitro.

The Separation of Tissues with EDTA and Trypsin

In previous sections, tissue isolation methods using trypsin or EDTA have been mentioned and their possible contribution to the experimental results. In addition, a less perfect alternative, mechanical dissection, is often used. In certain tissues such as older tooth germs (Huggins et al., '34) and adult epidermis (Woodley et al. '83), the mechanical technique may yield clean separations. This is a reflection of the tooth matrix already deposited in the older teeth which gives a natural plane of separation and the simple architecture of the epidermal-dermal junction in adult skin which make mechanical cleavage possible. However, when dealing with younger tooth germs, hair follicles, feathers, scales, salivary, mammary and other glandular structures, the junctional morphology is too complex to
permit using this technique.

The chief difference between trypsin and EDTA treatment is that the former digests the basement membrane, whereas EDTA preserves it in contact with the mesoderm or connective tissue. It was once thought that trypsin totally removed the basement membrane as seen in ultrastructural studies (Karcher-Djuricic et al., '78, Thesleff et al., '78). This has since been disproven by using indirect immunofluorescence staining (Lesot et al., '81). Residual antigenic sites were noted for type IV collagen and laminin on both the epithelial and mesenchymal components. Fibronectin was, however, totally removed with the trypsin treatment. Bernfield et al. ('73) demonstrated with Alcian blue staining that glycosaminoglycans were removed by trypsin. This was confirmed recently by Woodley et al. ('83) who showed with immunologic techniques that heparan sulphate was removed by this enzymatic treatment. Thus the actions of trypsin are to remove the fibronectin and heparan sulphate of the basement membrane, thereby weakening the union between heterotypic cell populations.

EDTA treatment leaves an electron dense layer and a fibrillar layer directly adjacent to the mesenchymal cells. Antigenic studies show that all major basement membrane components are still present after EDTA treatment, but they are confined to the mesenchymal surface only (Lesot et al., '81). The actions of EDTA are thought to be at the level of the lamina lucida adjacent to the epithelium. Lesot et al. ('81) postulated that since fibronectin requires Ca$^{++}$ and Mg$^{++}$ to bind to cells, the EDTA chelates these ions and weakens this cell-fibronectin complex.
It is important to verify that separation has been complete in any recombination experiment since the presence of contaminating cells could seriously affect the validity of the results. The common methods employed to check for lack of contamination are first, to visually inspect the tissue pieces under the dissecting microscope. Second, to fix the isolated components and examine serial sections for the presence of contaminating cells. Third, to grow the separated tissues under similar conditions to those of the experimentals. Fourth, to examine selected sites with transmission electron microscopy. Fifth, to use scanning electron microscopy in order to visualize the cleanliness of separated surfaces.

Although TEM and SEM methods give an extremely detailed view of the surface of separated tissues, they may not detect contaminating cells remaining after stripping procedures. TEM can only hope to sample a very small area of the specimen, and SEM cannot positively identify one cell type versus another. These shortcomings may be critical if one is performing recombination work, especially if the frequency of inductive events is low. Mackenzie et al., ('79) found that when they stripped leukoplakic epithelium from its connective tissue with EDTA and cultivated the isolated connective tissue in vivo, there was re-epithelialization. The authors attribute this to the deeper and irregular epithelial-connective tissue interdigitation of leukoplakic biopsy specimens which resulted in incomplete separations. In two out of four recombinations of leukoplakic connective tissue and normal mucosa there was suggestion of a change from a non-keratinized to a keratinized state. With the evidence of incomplete stripping, it does not seem reasonable to conclude that
connective tissue from leukoplakic areas always modifies normal mucosa to become hyperkeratotic.

Goldberg and Hardy ('83) using the same concentration of EDTA, 1mM, achieved excellent separation of vibrissae pads as seen with TEM and SEM, however they did note the presence of scattered epithelial cells in the deep pits of the hair follicles on light microscopic sections. This observation was not made with the trypsin separated tissues. These investigators did not grow the isolated tissues for a period of time either in vitro or in vivo, thus it is possible that enough epithelial cells remained after EDTA separation to permit hair follicle development. At the present, recombination studies have not been attempted by these investigators.

EDTA has been used in the separation of tooth germs, most notably by Ruch, and his colleagues. In some experiments they did not perform recombinations with heterotypic epithelium or mesenchyme. Rather they focused on the effects of basal lamina removal versus preservation on odontoblast differentiation (Osman and Ruch, '81). The controls consisted of serial sections of cultured components, transmission and scanning electron microscopy. Osman and Ruch ('81) made note of migrating cells which covered parts of the EDTA separated papillae. These cells were seen in both the light and electron micrographs and were only present on those papillae separated with EDTA, not those treated with trypsin. They cultured the papillae for varying lengths of time and the presence of migrating cells was noted at all times from three hours up to twenty-four hours. After a period of twenty-four hours the migrating cells accumulated in the clefts between the cusps and became stratified. Culturing the papillae in the presence of
colchicine did not inhibit this migratory cell population although the drug disrupts intracellular microtubules. The authors could not explain the origin of the migratory cells, but postulated the basement membrane aided their movements. A close examination of the figures in this paper leads to an alternative explanation for these mobile cells. The migratory cells could in fact be remnants of the enamel organ which adhered to the papillae. Certainly based on the appearance and distribution of these cells one should not rule out the possibility of epithelial contamination.

Perhaps the most accurate method of detecting homotypic tissue contamination is with a nuclear marker. Nuclear chromatin in quail cells has a distinctive pattern which easily distinguishes these cells from those of chick or mouse origin. Arechaga et al. ('83) found that trypsin treatment of molar tooth germs for less than 75 min. would result in homotypic tissue contamination. Quail limb buds also failed to separate cleanly if immersed for less than 135 min. The nuclear marker made contamination obvious and cast doubts on earlier similar recombinations by Hata and Slavkin ('78). As mentioned previously the latter group induced cartilage in molar mesenchyme under identical culture conditions. The difference in results could be due to imperfect tissue dissociations since Hata and Slavkin ('78) treated all their rudiments, including limb buds, for 75 min.

In summary, the most reliable method of detecting tissue contamination, short of nuclear markers, is by using in vivo culture of isolated tissues as controls. In this way contaminating cells in sufficient number to meet the critical mass for a tissue interaction, will have the opportunity to reveal themselves.
**Future Directions to be Followed in the Study of Tissue Interactions**

There has been very little experimentation utilizing heterochronal tissue recombinations. Therefore, the age at which cells and tissues are first determined is not known. Cells are multipotential up to a certain age, with continued development they become restricted to a number of pathways, and finally when only one option remains they are said to be determined (Wessels, '77). In the terminally differentiated state, each new generation of cells is identical to the one preceding it.

In the adult organism there is tremendous dependability in the maintenance of tissues most of the time. However occasionally this process of cell death and replacement goes awry and carcinomas arise. Carcinogenesis may be an example of embryonic tissue interactions taking place in the adult animal (Pierce, '78). There may be a reversion of the connective tissue to an inductive embryonic state which in turn instructs the epithelium to become neoplastic.

The analogy between carcinogenesis and embryonic tissue interactions implies that developmental biologists can make important contributions to the field of oncology. It is very likely the continued study of temporal patterns and instructive tissue interactions will clarify some of the basic requirements for normal tissue maintenance.
GENERAL OBJECTIVES

This study was undertaken to investigate the temporal effects on responsiveness of oral epithelium to an inductive stimulus. Embryonic tissues can dramatically alter their course of development under the appropriate experimental conditions. The adult epidermis is much more stable. Even though generations of cells are dying and are being replaced weekly, there is assurance that the next population of cells will be exactly the same as their predecessors.

The difference between the fetal and adult oral epithelium is similar to that of epidermis. Only slight modulations in keratinization pattern can be made in adult oral epithelium, but fetal epithelium can be induced to form teeth in addition to the characteristic keratinized stratum corneum. This study was designed to determine the age at which the oral epithelium is irreversibly stable. Does it have competence that extends past birth into the neonatal period or does it become restricted prior to birth?

SPECIFIC OBJECTIVES

The specific aims of this project were to determine at what age the palatal epithelium of mice will no longer form teeth when combined with molar mesenchyme; to investigate features of the epithelium that change in conjunction with the loss of competence to form teeth; to test the effectiveness of EDTA versus trypsin separation of tooth germs; to demonstrate the presence of a reconstructed basement membrane after trypsin treatment; and to examine the ultrastructure of the dental matrices comprising an induced tooth.
MATERIALS AND METHODS

CD-1 (Charles River) mice were used in this study. Fetuses were staged according to the morphologic criteria of Gruneberg ('43) and the timing of vaginal plugs. The day of the plug was taken as day zero. With the aid of a microscope at 10X to 20X magnification, 15- to 17-day mandibular molar tooth germs were dissected bilaterally from fetal mice. Palatal epithelium was excised from the center of the secondary palate, medial to the molar tooth germs and posterior to the incisor tooth germs, from mice aged 15 to 19 days. The palate fuses at 14 to 14 1/2 days. Thus within the age range used for these experiments there were only two palatal surfaces to be concerned with, the nasal and the oral side. All dissections were done in Maximov depression slides containing 10% fetal bovine serum (FBS) in Hank's salt solution.

Dissociation with Trypsin

Whole tooth germs and pieces of palate were immersed in a 1% solution of crude trypsin (Bacto-Difco, 1:250) for two hours at 4°C. The tissues were rinsed in a 1:1 solution of Hank's balanced salt solution and FBS. The epithelium was gently teased apart from the mesenchyme (fig. 1). Dissection of palatal epithelium was facilitated when older animals were used. Thus, in 15- to 16-day mice very small pieces of epithelium were recovered. There was a difference in the way the stripped epithelium curled depending on the age of the tissue. The 15-day and 16-day tissue curled with the basal side out, at day 17 it remained reasonably flat, and at 18 and 19 days of gestation it curled inwards. This tissue behavior helped in orienting the epithelium basal side down on the papilla and this is consistent with the observations
of McLoughlin ('61). In addition, at 18 and 19 days a stratum corneum was present in the form of a glistening, transparent layer which distinguished it from the more basal layers. One further criteria used to distinguish the oral from the nasal side was the characteristic rugae pattern of the oral surface.

Dissociation with EDTA

Some tooth germs were separated with EDTA and either of two protocols was followed. The first method employed was immersion in a 10mM solution of tetrasodium EDTA in Ca^{++}-Mg^{++}-free phosphate buffered saline (PBS) at pH 7.3, incubation for 10 to 15 min at 37°C, following which the tooth germs were rinsed twice in PBS (adapted from Osman and Ruch, '81). The second method based on the technique of Goldberg and Hardy ('83) utilized a solution of 1mM tetrasodium EDTA in Ca^{++}-Mg^{++}-free PBS. The tissues were rinsed twice and left in this solution for 1 hr and 30 min at 4°C with one change of EDTA during that period. The EDTA was replaced and the tooth germs were incubated at 37°C for an additional 1 hr and 30 min with one change of EDTA during that period. At room temperature, the enamel organs were carefully peeled away from the papillae. The papillae were transferred individually to agar-solidified complex media.

Recombination: *In Vitro* Culture

All tissues to be recombined or to be used as controls were transferred to agar-solidified medium consisting of Eagle's Basal Medium (Gibco, Grand Island, N.Y.), supplemented with 12% FBS, 1% gentamycin sulphate (Schering Corp. Kenilworth, N.J.), and 0.4% agar.

The separated components were visually inspected for signs of contamination prior to recombination. Palatal epithelium was
positioned basal side down over the trypsin treated papillae on the solidified complex medium (fig. 1). Isochronal combinations were made with 15-, 16- and 17-day tissue. Heterochronal combinations were made with 18-19-day palatal epithelium and 15- to 17-day molar papillae. A few neonatal combinations were made with EDTA and trypsin treated tissue, using oral epithelium from neonates up to one week postpartum. Isolated trypsin and EDTA papillae were cultured in a manner identical to that of the recombinants. The *in vitro* culture period was 24-48 hrs at 37°C in humidified, 5% CO₂-95% air mixture. After *in vitro* recombination, the tissue rudiments formed a cohesive mass.

**Intraocular Grafting: *In Vivo* Culture**

The combinations and controls were transferred to the anterior chambers of the eyes of adult host mice (Kollar and Baird, '69). The mice were anesthetized with .05 to 0.1 ml of ketamine diluted to 50 mg/ml (Ketaset, Bristol Lab, Syracuse, N.Y.) and 0.01 to 0.02 ml of xylazine solution diluted to 50 mg/ml (Rompun, Bayvet Lab., Shawnee, K.S.). An incision was made in the cornea with a scalpel blade and iridectomy scissors. A triangular flap was reflected, and the graft was placed in the anterior chamber just under the cornea and superior to the iris (fig. 1). The flap was replaced and combinations as well as EDTA and trypsin separated papillae controls were allowed to grow for 10-14 days.

**Harvesting of the Grafts**

The mice were killed by cervical dislocation, the eyes removed, and the grafts retrieved from the anterior chamber with the aid of a dissecting microscope (fig. 1).
Light Microscopy

Tissue combinations and control molar papillae were fixed in 10% neutral buffered formalin for 24 hours. They were decalcified with EDTA for one week, dehydrated and embedded in Paraplast (Sherwood Medical Industries, Inc. St. Louis, Mo.). The blocks were serially sectioned at 6 um and stained with hematoxylin and Bielbrach's scarlet.

Electron Microscopy Techniques

Some of the specimens were examined with transmission electron microscopy (TEM). They were fixed in a 2% glutaraldehyde solution for 2 hours, rinsed with sodium cacodylate buffer and post-fixed in 1% OsO₄ for 2 hours. The material was dehydrated and embedded in Epon 812. Semi-thin (1 um thick) sections were stained with toluidene blue and the block fine trimmed accordingly. Gold and silver thin sections were taken of this area and stained first with uranyl acetate and then lead citrate. Grids were examined and photographed with a Hitachi transmission electron microscope at an accelerating voltage of 75 kV. The specimens examined with TEM were not decalcified.

Representative specimens separated with EDTA and trypsin were examined with scanning electron microscopy (SEM). They were fixed in 2% glutaraldehyde and cacodylate buffer and post fixed in OsO₄. The tooth germs were critical point dryed and mounted on aluminum stubs with double sided tape, sputter coated with gold-palladium, and examined in a Hitachi scanning electron microscope at an accelerating voltage of 20 kV.

Indirect Immunofluoresence Staining for Laminin

The material stained for immunofluorescence consisted of combinations of molar papillae separated with either trypsin or EDTA.
and palatal epithelium separated with trypsin. In addition, tooth germs immersed in trypsin or EDTA, but only partially separated, were treated in a similar manner. Heterotypic/isochronal, heterotypic/heterochronal tissues and controls were fixed at 4°C in 95% ethanol for 2 hours (St. Marie, '62). The explants were then dehydrated in absolute alcohol for 1 hour and transferred to xylene, both at 4°C. The specimens were embedded in paraffin and serially sectioned at 6 μm. The sections were mounted on glass slides and deparaffinized in two, 30 second changes of cold xylene and cold 95% ethanol.

The slides were washed in three consecutive baths of phosphate-buffered saline for 4 min. each. After hydration, they were drained and rabbit anti-laminin antibody solution at a concentration of 80 μg/ml was pipetted over the sections totally wetting them. Control slides were wetted with preimmune rabbit serum. The slides were incubated at room temperature and 100% humidity for 30 min. Controls and experimentals were rinsed in PBS three times for 4 min. each, and stained with fluorescein-conjugated goat anti-rabbit antibody, for a period of 30 min. at 100% humidity. After three additional washes in PBS, the preparation was mounted under a coverslip in glycerin-PBS (9:1) and viewed with a Zeiss photo microscope equipped with an ultraviolet light.

After photographs were taken, some of the slides were immersed in PBS to remove the cover slips and routine staining for light microscopy was completed.
RESULTS

Number of Combinations and Controls Examined and Frequency of Tooth Induction

The total number of specimens examined for each group of experiments in each age category is shown in table 1. Each experiment used one to two litters of fetuses and resulted in approximately five isochronal and heterochronal combinations, and five controls. Isochronal combinations consisted of palatal epithelium and molar papillae of the same age, dissected from the same litter. This data, composed of experiments using 15-, 16-, and 17-day tissues, was divided into three age groups. Heterochronal combinations were composed of 18- and 19-day palatal epithelium and molar papillae ranging from 15 to 17 days of gestation. The heterochronal experiments were combined into one category because there was no induction of teeth in any case. All tissues were dissociated with trypsin.

There were many more controls grafted than combinations in all categories both to ensure contamination-free separation and because there was usually an excess of mesenchyme especially in the 15- and 16-day animals. Palatal epithelium was not cultured in isolation as it is well established that epithelium will not survive without an underlying mesenchyme or connective tissue.

Combinations of isochronal tissues always adhered after growth on solidified medium for 24–48 hours. The heterochronal combinations would infrequently separate due to improper orientation of the epithelium. However, the majority of these recombinants did form a cohesive mass and epithelial growth was present in the harvested graft.
The incidence of tooth induction in the combinations is expressed as the numbers of whole teeth formed over the number of combinations grafted (Table 2). The frequency of tooth formation in the controls in each age category is listed as zero over the number grafted since teeth were never formed.

There was more induction in 17-day tissues where 12 teeth were formed from 39 combinations. At 15 days, 5 out of 23 combinations produced teeth, and at 16 days, 2 teeth were induced from a possible 26 combinations. The total frequency of induction for isochronal combinations was 21.5%. Palatal epithelium of 18 or 19 days of gestation would not respond to the inductive stimulus, thus, the frequency of induction in heterochronal combinations was 0%.

The incidence of epithelial contamination was 19% when EDTA separation was used. This percentage included both grafts that contained epithelium and teeth or both.

Descriptive Results

A. Controls

i. Trypsin Dissociated Molar Papillae

a) Ultrastructure

Trypsin-separated molar papillae showed no evidence of a basal lamina overlying the odontoblast layer. The odontoblasts were seen to have irregular cell borders or blebbing as a result of enzyme treatment. Both the light microscopic sections (fig. 2) and ultrastructural appearance (fig. 3) illustrate this feature. Other than the absence of the basement membrane the odontoblasts were normal in every respect.

b) Appearance After in vitro Culture
Molar papilla cultured for two days on agar-solidified complex media lost all cuspal morphology. The mesenchyme formed a solid, round mass of cells and no epithelium could be detected (fig. 4).

c) Appearance After in vivo Culture

After one to two weeks of intraocular culture, explanted trypsin dissociated papillae were variable in appearance. In the absence of an organizing epithelium, the grafted papillae occasionally dissipated completely such that the anterior chamber of the eye was empty upon harvesting. The second and far more common appearance was a solid mass of bone spicules and loose connective tissue (fig. 5).

ii. EDTA Dissociated Molar Papillae

a) Ultrastructure

In semi-thin section, the basement membrane is represented by a fine line overlying the surface of the papilla (fig. 6). In these specimens there was no evidence of stray epithelial cells lying upon the basal lamina. The ultrastructural features of the basement membrane covering the EDTA-separated papillae consisted of a distinct electron dense layer and a cell-free zone containing fibrillar material (fig. 7). A scanning micrograph of a stripped molar papilla gives an overall view of the cuspal morphology and the velvety surface imparted by the EDTA treatment (fig. 8).

b) Appearance After in vitro Culture

EDTA-treated molar papillae lost their cuspal morphology as did the trypsin-dissociated tissues. However, in serial sections, significant epithelial growth was detected (fig. 9).

c) Appearance After in vivo Culture

The grafts consisting only of isolated molar papillae exhibited
two predominant features. Most consisted of bone fragments and connective tissue. However, 19% contained keratin cysts and teeth.

iii. Normal Development of Palatal Epithelium

Advanced keratinization of palatal epithelium similar to that seen in vivo, was supported by the tooth mesenchyme. Normal differentiation of palatal epithelium at the ages used in this study is shown in figures 10-13. At 15-days the epithelium is only two to three cell layers thick and all are cuboidal in shape (fig. 10). At 16-days the surface cells are beginning to flatten and the thickness has increased to four to five cell layers (fig. 11). Seventeen-day epithelium exhibits full stratification including; stratum basale, stratum spinosum, a distinct stratum granulosum and stratum corneum on the surface (fig. 12). Eighteen-day epithelium has added a significant layer of orthokeratin to the surface (fig. 13).

B. Experimental Tissue Combinations

i. Isochronal

a) Light Microscopic Appearance

The combinations of epithelium and mesenchyme of the same age contained teeth in 21% of the explants. The teeth that were formed were normal in appearance and the degree of development depended directly on the duration of the intraocular culture period. After 14-15 days the full complement of cell and matrix layers was present and included: odontoblasts, predentin, dentin and enamel matrices, enamel space (in some more mineralized specimens the decalcification process totally removed the the enamel), and inner enamel epithelium comprised of post-secretory ameloblasts. The tooth illustrated in fig. 14 was formed from a combination of 17-day epithelium and 17-day papilla. All
appropriate structures are present in normal configuration in this specimen. The enamel matrix is newly secreted, unmineralized and is retained in this specimen. The space between the enamel and dentin is an artifact produced in processing. A semi-thin section taken from a similar combination also does not show an enamel space because the matrix is not fully mineralized (fig. 15). The intercellular spaces in this section are exaggerated due to a fixation artifact. In both combinations the tapering of the ameloblast layer at the future cemento-enamel junction is seen. Thus, full crown morphology has been achieved and root development is commencing.

An immature bell stage is seen in a combination of 15-day tissues that was cultivated for 5 days (fig. 16). Another graft of 15-day tissues grown for 7 days has reached late bell stage with predentin and dentin already deposited. The ameloblasts have just entered the secretory phase hence they are extremely elongated with the nuclei placed basally next to the stratum intermedium (fig. 17).

All of the harvested combinations contained keratinizing epithelial cysts. These structures were always closely associated with the cultivated tooth and conversely a tooth was never observed without a cyst nearby. Figure 18 illustrates a tooth grown from a combination of 16-day tissues whose stellate reticulum seems to be fused with the basal layer of a cyst. A higher magnification of this area of contact is seen in figure 19.

Occasionally a piece of nasal epithelium from the opposite side of the palate was included with the graft. Figure 20 shows nasal epithelium in continuity with the epithelium forming the keratin cyst. A higher magnification of this region illustrates the typical
appearance of respiratory epithelium which consists of pseudostratified, ciliated, columnar cells containing mucous secretions (fig. 21).

b) Ultrastructure

The ultrastructure of the enamel in an induced tooth displays the typical pattern of enamel crystals as deposited in rodent teeth (fig. 22). The needle-like crystals conform to the prism pattern described by Tomes' processes. The irregular dentin-predentin junction is visible in figure 23 with the crystalite pattern being much less organized than that of enamel.

ii. Heterochronal Combinations

a) Structure of Keratin Cysts

The appearance of heterochronal combinations was similar to that of the combinations using isochronal tissues, in that keratin cysts were formed. Figure 24 shows a keratin cyst formed in a combination of 15-day molar mesenchyme and 18-day palatal epithelium. However, in contrast to the isochronal combinations, there was not a single instance of tooth induction. Since the intraocular culture site does not encourage cell spreading, round, cyst-like structures are formed. The center of the cyst is typically filled with orthokeratin and is surrounded by a layer of cells rich in keratohyalin granules (fig. 25). Stratum spinosum is directly beneath the granular layer and finally the outermost cells form a stratum basale. The ultrastructural appearance of the keratohyalin granules can be described as electron dense bodies within the epithelial cells (fig. 26). Surrounding the cysts, are bone spicules and loose connective tissue (fig. 24).

Control grafts of intact tooth germs also contained keratin
cysts, but their structure varied from that just described. The origin of these cysts was the enamel epithelium, a tissue that does not normally keratinize. Thus, there were far fewer keratohyalin granules in the stratum granulosum (fig. 27).

C. Immunofluorescence Microscopy

i. Controls
a) Trypsin-treated Tooth Germs

Tooth germs treated with trypsin demonstrated fluorescence at both the epithelial and mesenchymal surfaces adjacent to the plane of cleavage (fig. 28). The staining is less brilliant than that see with EDTA treatment and is interrupted.

b) EDTA-treated Tooth Germs

EDTA treatment resulted in a very brightly staining layer of laminin in contact with the papilla and no fluorescence associated with the enamel organ (fig. 29). An incomplete cleavage of the epithelium from the mesenchyme is witnessed by the presence of epithelial cells clinging to the fluorescein stained basement membrane (fig. 30).

ii) Heterochronal/heterotypic Recombinants
a) Trypsin-separated Molar Papilla in Combination with Trypsin-separated Epithelium.

Epithelial cysts filled with keratin were formed in these combinations (fig. 31). The basement membrane component laminin, was labeled by indirect immunofluorescence staining. The labelling was distinct, and described a very fine line between the epithelium and mesenchyme. The endothelium of the vasculature was also decorated with the antibody and fluoresces.
b) EDTA-separated Molar Papilla in Combination with Trypsin Separated Epithelium.

Keratin cysts were usually formed in these grafts similar to those already described for the trypsin dissociated tissues. The presence of laminin was verified by a bright line of fluorescence at the junction of the epithelium and mesenchyme (fig. 32). The brilliance of this label was much greater than that seen in the trypsin treated recombinants. The endothelium of adjacent blood vessels was also stained with fluorescein-labeled antibodies. No other areas acquired the stain, and background fluorescence was low.
DISCUSSION

The results demonstrate that palatal epithelium in the mouse cannot be induced to become an enamel organ at eighteen days of gestation or one day before birth. Prior to this time, seventeen days of gestation or younger, palatal epithelium is capable of forming an enamel organ and dental matrices. When tooth formation was not elicited, full histogenesis in the form of keratin filled cysts occurred.

Combinations with similar tissues were made by Tyler and Koch ('77). These investigators combined sixteen-day incisor papillae with twelve- to fourteen-day palatal epithelium. The deposition of enamel or dentin was not observed nor did the epithelial cells progress to the fully differentiated state. Two other heterotypic combinations were made with tongue and salivary gland mesenchyme. In both these recombinants, keratinization of the epithelium was supported. The incomplete cytodifferentiation of palatal epithelium in the presence of tooth mesenchyme may have been related to the age of the epithelium according to the authors. There was a subtle difference between the response of the twelve- and fourteen-day palate in that there was slightly more keratinization in the fourteen-day epithelium. Perhaps the cells required a certain degree of specialization in order to continue differentiation in the presence of tooth mesenchyme. This, however, was not a requirement of tongue or salivary gland mesenchyme both of which supported full histogenesis in twelve-day epithelium. In addition, Tyler and Koch postulated that the age of the incisor they used, sixteen-days, was perhaps too old to be inductive. At this time the odontoblasts are beginning to secrete dentin. Incisors of
this age were used in recombination experiments by Kollar and Baird ('70a) and they found them to be inductive. Thus it is unlikely that the age of the incisor hindered induction and in the very least the mesenchyme should have promoted keratinization. The conclusions drawn by Tyler and Koch ('77) were that palatal epithelium is similar to pancreatic epithelium and that it will continue to follow an inherent pattern of histogenesis with some heterotypic mesenchymes (except tooth papilla).

The disparity in the results of the present study as compared to those of Tyler and Koch ('77) is possibly related to culture technique. The previous investigators used a millipore filter substrate which promotes mesenchymal spreading and an in vitro system (Lawson, '74), that provides a nutritionally limited environment. A comparison can be made with the work of Kollar and Baird ('70b) in which plantar epithelium, also a keratinizing tissue, and molar mesenchyme were recombined. The anterior chamber of the eye served as their in vivo explantation site. Well differentiated keratin cysts were formed in all grafts as well as teeth in several of the recombinants. This demonstrated that tooth mesenchyme can support histogenesis of a keratinizing epithelium and when cell spreading does not occur, the epithelium assumes a round, cyst-like morphology. These results are identical to the ones obtained in the present study. Thus, it seems that the intraocular culture site is optimal for promoting tooth formation as well as the differentiation of an epithelia that keratinize.

The present study also supports the notion that there is a link between histogenesis and morphogenesis. Accompanying the dental
morphology there is secretion of a significant quantity of enamel and dentin matrix. The prolonged culture period allowed mineralization of enamel and dentin to take place unlike the work done with in vitro techniques (Koch, '67; Thesleff et al., '77; Slavkin et al., '82). In addition, the ultrastructural appearance of both matrices is normal. This is an indication that the induced structure not only looks like a tooth but that the cells participating in odontogenesis are expressing new genetic information and are performing properly. Thus, the inductive message is a complete one as seen in the studies of Cunha et al. ('83) and Karkinen-Jaaskelainen ('78). Tyler and Koch ('77) found palatal epithelium incapable of altering histogenesis despite its new glandular morphology. On cross section, the cells lining the lumen were stratified and demonstrated birefringence which they interpreted as keratin. However, a granular layer was absent and no other evidence for the presence of keratin was presented. Once again the in vitro culture system may have prevented the complete effect of the inductive stimulus.

In the present study, the inductive ability of the molar mesenchyme was held constant, since all ages used, from 15 to 17 days of gestation are inductive. By varying the age of the epithelium in combination with the 15- to 17- day papillae only one variable, the responsiveness of the epithelium, was tested. The results of the present study reveal a somewhat sudden loss of competence in the palatal epithelium at 18 days of gestation. Whether this represents a true biologic timetable of development is uncertain. A similar pattern of development occurs in the avian anterior shank dermis where there is a precise period during which it is capable of instructive
induction. During this phase of development the dermis can induce middorsal epidermis, a feather bearing area, to form scales (Rawles, '63; Sengel, '76). The younger dermis from five- and six-day embryos fails to produce any sort of appendage and at nine days it acquires inductive capabilities which result in feather formation. At day twelve the first hint of new inductive powers is expressed and feathered scales are induced. Finally a peak of inductive activity is reached at thirteen days of development when scales are produced. This inductive power is only expressed with epidermis from five and one half to seven and one half days old. When the epithelium ages an additional day, scale formation is not possible even with the optimal inducer, 13- to 15-day dermis. Thus, in avian embryogenesis the epidermis displays a rather sudden end to responsiveness.

The absence of induction in eighteen- and nineteen-day palatal epithelium might be rationalized in at least two ways. Ruch et al. ('82) suggested that odontoblasts must go through a minimum number of cell cycles before they will polarize and secrete dentin. The basal cells of palatal epithelium may have a similar program which determines when they become unipotential. At day eighteen the last cell division before the epithelial cells become fully functional has been completed. The synthesis of keratin is accelerated and enamel protein synthesis cannot be switched on no matter how inductive the tooth mesenchyme may be. Thus, the simultaneous histodifferentiation of a keratinized stratum corneum and loss of competence may be more than coincidental.

The second explanation for determination of eighteen-day palatal epithelium is also linked to the appearance of a less pliable,
keratin-rich stratum corneum. For example, with increasing age, avian mid-ventral epidermis loses its ability to form feathers in combination with dorsal dermis (Rawles, '63). Sengel ('76) proposed that the physical constraints of the outermost horny layer of the epidermis precluded the evagination necessary to form feathers. Histologic support is taken from the appearance of the basal layers of the mid-ventral epidermis. These cells appear to be engaged in placode formation, while the higher strata are already beginning to keratinize. In the development of the tooth germ, the dental lamina cells invaginate. The keratinization of the stratum corneum should not prohibit this downward growth of dental lamina cells. However, the direction of cellular migration in the basal layers has not been fully determined. It is possible that keratinization could impede the recruitment of distal cells into the invagination. This would provide a causal link between keratinization and cellular determination. Based on this study, such a relationship cannot be established although the correlation may be valid.

In the present investigations, the cumulative frequency of induction in palatal epithelium of seventeen days gestation or less is 21%. With tooth formation occurring in only one of five combinations, it is critical to avoid contamination of the dental papilla with enamel organ. The results of trypsin dissociation are excellent. The presence of epithelium or teeth was not observed in any of the control explants. Instead the mesenchyme formed bone when grown intraocularly confirming the observations of previous investigators (Kollar and Baird '69; Yoshikawa and Kollar, '81). Thus the cells of the future pulp have the capacity to form bone in the absence of an epithelial
interaction. In addition to the numerous controls, the inclusion of respiratory epithelium in one of the explants gives further support for the cleanliness of separation procedures. The only place this specialized epithelium could have originated is the nasal side of the palate.

The comparison between EDTA and trypsin separation of tooth germs demonstrated that EDTA-separated papillae were capable not only of bone formation but also tooth development. This finding was surprising based on previous reports in the literature that had used EDTA for tissue separation. Osman and Ruch ('81) used this agent on eighteen-day molar tooth germs and claimed not to have contamination. However, the published light micrographs show epithelial-like cells in contact with the papilla. In the present paper, material fixed immediately after EDTA treatment was examined with immunofluorescence and transmission electron microscopy. In addition, specimens that were cultured either for two days in vitro or two weeks in vivo were sectioned serially. Utilization of these techniques reduced the chances of not observing adherant enamel epithelium. It is possible that Osman and Ruch ('81) also had incomplete separation with EDTA but it did not express itself under the brief, 24 hour in vitro culture conditions. Within the bounds of their experimental method, and for the purpose of short-term observations on odontoblast differentiation, EDTA may be a useful separation technique. However in recombination work where the necessity for clean tissue separation is paramount, EDTA in 1 or 10 mM concentrations, is not the method of choice.

The 21% frequency of tooth induction in recombinations in the present study is reasonable. Even when purely dental tissues are
recombined under optimal culture conditions, 100% success is not attained. Yoshikawa and Kollar ('81) combined dental papillae and dental sac with enamel organs and found tooth formation in only 70% of the grafts. The 70% induction frequency in Yoshikawa and Kollar's study and the 21% incidence of tooth formation in the present study could be reflections of; critical mass, tissue damage during the dissection and stripping procedures, and improper positioning of the epithelium with respect to the mesenchyme.

There may be other explanations for the induction of teeth in addition to the formation of cysts in some recombinants. Perhaps there is a change in the basement membrane. Trypsin treatment removes portions of this structure and it must be replaced prior to continued development. Any delay in replacement or alteration of the basement membrane structure would favour a negative result. The presence of a reconstructed basal lamina between epithelium and mesenchyme has a definite effect on polarization of odontoblasts and basal epithelial cells. It has been shown that they will not polarize and hence will not become functional in the absence of a basal lamina (Thesleff et al., '78; Thesleff and Pratt, '80; Dodson, '67a; Gospodarowicz et al., '78). The sequence of differentiation in dental tissues requires the odontoblasts to polarize before the inner enamel epithelial cells become ameloblasts. Based on this principle, if the basement membrane was absent or abnormal, odontoblast differentiation and induction of ameloblasts would have been prevented. Without the contact of a differentiated layer of odontoblasts the palatal epithelium can only continue its original program of development resulting in keratin filled cysts.
The results of indirect immunofluorescence staining clearly demonstrate the presence of laminin at the interface between the epithelium and mesenchyme after trypsin treatment. This is consistent with the observations of Lesot et al. ('81). It is not surprising therefore, that laminin is also present between heterotypic combinations of oral epithelium and molar mesenchyme. The data of the present study cannot confirm that fibronectin, heparan sulfate are deposited. It is still possible, therefore, that differences in composition in the reconstructed basement membrane were present, but not detected by the methods used in this study. These alterations could have influenced whether a tooth was formed versus an epithelial cysts.

An alternative to compositional changes in basement membrane affecting induction, is the possibility of a slight delay in basement membrane deposition within some combinations. The time lag may be just enough to prevent odontoblast differentiation and subsequent ameloblast induction. The basement membrane could be involved in transmission of the inductive message but the key may not lie in its molecular composition. Rather, it may act as a coordinator and arrange the correct developmental sequence for normal tooth formation.

In every experimental combination of palatal epithelium and tooth mesenchyme, keratin cysts were formed. In those explants containing teeth, the dental epithelium was invariably in close association with a cyst. The corollary to this observation is also true; teeth were never formed in the absence of a keratin cyst. This would infer that the palatal epithelium can both participate in dental lamina construction and histodifferentiation typical of masticatory
epithelium. It is unclear based on these results whether epithelial cyst formation must precede enamel organ differentiation. The two types of structures may form simultaneously but independently, or the enamel organ may derive from the basal cells of the cyst. The most important aspect of the observation of keratin-filled cysts is that the tooth mesenchyme did support full histodifferentiation of palatal epithelium in a manner indistinguishable from homotypic mesenchyme. Surrounding all keratin cysts was a distinct granular layer. This stratum, rich in keratohyalin granules, is a well recognized feature of palatal epithelium (Squier et al., '76). The dental lamina does not keratinize, instead, it differentiates into a specialized enamel secreting epithelium. In the epithelial cysts formed adjacent to control grafts of intact molar tooth germs an indistinct stratum granulosum was present. Presumably these cysts originated from a part of the dental epithelium. Thus, the presence or absence of large numbers of keratohyalin granules in the keratin cyst may be a diagnostic aid in determining the source of the epithelium (Heaney and Jones, '78).

The results of the present study limit the feasibility of tooth induction in post-natal oral epithelia. The loss of developmental plasticity in the palate of the mouse prior to birth is likely to be irreversible. Based on experimental data from adult epidermis and oral mucosa this is not unreasonable. All that has been achieved so far is an alteration in keratinization pattern (Billingham and Silvers, '68; Briggaman and Wheeler, '68; Karring et al., '75; Heaney, '77; Heaney and Jones, '78). New cutaneous appendages such as hair follicles, or glandular structures have not been induced in adult skin. Thus, once
the palatal cells have become determined, they cannot become enamel epithelium in the neonate or adult.

The resistance of adult tissues to instructive messages from connective tissue is fortunate in that unwanted metaplasia is prevented (Pierce, G.B. et al. '78). From the results of this study epithelial stability appears to be established before birth in mice. The degree of this restriction has been confirmed by experimentally arranging intimate contact with a previously determined, instructive inducer.
SUMMARY AND CONCLUSIONS

The purpose of this project was to examine the effect of cellular aging on tissue interactions. Combinations were made between molar papillae of fifteen-seventeen days gestation and palatal epithelium from fifteen-nineteen-day mice. The tooth formation in the seventeen-day and younger tissues has demonstrated that these ages are still responsive to an inductive stimulus while the eighteen-day epithelium has lost this plasticity.

In summary the following conclusions can be made:

1. Molar mesenchyme supports full histodifferentiation of palatal epithelium.
2. Laminin is not completely removed after trypsin dissociation of tissues.
3. EDTA is not an effective method of epithelial-mesenchymal separation in tissues with complex junctional morphology.
4. Palatal epithelium is determined histogenically and morphogenically at eighteen days of gestation in mice.
5. Palatal epithelium up to and including seventeen days of gestation, is capable of responding to an inductive message of an instructive type. It can become a dental epithelium and alter its pattern of gene expression. The induced enamel matrix has a normal appearance at the light and ultrastructural levels.
6. The loss of competence of the epithelial cells of the palate to respond to an inductive stimulus coincides with the establishment of stratum corneum and the attainment of the fully differentiated state.

Future Relevant Investigations

The present experiments could be extended to investigate the
reason for cellular determination in the palatal epithelium at eighteen days gestation. Recombinations performed with other keratinizing epithelia just prior to and after the secretion of orthokeratin, would establish whether or not cellular determination at the time of full histodifferentiation is a generalized feature of keratin secreting cells. In this way evidence for a link between terminal cytodifferentiation and loss of competence to respond to an inductive signal would be established.

Reciprocal combinations of palatal epithelium and other mesenchyme such as salivary gland would help confirm that seventeen days is the last age this epithelium can be described as labile. Tyler and Koch (’77) did perform this combination with twelve day epithelium and produced glandular structures. The micrographs show unusual morphology for a gland as well as birefringence in the cells lining the lumen. Repeating the experiments with the optimal conditions of the intraocular site as opposed to the millipore filter should clarifying the previous results. The difference in technique could change the birefringence in the epithelial cells to an appearance more consistent with glandular secretions. This would offer more support for a difference in histogenesis in response to altered morphology. In addition older, post-fusion epithelia should be combined with the inductive submandibular mesenchyme to see when it is restricted to keratinization.

The peak inductive capacity of seventeen day tissues could be tested further by making more combinations and increasing the tissue masses obtained from the fifteen and sixteen day old animals. In this way, critical mass problems could be overcome. It should be possible
to use progressively younger epithelium and test pre-fusion epithelium of the ages used by Tyler and Koch ('77). One would expect that twelve-day tissues could respond to an inductive stimulus consisting of molar papillae at their maximum potential, and cultivation in the nutritionally superior anterior chamber of the eye. These modifications should produce teeth in pre-fusion palatal epithelium and support histodifferentiation in the absence of tooth formation.

Indirect immunofluoresence staining with antibodies to the other basement membrane components, fibronectin, heparan sulphate, and type IV collagen could be done on the combinations that produced keratin cysts. This would establish if any of these molecules were not replaced in the reconstructed basement membrane which could be correlated with incidence of tooth induction. Any differences in composition may explain the lack of induction. Similarities would make one think that the timing of reconstruction or spatial arrangement of the molecules is significant rather than the composition.

The final experiment would try to overcome the loss of competence and extend the period of responsiveness to the neonate and perhaps adult animal. It is possible that by dissociating the epithelial cells and culturing them for several generations in vitro, a cell line comprised of dividing basal cells will be obtained. Combinations would then be made with a pellet of eighteen-and nineteen-day basal cells and molar mesenchyme. Based on the success at these ages experimental tissues could be taken from the neonate. Perhaps using only dividing basal cells that have not begun histogenesis will result in tooth induction.
<table>
<thead>
<tr>
<th>EPITHELIUM</th>
<th>MESENCHYME</th>
<th>( n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>(palatal)</td>
<td>(molar papilla)</td>
<td></td>
</tr>
<tr>
<td>15 DAY</td>
<td>15 DAY</td>
<td>23</td>
</tr>
<tr>
<td>16 DAY</td>
<td>16 DAY</td>
<td>26</td>
</tr>
<tr>
<td>17 DAY</td>
<td>17 DAY</td>
<td>39</td>
</tr>
<tr>
<td>18-19 DAY</td>
<td>15-17 DAY</td>
<td>43</td>
</tr>
</tbody>
</table>

**TOTAL:** 131 236

* TRYPsin DIssoCIAted
<table>
<thead>
<tr>
<th>EPITHELIUM (palatal)</th>
<th>MESENCHYME (molar papilla)</th>
<th>CONTROLS</th>
<th>TEETH GROWN COMBINATIONS GRAFTED</th>
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</thead>
<tbody>
<tr>
<td>15 DAY</td>
<td>15 DAY</td>
<td>0/60</td>
<td>5/23 = 22%</td>
</tr>
<tr>
<td>16 DAY</td>
<td>16 DAY</td>
<td>0/68</td>
<td>2/26 = 8%</td>
</tr>
<tr>
<td>17 DAY</td>
<td>17 DAY</td>
<td>0/46</td>
<td>12/39 = 30%</td>
</tr>
<tr>
<td>18 - 19 DAY</td>
<td>15 - 17 DAY</td>
<td>0/62</td>
<td>0/43 = 0%</td>
</tr>
</tbody>
</table>

TOTAL: 19/88 = 21%
Figure 1.

A schematic diagram of the experimental procedures involved in recombination experiments.
recombine on solidified media for 24 hours

graft to the anterior chamber of the eye for 10 - 14 days

harvest and process for microscopy

epi. epithelium
e.o. enamel organ
l.p. lamina propria
d.p. dental papilla
PLATE I

Figure 2. A photomicrograph of a semi-thin section of a 17-day molar papilla separated with trypsin treatment. The rough surface of the papilla is a result of digestion of the basement membrane (→). 260 X

Figure 3. A transmission electron micrograph of the same specimen in fig. 1. The odontoblasts have irregular borders or blebbing and the basement membrane is not visible. 13,000 X
PLATE II

Figure 4. A photomicrograph of a 16-day molar papilla isolated cultured for 2 days in vitro. All cuspal morphology has been lost and there is no epithelium present.
260 X

Figure 5. A photomicrograph of a 16-day molar papilla isolated with trypsin after 7 days of intraocular cultivation. Bone spicules (B) are present within a mass of connective tissue.
200 X
PLATE III

Figure 6. A photomicrograph of a semi-thin section of a 17-day molar papilla separated with EDTA. The basement membrane can be seen overlying the surface of the odontoblasts (→). 260 X

Figure 7. A transmission electron micrograph of the same section as seen in fig. 6. The electron dense basement membrane (→) has a cell-free zone beneath it which contains fibrillar material. Odontoblast processes are not visible in this section. 13,000 X
PLATE IV

Figure 8. A scanning electron micrograph of a 17-day molar papilla that has been separated with EDTA. The velvety surface of the cusps was imparted by the EDTA treatment. 3150 X

Figure 9. A photomicrograph of a 16-day molar papilla dissociated with EDTA and grown in vitro for 2 days. Cuspal morphology is absent and the presence of epithelium (E) over the periphery of the mesenchyme (M), should be noted. 420 X
Figure 10. A photomicrograph of 15-day palatal epithelium. The epithelium is 2 to 3 cells thick and all cells are cuboidal in shape. 660 X

Figure 11. A photomicrograph of 16-day palatal epithelium which is 4 to 5 cells deep. The surface cells are beginning to flatten. 660 X
Figure 12. A photomicrograph of 17-day palatal epithelium. Full stratification is evident including: stratum basale (b), stratum spinosum (s), stratum granulosum (g), and stratum corneum (c). The parakeratin layer overlying the surface is thin. 660 X

Figure 13. A photomicrograph of 18-day palatal epithelium. A much thicker stratum corneum is present (c) with some orthokeratin deposited on the surface. The keratohyalin granules form a distinct granular layer (g) beneath the stratum corneum. 660 X
PLATE VII

Figure 14. A photomicrograph of a tooth formed in a combination of 17-day trypsin separated molar papilla and 17-day palatal epithelium cultured for 15 days in vivo. The shortened post-secretory ameloblasts (A) overlie the unmineralized enamel matrix (E), beneath which is the mineralized dentin (D) and predentin (P). The beginning of root development is noted where the ameloblast taper into the cemento-enamel junction (CEJ). 420 X
PLATE VIII

Figure 15. A photomicrograph of a semi-thin section of a non-decalcified tooth produced from 17-day papilla and epithelium. The odontoblast layer is present (O), the predentin (p), dentin (d), mineralized enamel (E) and post-secretory ameloblasts (A). The beginning of root development is noted where the ameloblast taper into the cemento-enamel junction (CEJ). 420 X
Figure 16. A photomicrograph of an early bell stage tooth formed from a combination of 15-day tissues and cultured for 5 days. Keratohyalin granules (g) are prominent in the granular layer adjacent to the cyst lumen. 260 X
Figure 17. A photomicrograph of a combination of 15-day molar papilla and 15-day palatal epithelium that has been cultured for 7 days in the anterior chamber of the eye. The late bell stage has been reached in which the ameloblasts (a) are polarized and ready to begin secretion of enamel protein. The first layer of predentin and dentin has already been deposited (p). 260 X
PLATE XI

Figure 18. A photomicrograph of a tooth grown in a combination of 16-day palatal epithelium and 16-day molar mesenchyme. The stellate reticulum (s) is intimately associated with the cyst epithelium (e). An enamel space (→) is evident which has resulted from decalcifying the specimen. 170 X

Figure 19. A photomicrograph of the junction between the stellate reticulum (S) and the epithelium (e) comprising a keratin cyst. The enamel space (e.s.) and dentin (d) of the cusp tip are seen below. 670 X
Figure 20. A photomicrograph of a tooth and keratin cyst formed from a combination of 16-day tissues. Respiratory epithelium (R) taken from the nasal side of the palate is continuous with the oral epithelium of the cyst. 260 X

Figure 21. A photomicrograph of the respiratory epithelium originating from the nasal side of the palate. The pseudostratified, ciliated, columnar respiratory cells appear to be filled with mucus. 835 X
PLATE XIII

Figure 22. A transmission electron micrograph of the mineralized enamel deposited in a tooth formed by 17-day tissues that had been cultured for 15 days \textit{in vivo}. The needle-like crystals conform to the prism (rod) pattern described by Tomes' processes. 13,000 X

Figure 23. A transmission electron micrograph of the predentin (P)-dentin (D) junction in a tooth induced in a combination of 17-day tissues that had been cultured for 15 days in the anterior chamber of the eye. 13,000 X
Figure 24. A photomicrograph of a combination of 15-day molar papilla and 18-day palatal epithelium that has produced a keratin cyst (C). Surrounding the cyst are bone spicules and loose connective tissue. 170 X

Figure 25. A photomicrograph of semi-thin section taken from a combination of 15-day molar papilla and 18-day palatal epithelium. Note the cyst lumen filled with orthokeratin (K), keratohyalin granules prominent (G) in the granular layer, and the basal layers beneath (B). 830 X
Figure 26. A transmission electron micrograph of the granular layer of a keratin cyst formed in a combination of 15-day molar papilla and 18-day palatal epithelium. Electron dense keratohyalin granules are seen (G) beneath anucleated cells of the stratum corneum (C). 13,000 X

Figure 27. A photomicrograph of keratin cyst which arose from dental lamina cells adjacent to a grafted, whole tooth germ. The granular layer is indistinct. (G). 330 X
Figure 28. Photomicrograph of a section indirectly stained with antibodies to laminin. This 17-day trypsin treated tooth germ has been partially separated to demonstrate the plane of cleavage. Fluorescence can be noted on both the papilla (P) and enamel organ surfaces (E.O.). 260 X
Figure 29. A photomicrograph of a section indirectly stained with antibodies to laminin. The fluorescent label has adhered to the surface of the papilla only (P) in this EDTA-treated tooth germ. There are numerous blood vessels in the papilla which exhibit fluorescence of the endothelial layer. Incomplete separation of the enamel organ from the papilla can be noted in several places. A higher magnification of area labeled C is shown in figure 30. 260 X

Figure 30. The basement membrane containing the indirectly labeled laminin (b.m.) covers the dental papilla (P). Several epithelial cells are clinging to the basement membrane (→). 670 X
Figure 31. A photomicrograph of an indirectly laminin-labeled keratin cyst. The cyst was formed from a combination of trypsin-separated molar papilla and trypsin-dissociated neonatal oral epithelium. Laminin staining is present as a very fine line between the epithelium (E) and mesenchyme (M). The endothelium of the vasculature is also decorated with the antibody and fluoresces. 670 X

Figure 32. A photomicrograph of a keratin cyst formed in a combination of EDTA-treated molar papilla and trypsin-separated neonatal oral epithelium. The basement membrane contains antibodies to laminin and fluoresces (---->). The brilliance of the fluorescent stain is much greater than in the trypsin-treated recombinant (fig. 31). 670 X
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