Spatial and Temporal Distribution Of Desert Hedgehog mRNA in the Murine Craniofacial Complex

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FORMAT

SUMMARY

Hedgehog genes are involved in the development of pattern formation during embryogenisis. This study reports the spatial and temporal distribution of *Desert hedgehog* (*Dhh*) transcripts of gestational day 10 - 14 in the mouse craniofacial complex. Trancripts are present in the craniofacial complex (reverse transcription / polymerase chain reaction analysis). *Dhh* is present in the medial edge epithelium of the developing murine palate at days 12 and 14 (in situ hybridization). *Dhh* may be involved in the later stages of palatal development, during the fusion and degeneration of the palatal epithilial seam.

INTRODUCTION

Development and patterning in the vertebrate is a complex phenomenon involving many interactions on a cellular level. Even more puzzling is the control of these interactions on the molecular level. Mechanisms of these interactions are beginning to be investigated with the use of studies focusing on molecular events that may drive morphogenic regulation (Edelman, 1992).

Craniofacial development consists of a multitude of complex processes, whose molecular mechanisms are now just beginning to be understood. Of primary importance to the dental profession is the development of the maxilla and mandible, as well as the events of odontogenesis that occur within them. Odontogenesis consists of many molecular interactions between tissues that allow for this process to occur. With respect to these processes one can ask three basic questions. First, what are the molecular signals that initate tooth formation? Second, what are the molecular signals that determine the location of a tooth? And thirdly, what are the molecular signals that determine the morphology of the tooth being formed (i.e.. molar vs. incisor).

The first question posed has been investigated by several investigators (Kollar, 1983, and Kollar and Baird 1970). Collectively they have found that interactions between the first branchial arch derived embryonic epithelium, and mesenchyme of neural crest are required for the initiation of tooth formation. These interactions allow for a proliferation of site specific epithelium, the first step in odontogenesis. Epidermal growth factor (EGF) has been found to be

necessary for this proliferation of epithelium which occurs in the mouse mandible at embryonic days 11-12 (Kronmiller, Upholt and Kollar, 1991). It has also been found by this group that EGF mRNA is expressed in the mouse mandible only at day 9-10, just before epithelial proliferation. Blocking the expression of EGF using antisense oligonucleotides inhibits odontogenesis (Kronmiller, Upholt and Kollar, 1991). This indicates that the EGF signal plays an important role in the initiation of odontogenesis. Studies done by MacKenzie *et al* (1992) have shown that *Hox-8* a homeobox gene can also play a role in the initiation of tooth development in the mouse. Experiments performed by Kronmiller *et al* (1995) have also shown that the segment polarity gene *Sonic hedgehog* (*Shh*) may as well play a role in initiation as it has been found to be transcribed during embryonic day 10 and 11, the time in which proliferation of mandibular epithelium occurs in the mouse embryo. Expression of *DVR/BMP 4,6* genes has been shown in human fetal teeth by Heikinheimo (1994), and may function to initiate odontogenesis. However other candidate signals are likely to play a role as well.

The signals that determine the location of tooth formation seem to be due to the effects of retinoic acid (RA). Studies by Kronmiller *et al* (1995) have shown that all trans retinoic acid can induce incisor bud formation in diastema areas of the mouse mandible where teeth do not normally form. Furthermore, these diastema regions have been shown to express transcripts encoding for CRABP or retinoic acid binding proteins (Kronmiller *et al.* 1995). These proteins bind RA thereby reducing retinoic acid necessary for tooth formation. These preliminary results have identified retinoic

acid as a primary candidate for the signal controlling location of tooth formation.

The signals that determine morphology of tooth formation have also been studied. In the mouse mandible there are only two basic morphologies of teeth, the incisor, and molar. Preliminary *in situ* experiments by Kronmiller *et al.* (1995) have shown that Sonic hedgehog has been found to be expressed only in the incisor region and not in molar regions at days 10 and 11 during the initiation of the dentition (Mina and Kollar, 1987), perhaps then signaling incisor morphology. A homologue of *Shh*, *Indian hedgehog* (*Ihh*) *in situ* has been shown to be expressed in molar bud epithelium and in the incisor region, and may control proliferation of dental epithelium in both these areas (Kronmiller *et al,* 1996). Kronmiller and Beeman (1994) have found that retanoic acid levels in the incisor region are significantly higher than in the molar region. Retinoic acid has also been shown to induce incisor formation by Kronmiller *et al* (1995). They have also shown that exogenous all trans RA can change areas of the mouse mandible that specify molar formation into areas that produce incisors. The regulation of *Shh* expression may be part of this signalling mechanism. (Kronmiller *et al*, 1995).

In situ experiments performed by MacKenzie *et al.* (1992) have identified the homeobox gene *Hox 8* as another candidate for signaling morphology. Chai *et al* (1994) have found that transforming growth factor B2 may also regulate tooth formation. Using antisense TGF-B2 therapy these investigators found that tooth size increase was threefold and that these teeth showed precocious development. EGF, as stated earlier seems to be necessary for

initiation of odontogenesis but is also thought to regulate tooth size as well. Shum *et al* (1993) found that treating day 10 murine mandibles with EGF antisense oligonucleotides led to a decrease in tooth bud size and a total reduction in tooth volume of 48%. The same study also investigated the effects tyrophostin RG 50864 on tooth development. Cultures treated with this agent showed an increase in tooth size of 20%, and also induced a precocious molar cap stage.

Development of the maxilla and mandible are important procesess to understand as one sees many aberations in these developmental process that manifest themselves clinically. Specifically, alterations in the development of the maxilla and the palate may lead to various forms of cleft palate. A summary of palatal development from Ferguson, (1988) is as follows; mesenchymal cells from the neural crest migrate into the bilateral maxillary processed where they interact with craniopharyngial ectoderm. Bilateral palatal shelves arise from these structures at day 12 in the mouse. The palatal shelves first grow vertically down the sides of the developing tongue but at a precise moment elevate to a posistion superior to the dorsum of the tongue. The medial edge epithelia of the approximating palatal shelves then around days 14-15 fuse with each other to form a midline epithilial seem. This epithilial seem then degenerates establishing mesenchymal

continuity across the intact horizontal palate by the end of day 15. Cleft palate can result from disturbances at any of these stages of development. These mechanisms include; defective palatal shelf growth; delayed or failed shelf elevation; defective shelf fusion; failure of medial edge cell death; and/or postfusion of MEE into the underlying palatal mesenchyme. There have been numerous studies that have identified many teratogens that affect palatal develpment and can induce cleft palate, these include caffeine and theophylline (Kosazuma and Kawauchi, 1994), fluorouracil (Shuey *et al*, 1994, Young *et al*,1994), and retanoic acid (Luning *et al*, 1994, Abbott and Pratt, 1991).

Studies by Satokata and Maas (1994) have shown that mice lacking *Msx1* gene function have clefts of the secondary palate as well as abnomalities in mandibular development and failure of tooth development. These mutant strains of mice manifest a complete cleft of the secondary palate and the result is incomplete palatal development. There were also deficiencies in development of bone in the premaxilla where the upper incisors normally are found. These mice did not have maxillary incisor buds as well as the alveolar processes of the maxilla surrounding the maxillary molar tooth buds. Analysis of the mandible shows a deficency of the mandibular alveolar process as well as abscence of mandibular incisor tooth buds. There also seems to be a reduction in overall length of the mandible as well as abnormal anterior lower borders

including the symphysis menti. These authors believe that the function of *Msx1* is to mediate complex epithileal-mesenchymal interactions during the development of the crainiofacial complex. and tooth development.

Still, however, there are many other genes thought to play a role in regulation of craniofacial development. By looking at other developmental processes in vertebrates and their associated molecular candidates, one can look for analogous roles in the regulation of development of the jaws and the patterning of the dentition in vertebrates. Many of the genes responsible for development and patterning of the vertebrate CNS and limb may have a conserved function in the development and patterning of the jaws and dentition.

Molecules that may affect the regulation of patterning and development have been termed morphogens. These molecules, as well as the genes that encode and regulate them, have been investigated. The list of molecules suspected of regulating and determining pattern in the vertebrate is ever increasing. Even more complex are the hypothesized interactions and regulatory events between these molecules.

Many studies have implicated the homeobox family of genes, *Hox D* (Tabin, 1991; 1989; Oliver *et al,* 1988, 1990; Ilzpisua-Belmonte, 1991; Helms, 1994) in playing a role in the regulation of development. Others have determined that retinoic acid genes are important factors (Helms, 1994; Tabin, 1991). Still others note the importance of *FGF-4* (Laufer, 1994), bone morphogenic proteins

(BMP's), or most recently the *Wnt* family of genes (Yang and Niswander, 1995). *Sonic hedgehog* (*Shh*) and it's various homologs, *Indian* (*Ihh*) and *Desert* (*Dhh*) hedgehog have also been identified and have received attention. *Hedgehog's* (*hh*) inductive signals were first discovered in developing Drosophila embryos (Ingam, 1988). These signaling transduction pathways seem to confer positional specification along an anteroposterior axis of development of a body segment in the embryo. *Hedgehog* along with its vertebrate homologs have been termed segment polarity genes. These seem to allow for the encoding of protein products confiring specific cell to cell interactions resulting in patterning of the embryo. This process is essential to development of all multicellular organisms.

The cloning of Drosophila *hedgehog* (*hh*) by several investigators (Mohler, 1988; Moller and Vani, 1992; Ingham and Martinez, 1992) led the way for the search of *hh* homologs in vertebrates and to determine whether they were serving similar functions in patterning as they did in Drosophila. These vertebrate homologs hh have been identified in several species. Studies with zebra fish (Krause *et al* 1993) have shown expression of *Shh* whose predicted amino acid sequences are 47% homologous with those of drosophila *hh*. According to these authors, this confirms that *Shh* is a true homolog of the drosophila *hh* gene. Since this striking similarity between the two genes exists it is likely that this gene may be essential to patterning and development, and that this function has been conserved throughout evolution. These investigators have also found restricted areas in the zebra fish embryo during early somatogenesis in which *Shh* is being expressed. This along with the

finding that induction of increased expression of the gene causing abherrent development in the zebrafish points to an important role the gene may play in development. Studies done with the mouse (Echlerd, 1993) have also identified 3 members of a mouse gene related to Drosophila hh which like hh encode for secreted proteins. *Shh* expression was temporally limited to day 13.5 embryo and is suggested to regulate patterning along the entire ventral CNS. It has also been shown to be tightly expressed in notochord, limb bud, and hindgut. Studies have also been performed looking at limb development in both the chick and mouse. *Shh* has been shown to be expressed during initiation of limb bud development in the zone of polarizing activity (ZPA) within the limb bud. This expression also was found to be temporally regulated. Over- expression of *Shh* by induction methods causes digit duplications with in the limb thereby indicating that the gene is an important signal in limb development. Similarly, mouse *Shh* has been shown to play a role in patterning of the mouse limb (Chang, 1994; Laufer, 1994) and the central nervous system (Riddle, 1993; Echlard, 1993) and has most recently been found to be expressed in the mandible (Kronmiller, 1995). *Shh* has been implicated in providing signals to organizing centers like the ZPA and inducing patterning in the CNS and limbs of developing organisms. Consistent homology between species implicates an important function for the gene since its constancy has been passed down through evolution. With a basic overview of the aforementioned genes we can now delve into information with respect to hypotheses regarding roles these genes may play in regulation of pattern formation and each other.

The complex interactions that occur in the limb have been eluded to earlier. Many authors have hypothesized how these patterning genes are regulated within the limb. With these hypotheses and gene pattern data obtained from the developing murine mandible studies one can perhaps begin to propose a model for craniofacial development.

A recent limb development model has been proposed by Laufer *et al* (1994). In this model for coordinated patterning and growth of the limb (see fig. 1), results indicate that *Shh* can act as a signal that begins a cascade of secondary signals whose subsequent interactions lead to pattern formation. After *Shh* is induced, it signals both to the mesoderm and limb ectoderm. It acts in the mesoderm to induce expression of patterning genes such as *Hoxd* and *Bmp-2* genes (and perhaps others) as long as permissive signals are obtained from the overlying ectoderm. The authors have shown that *Bmp-2* expression is solely dependent upon *Shh* expression, while *Hoxd* is initially, but later becomes independent upon the *Shh* signal. Reciprocally this activity in the mesoderm has to be maintained by the apical ectodermal ridge (AER). This is accomplished by expression of *Fgf-4* in the posterior 1/2 of the AER which provides competent signals to the mesoderm maintaining Shh expression via a positive feedback loop. *Shh* also seems to induce a polarity of the AER by only inducing *Fgf-4* expression in the posterior 1/2 of the AER, thereby setting up a anterior-posterior area of development. *Fgf-4* not only provides this positive feedback loop to the mesoderm thereby regulating *Shh* expression, but also promotes mesodermal proliferation as well. Thus, a signaling

pathway exist from the ZPA to the AER via *Shh* , and a maintenance pathway exists from the posterior 1/2 of AER to the ZPA via *Fgf-4*. This mechanism allows for outgrowth and patterning of the limb to be coordinated. Another model proposed by Yang and Niswander 1995 (see fig. 2) propose interactions between *Wnt7a*, *FGF4* and *Shh* in establishing the ZPA. They hypothesize that while the ZPA requires signals from the AER, posterior mesenchyme and the dorsal ectoderm, they also need local signals from the gene products *FGF4*, *Wnt7a*, and *Shh*. A positive feedback loop exists between *FGF4*, and *Shh* (Laufer, 1994, Niswander, 1994) in the posterior area of the limb bud, this begins proximally and then moves more distally as the outgrowth of the limb occurs. They believe that this triad of signal interaction is what defines the ZPA and subsequently leads to downstream expression of patterning genes in the developing limb bud. The ZPA then once developed can coordinate signals for positional development of the limb in the primary body axes. They believe that *FGF* provides proximodistal information, *Shh* anteroposterior information, and *Wnt7a* dorsoventral signals.

This investigation will attempt to locate expression of the vertebrate homologue of *Shh - Desert hedgehog* in the day 10,12 and 14 murine embryonic head at the time in which initiation of the dentition begins and ends. This information once gathered, may help in combination with existing data on tooth development as well as limb development to provide models for craniofacial development of the mouse and patterning of the dentition.

MATERIALS AND METHODS

Preparation of biotin labeled probes

Gestational day 10 CD-1 mice were sacrificed by cervical dislocation and embryos removed in a solution of Hank's Balanced salt solution. The mandibular processes were dissected and placed in guanidine isothiocyanate buffer and then homogenized for use in RNA isolations. Isolation of RNA was performed using the cesium chloride (Cs/Cl) gradient technique. Approximately 8-10 mandibular processes were used per RNA preparation. The homogenized mandibular processes were dispensed over equal amounts of 5.7 M cesium chloride. The mixture was then placed into an Eppendorf 5420 centrifuge for 2 hours @55,000 rev/min. and at 20°C. The supernatant was then removed leaving the RNA pellet. The pellet was resuspended with 10µl of DEPC water and dispersed into sterile RNAse free eppendorfs. Samples were then ethanol precipitated by adding 1/10 volumes of 3M Na Ac and 2.5 times volume of cold 100% ethanol. This was kept at -70°C for 30 minutes and then centrifuged for 20 minutes @12,000 rev/min. at 4°C. Supernatant was removed and any remaining ethanol was left to evaporate. The RNA sample was then resuspended in 10µl of DEPC H_2O. 5µl of RNA sample was used for production of cDNA by reverse transcription. To the RNA sample was added 2.5 µl of PCR buffer; 2.5µl 25 mM $MgCl_2$; 1µl of Reverse transcriptase; 2µl RNAse inhibitor; 10µl DNTP's and 1µl of random hexomer. This mixture was left at room temperature for 10 minutes. It was then placed in a 42°C water bath for 1 hour, then at 95°C for 5 minutes then placed into -20°C freezer for use in DNA amplification.

DNA amplification was performed using *Dhh*-specific primers to amplify the *Dhh* cDNA within the reverse transcription mixture. The sequence of *Dhh* was obtained from the Genebank data source (McMahon, 1993). The primers were designed using the computer program AMPLIFY 1.2. The nucleotide sequences for the *Dhh* primers were 534 - 553 (5' primer) and 812 - 831 (3' primer). Primers were then synthesized using the Special Gene Assembler (Pharmacia) and then purified using Pharmacia's EZE prep oligonucleotide purification kit. The reaction mixture contained 5µl of reverse transcription product, 1µg of each primer, 4.5 µl of PCR buffer, 1.8µl of $MgCl_2$, 3.7 µl of dNTP, and 0.5 µl of TAQ polymerase for a total volume of 50µl. Each sample was amplified by 40 cycles of polymerase chain reaction. Each cycle consisted of a denaturing at 95° for 30 seconds, followed by annealing at 65° for 30 seconds, and then extension at 72° for 30 seconds, with a beginning denaturing of 5 minutes at 95° prior to cycling and a final extension of 72° for 5 minutes after cycling. PCR products were then run on a 15 % agrose gel and run @ 90 volts for 60 minutes. The cDNA was then cut out of the gel and was removed and purified using the PCR plasmid purification kit from Qiagen. Gene specific amplification was confirmed by a restriction digest. These oligomers were then biotin labeled using the BlueGENE non radioactive Nucleic detection system (GibcoBRL) and stored at -20° C.

Isolation of whole Murine heads for *In situ* hybridization

Gestational day 10 and 12 CD-1 Swiss mice embryos were used. The embryonic age was determined by the appearance of a vaginal plug after mating and then also confirmed by identification of

morphologic criteria according to Thieler (1989). Pregnant mice were sacrificed via cervical dislocation and embryos dissected out of the uterus in a solution of Hank's Balanced salt solution (HBSS, Gibco, Grand Island, New York). Whole embryonic murine heads were then placed in fresh solution. Heads were then fixed in a solution of 4% paraformaldehyde and placed in a 4° C refrigerator for 12 hours. Heads were then rinsed 2X in PBS for 5 minutes. They were then subjected to a series of ethanol treatments according to this series: 50% EtOH 15 min.: 70% EtOH 20 min.: 95% EtOH 20 min. and 1 drop Biebrichs solution: 2 rinses in 100% EtOH for 20 minutes each. They were then rinsed in xylene for 15 minutes. Heads were then embedded in paraffin for 1 hour with this paraffin changed every 15 minutes. Heads were then finally embedded in paraffin and then placed at 4° C until sectioning.

Preparation of Murine Head sections

Sections were prepared on a Riechert-Jung Model 2040 microtome at a thickness of 7µm. Frontal sections taken from approximately the beginning of the maxillary process to the posterior of the mandibular process were placed on RNAse free slides and warmed at 56° C for 2-3 hours to allow for adherence of sections to the slides.

Colormetric *In Situ* Hybridization

Slides were then deparaffinized 2X in xylene for 5 minutes. They were then dehydrated in 100% ethanol for 5 minutes and let to air dry for approximately 40 minutes until frosty white. They were then digested with protinase K (20µg/µl) for 5 minutes at 37° C. This was followed by a PBS rinse and a post-fixative treatment consisting

of 4% formaldehyde for 1 minute and a rinse in PBS. Dehydration of the slides then took place using ethanol at the following concentrations; 50%, 70%, 95% for 3 minutes each, followed by twice by 100% for 5 minutes. The slides were then air dried for approximately 1 hour and 40 minutes.

Hybridization consisted of preparation of the *Dhh* probe (0.2 μg/μl) with 20% dextran sulfate and denaturization in 100° C H_2O for 5 minutes. Then 250 μl of probe was added to each slide evenly over the tissue sections. The slide was sealed with rubber cement over the edges of a cover slip and placed in a humidor to allow for hybridization. Hybridization took place for 16-18 hours at 42° C. A Puc-19 DNA probe was used for a negative control. For a positive control a previously prepared slide containing Hela cells (GibcoBRL *in situ* hybridization kit) were also used. The Puc-19 probe was prepared by our laboratory using the previously mentioned BioNick labeling technique. Detection was then performed by rinsing slides in 0.2X SSC three times and removing the coverslip in the process. They were then rinsed in 0.2X SSC twice for 15 minutes. The slides were then covered with 500 μl of blocking solution (GibcoBRL) and incubated in a humid chamber at room temperature for 15 minutes. Then blocking solution was removed and 200-300 μl of Steptavidin-alkaline phosphatase/conjugate solution was added and incubated in a humid chamber at room temperature for 15 minutes. Slides were then washed 3 times in 2X TBS (pH 7.5) for 15 minutes. Slides were then washed in Alkaline substrate buffer for 5 minutes. They were then incubated in a solution of NBT/BCIP at 37° C for 10 minutes to 3 hours until probe signal was detected. Color development was then

stopped with H_2O rinses. They were then mounted with Crystalmount.

Isolation of RNA from Maxillary process for PCR

Maxillary processes from gestational day 10, 12, and 14 embryonic CD-1 Swiss mice were removed and prepared similarly as stated above. The reverse transcription and DNA amplification procedure was also executed under the same conditions as stated previously. The nucleotide sequences for the PCR primers were obtained from Echlerd *et al* (1993) A sample of each reverse transcription product was also used for B-Actin cDNA amplification (nucleotide sequences 25-44 and 245-269) (Alonso et al., 1986). Reaction products were fractionated using electrophoresis on a 5% polyacrylamide gel and then stained with ethidium bromide. The predicted uncut fragment size was 299 bp. A restriction digest using *Pst 1* was also performed to confirm the amplification of the desired reaction products. The predicted fragment sizes were 210 and 89 bp.

RESULTS

Dhh transcripts were found at embryonic days 10, 12 and 14 see (fig. 3). PCR fragments of predicted size (299 base pairs) were observed and were identical in size to PCR products from day 10 whole body murine embryos which were used as positive controls throughout the experiment. Restriction digests (Pst 1) of day 10, 12, and 14 showed predicted PCR fragments of 210 and 89 base pairs (data shown only for day 12, see fig. 4). These were identical to those restriction fragments of the day 10 whole body (*Pst 1*) digest. Triplicate assays were performed for each gestational day and produced identical positive results.

Dhh transcripts were not localized to any tissue in day 10 heads by *in situ* hybridization techniques however they were detected by the (RT/PCR) assay. *Dhh* mRNA transcripts were not found to be present in the dental lamina or tooth bud areas of the mandible at day 10, 12 and 14 via *in situ* hybridization. However *Dhh* mRNA transcripts were detected in the developing eye area of day 12 and 14 embryos. Trancripts were also found in the lower border and midline areas of developing mandibles in day 12 embryos, however this finding was not seen in the day 14 samples. Detection of *Dhh* transcripts were also found in the medial edge epithelium of the maxillary processes of day 12, and 14 embryos (see fig. 5).

DISCUSSION

Dhh and Odontogenesis

This is believed to be the first study to characterize the temporal and spatial expression of *Dhh* in the mouse craniofacial complex at days 10, 12, 14. From the *in situ* hybridization analysis data we report no expression of *Dhh* mRNA within or around the mesenchyme of the developing tooth buds at the time when initiation of the dentition is occurring. This data suggests that *Dhh* may not have a direct influence on molecular mechanisms involved in initiation, location, and morphology of the developing dentition of the mouse. This is in contrast to the possible roles found of other members of the *Shh* gene family. *Shh* has been found to be expressed in the presumptive mandibular incisor region (Kronmiller *et al* 1995), and another homolog *Ihh* has found to be expressed in the presumptive molar and incisor region (Kronmiller *et al*, 1996) . Therefore it is suspected that *Dhh* may mediate other events in craniofacial development other than odontogenesis.

Dhh and Palate formation

In situ hybridization data of day 12, and 14 murine heads showed expression of *Dhh* mRNA transcripts located exclusively in the medial edge epithelia (MEE) of the developing palatal shelves. Transcripts were not detected in the underlying mesenchyme. This may indicate a possible role of *Dhh* in palatogenesis. Palatogenesis is a complex and poorly understood event, even though it has been the subjects of numerous studies. These have mainly been restricted to the prevention or treatment of cleft palate in humans, but also the

developing palate serves as a model system for the investigation of embryological mechanisms common to many developing organ systems. To elucidate any possible role of *Dhh* in palatal development one can discuss the various stages of development of the palate to see if there is a possibility *Dhh* is regulating these events. With respect to initial palatal outgrowth, there is a possibility that *Dhh* plays a role. It has been shown that there are two peaks of DNA synthesis at the time of initial palatal development corresponding with initial shelf outgrowth, and elongation of the shelf in a vertical direction (Burdett, Waterfield and Shah, 1988). However since the mRNA transcripts are active in the MEE and not in the underlying mesenchyme where most of the growth of the shelves is occurring. And the fact that the usual flow of influence is from the underlying mesenchyme to the overlying epithelium, I believe *Dhh* is not playing a role in initial palatal outgrowth. The mechanism of palatal shelf elevation is the next process which *Dhh* may play a role. The force for elevation is generally thought to be generated intrinsically within the palatal shelf. It most likely a quickly occurring event, taking only from a matter of minutes to hours. The principle elements driving this process have been identified as the regional accumulation of glycosaminoglycans, namely hyaluronic acid (Ferguson, 1978). Many studies have shown that EGF and TGFβ stimulate synthesis of hyaluronic acid by the palatal mesenchyme cells (Turley, Hollenberg and Pratt, 1985; Dixon, Foreman, Shor, and Ferguson, 1988; Sharp and Ferguson, 1988). Since *Dhh* mRNA was not detected in the palatal mesenchyme and that mesenchyme usually influences overlying epithelium the possibility of *Dhh*

regulating shelf elevation via hyaluronic acid synthesis is low. After approximation of the palatal shelves which occurs first in the middle third of the palate then spreads out anteriorly and posteriorly. The MEE of the opposing palatal shelves adhere to one another via a sticky cell surface glycoprotein coat and desmosomes (Ferguson, 1988). This adherence is specific i.e. the MEE will not normally fuse with any other epithelium of structures in the surrounding oral cavity (Ferguson *et al*, 1984). The same author believes that the turnover rates of various desmosomal components prior to during and after MEE adherence may be an important controlling mechanism in palatal fusion. I believe that it is quite possible for *Dhh* to play an important role in this developmental process since these events are occurng in the MEE where the fusion occurs. It could be possible for *Dhh* to upregulate the synthesis of desmosomal plaque proteins, and cell surface glycoproteins necessary for fusion. It has been found that expression of TGFα and EGF receptors are also upregulated in the MEE around the time of fusion (Citterio and Gaillard, 1994; Dixon, Garner, and Ferguson, 1991) and there exists a possibility of coordination of signaling at this stage of development. The effects of RA have been studied at this stage (Abbot and Pratt, 1991). RA alters the expression of EGF receptors sustaining DNA synthesis, proliferation, survival, and shift in phenotype of the MEE when these cells are supposed to be undergoing programmed cell death. The authors believe this is the mechanism that causes interference with adhesion and fusion of the opposing palatal shelves resulting in cleft palate. The last stage of palatal development is the disruption of the epithelial seam and resulting continuity of palatal

mesenchyme to form the intact palate. The epithelial seam almost as soon as it has formed starts to degenerate. It is initially thinned due to an increase in palatal height and epithelial cell migration. The fate of MEE cells is of some debate. Some investigators believe that a programmed cell death of the MEE occurs (Abbott and Pratt, 1991), whilst others believe that seam disruption is due to migration transformation of MEE into the palatal mesenchyme (Shuler, Majumder, Guo, and Luo, 1991), while others believe both processes are occurring (Ferguson, 1988). Unfortunately this study does not have data to support that *Dhh* is active at this stage in palatal development. Therefore, it is difficult to speculate what function *Dhh* plays at this stage of palatal development.

CONCLUSION

The results of this preliminary study are as follows; It appears that *Desert hedgehog* may not play a regulatory role in odontogenesis. It's expression is not detected by the methods used in this study in tooth bearing areas of the mouse when initiation and development of the dentition occurs. However *in situ* hybridization analysis did show the expression of *Dhh* mRNA transcripts in the medial edge epithelia of the developing murine palate at day 12 and 14. This finding may allow *Dhh* to be among those genes whose function plays a role in palatogenesis. While the study did not show the exact role of *Dhh* in palatal development, it can be speculated that it may play a role in the later stages of palatogenesis, during the fusion and degeneration phases of the epithelial seam. More studies are needed to identify the role, if any, *Dhh* plays in the regulation of palatal development, and in overall craniofacial development.

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FIGURES

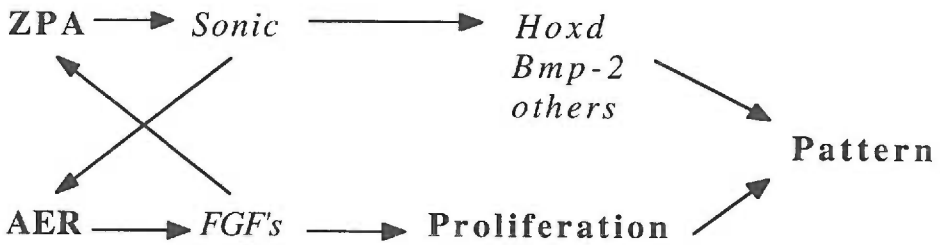

Figure 1. Model for the Coordinated Growth and Patterning of the Limb. (from Laufer *et al.* 1994.) see text for discussion.

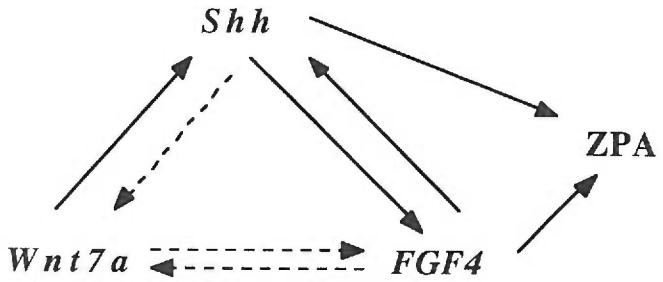

Figure 2. Interaction among *Wnt7a*, *FGF4*, and *Shh* serves to establish the ZPA. (from Yang and Niswander, 1995.) see text for discussion.

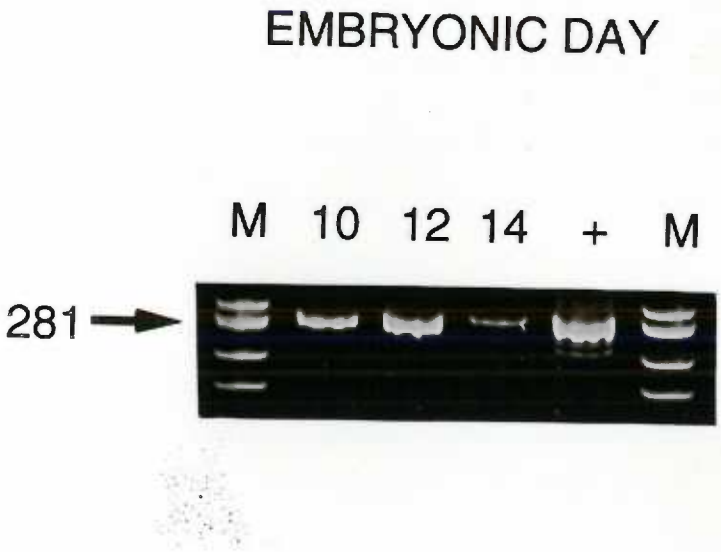

Figure 3. *Dhh* expression in gestational days 10- 14 mouse maxillae. 8% polyacrylamide gel electrophoresis of RT/PCR product, 10 µl/lane. Marker lane (M) contains *Hinf* I digest of Phi-X174. Positive control lane (+) from *Dhh* RT/PCR product from gestational day 10 whole mouse embryos.

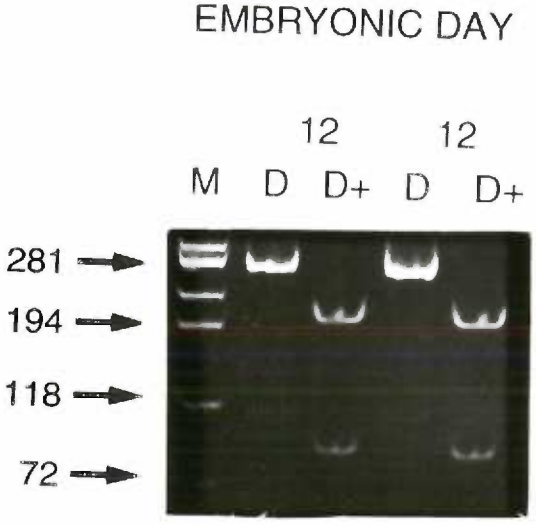

Figure 4. Restriction digests of day 12 *Dhh* mouse maxillae RT/PCR product. 8% polyacrylamide gel electrophoresis. M, marker lane contains *Hinf I* digest of Phi-X174. Lanes D, PCR product from day 12 mouse maxillae (fragments of 300). Lanes D+, PCR product from day 12 mouse maxillae cut with *Pst I* resulting in fragments of 210 and 89.

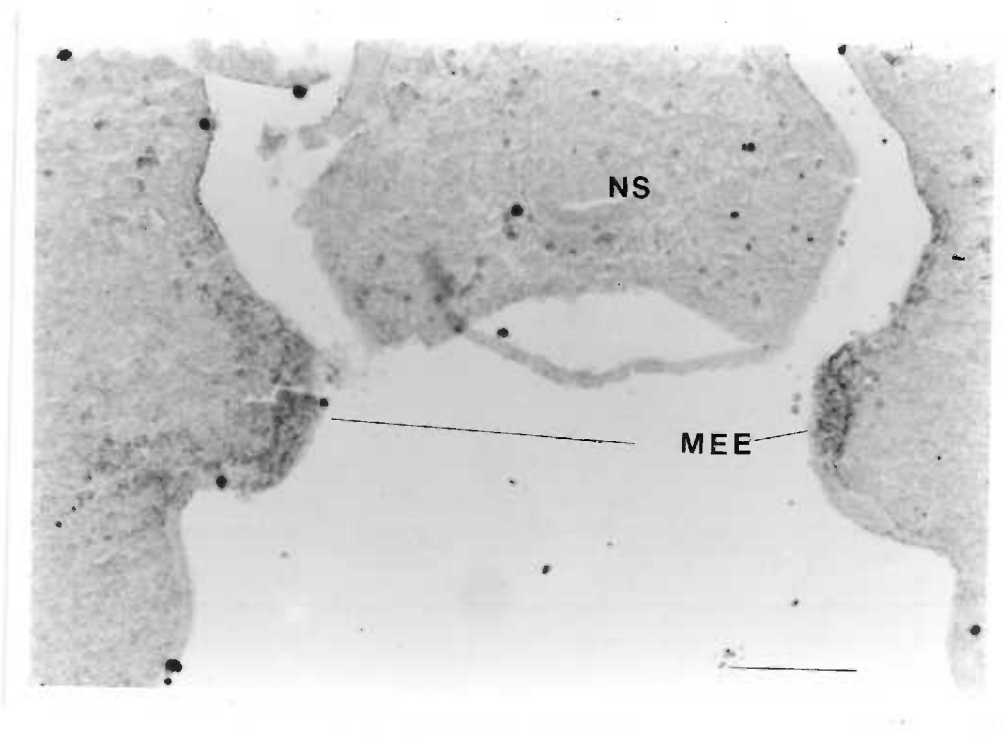

Figure 5. *In Situ* hybridization of detected *Dhh* trancripts located to the medial edge epithelia (MEE) of the developing murine palate (frontal section) at gestational day 12. X 200 (Bar = 100μm)

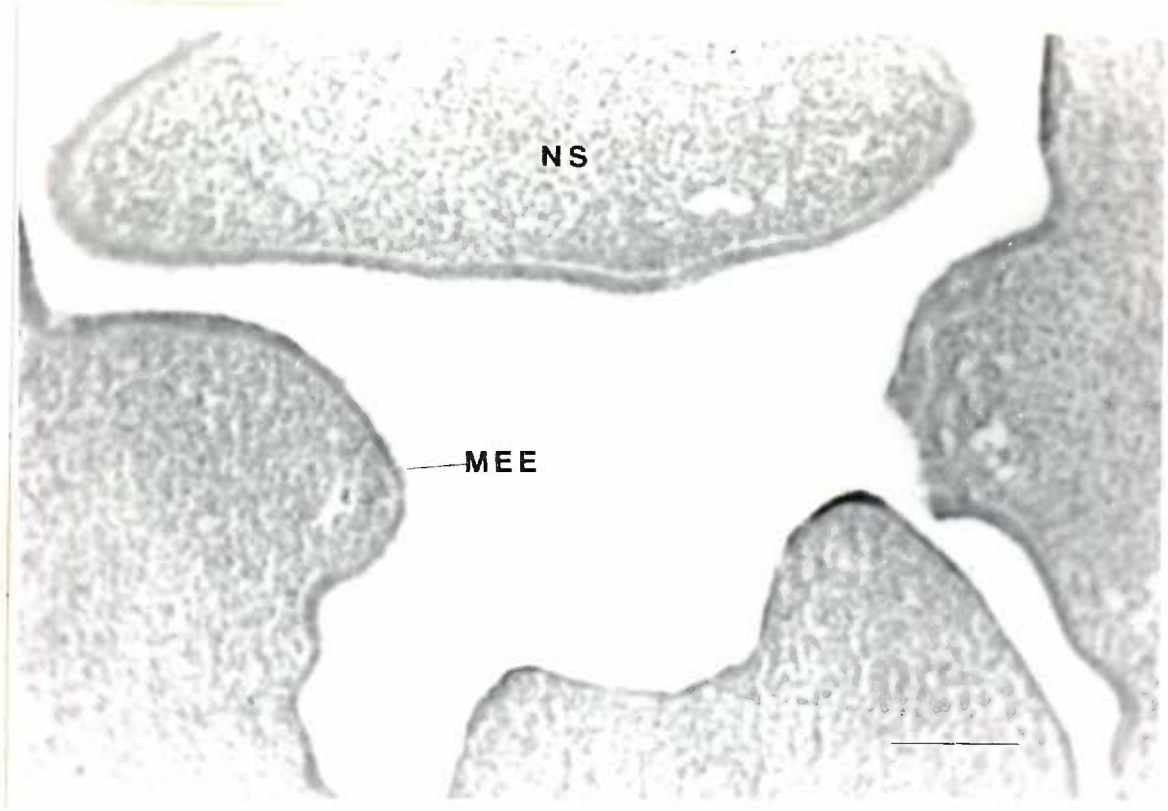

Figure 6. *In situ* hybridization/ negative control (PUC-19 cDNA probe) of the medial edge epithelium of the mouse maxilla at day 12 (frontal section). MEE= medial edge epithelium. No signal backround. X = 200 (Bar = 100μm)