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Original Article

Characterization of desmosomal component expression during palatogenesis

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ABSTRACT Adhesion of the opposing palatal shelves is a critical first step in the mechanism for palatal fusion. Formation of desmosomal junctions between the two medial edge epithelia provides a mechanism for palatal shelf adhesion. RT-PCR and immunohistochemistry were used to determine the pattern of expression of desmosomal components during palatogenesis. Desmosomal expression was specifically upregulated in the medial edge epithelia (MEE) at the early stages of palatal fusion as detected by both immunohistochemistry and electron microscopy. RT-PCR characterization of the desmosomal components detected all known elements, except desmocollin 1 (DSC1). Desmocollin 2 (DSC2) was expressed as both the DSC2a and DSC2b variants. The two variants are expressed at the same level. Western analysis of desmoglein expression paralleled the RT-PCR result. The temporal and spatial upregulation of desmosomal gene expression is evidence that the MEE induce new gene expression required to accomplish palatal shelf adhesion and initiate the first stage of palatal fusion.

KEY WORDS: *Desmosome, palate development*

Introduction

Development of the mammalian secondary palate is a complex and critical event, which in humans is frequently disturbed, resulting in a birth defect of the palate. Thus, palate embryogenesis has been the target of research with the aim of preventing or improving treatment for a cleft palate (Ferguson, 1988). The palate is often used as a model system for investigating fundamental mechanisms common to the embryogenesis of many structures since it develops relatively late in embryogenesis and can be easily excised and cultured under chemically defined serum-free conditions (Ferguson, 1984).

Bilateral palatal shelves arise from the maxillary process of the first branchial arch at embryonic day 12 (E12) in mice and day 45 in humans (Ferguson, 1988). The two palatal shelves at first grow vertically down along the side of the tongue, but at the precise developmental stage they rapidly reorient to a horizontal position above the dorsum of the tongue. The medial edge epithelia (MEE) of the opposing palatal shelves then adhere with each other to form a midline epithelial seam, which rapidly disappears to establish mesenchymal continuance and complete the process of palatal fusion. (Ferguson, 1987; Shuler *et al.,* 1991; Hay, 1990).

Desmosomes consist of cadherin-like glycoproteins that are involved in cell-cell adhesion and plaque formation. They are localized in discrete spot-like trilaminar plaque structures of the lateral plasma membrane of adjacent epithelia cells (Schwartz et al. 1990). Desmosomes are composed of transmembrane and plaque proteins. Plaque proteins are linked to keratin intermediate filament and transmembrane proteins (Schwartz et al. 1990). This linkage generates a structural continuum throughout the epithelium that may be required for the maintenance of tissue integrity. There are two types of desmosomal transmembrane glycoproteins: Desmoglein (DSG) and Desmocollin (DSC). Both DSG and DSC occur as three distinct isoforms, the product of separate genes (reviewed by Garrod, 1993). Each isoform has alternatively spliced "a" and "b" forms (reviewed by Garrod, 1993; Buxton *et al.,* 1993; King *et al.,* 1993; Legan *et al.,* 1994). There are at least three plaque proteins, Desmoplakin, plakoglobin and plakophilin, (reviewed by Koch and Franke, 1994; Schmidt *et al.,* 1999). Plakoglobin and desmoplakin have been identified in all desmosome forming cells (reviewed by Koch and Franke, 1994). Desmoplakin occurs in most cells in two isoforms generated by differential splicing although in widely variable ratios (reviewed by Koch and Franke, 1994). Plakophilin represents a distinct subfamily of arm-repeat proteins comprising three different members, plakophilin 1, plakophilin 2 and plakophilin 3 (Schmidt *et al.,* 1999). Plakophilin 1 and plakophilin 2 exist in two splice variants: plakophilin 1a and 1b, and plakophilin 2a and 2b. Both plakophilin 1 and 2 are

Abbreviations used in this paper: DP, desmoplakin; DSC, desmocollin; DSG, desmoglein; MEE, medial edge epithelium; RT-PCR, reverse transcription polymerase chain reaction; TGFβ, transforming growth factor β.

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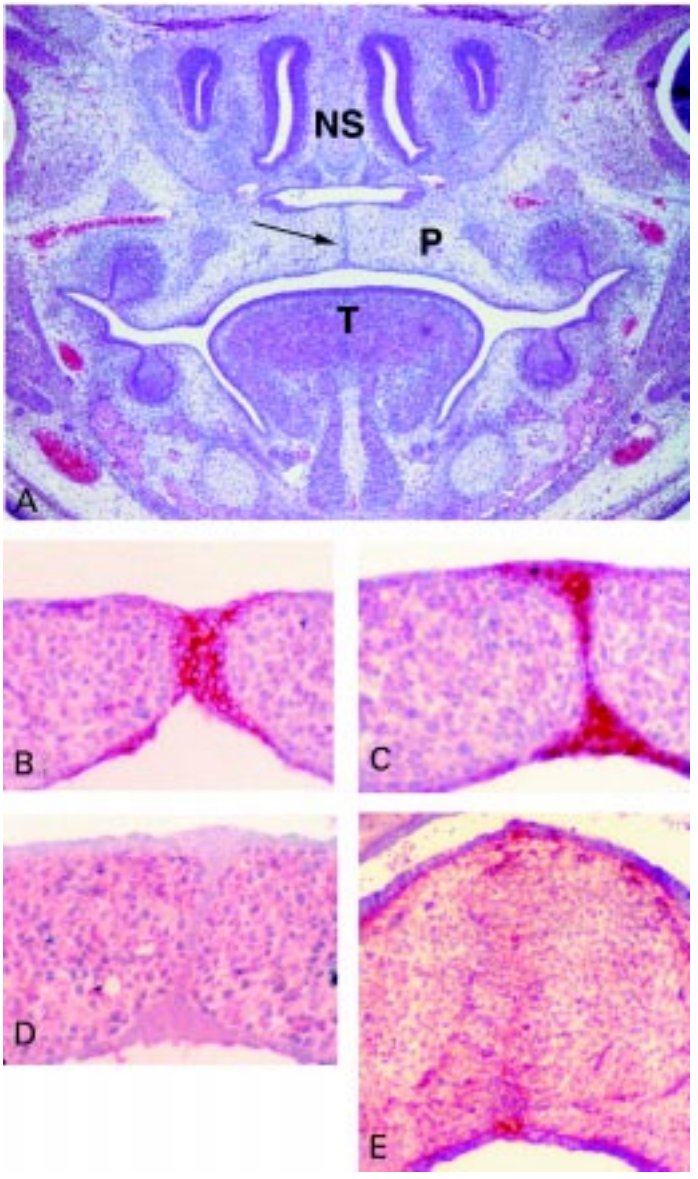

Fig. 1. Immunohistochemical localization of desmosome in the developing palate. (A) *Photomicrograph of an E 14.5 mouse palate. The MEE of opposing shelves have adhered to one another and a mid-line seam has formed (arrow) (P, palatal shelf, T, tongue and NS, nasal septum).* **(B)** *Photomicrograph of histological section stained with multiepitope desmoplakin during palatal shelve fusion. Anti-desmoplakin immunostaining was concentrated in the MEE during palatal touching. The MEE have a marked increase in the quantity of desmosome.* **(C)** *Photomicrograph of a section immunostained with same antibody after palatal fusion. The desmosome concentration remained high as the midline degenerated.* **(D)** *Negative control section, illustrating the absence of staining for desmoplakin.* **(E)** *Following epithelial-mesenchymal transformation of the MEE, there was no significant concentration of desmosome in the mesenchyme.*

constitutive nuclear proteins in diverse cell types but in addition are specifically recruited to desmosomal plaque in a highly regulated cell type specific manner (Mertnes *et al.*, 1996).

Different isoforms of desmosomal components show differential distribution in epithelia and also different expression patterns within the same epithelium (reviewed by Garrod, 1996). Desmosomal junctions have been implicated in the initial adhesion between the two palatal shelves (reviewed by Ferguson, 1988). The purpose of this study was to characterize desmosomal component expression during palatogenesis and provide insight into the role of desmosomal organization and function for MEE cells during palatal fusion.

Results

Desmosome formation in medial edge epithelia

The temporal and spatial pattern of desmosome formation during palatogenesis was characterized with an anti-desmoplakin antiserum with a multi-epitope specificity (Fig. 1). This antiserum recognizes components of desmosomes that are common in a number of different tissues and this antiserum has been used to detect the presence of desmosomes in transformed epithelial cells to determine their origin (reviewed by Garrod, 1996). This immunohistochemistry analysis demonstrated a developmental stage-specific pattern of desmosome formation in the medial edge epithelium (Fig. 1). There was a marked increase in desmosomes

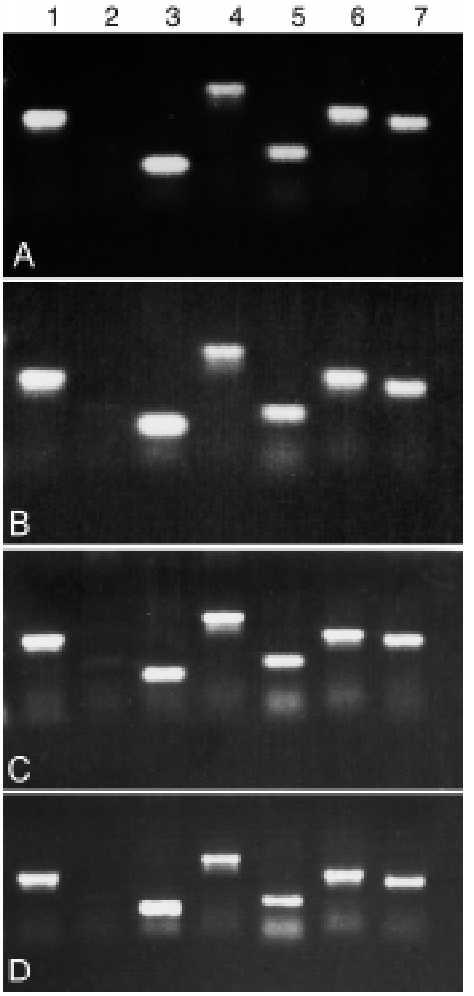

Fig. 2. RT-PCR amplification of desmosomal components expressed during mouse palate development. *Total RNA isolated from E13-E16 mouse palate was subjected to RT-PCR analysis and amplified products were analyzed by agarose gel electrophoresis. Lanes (1) DP, (2) DSC1, (3) DSC2, (4) DSC3, (5) DSG1, (6) DSG2, and (7) DSG3.*

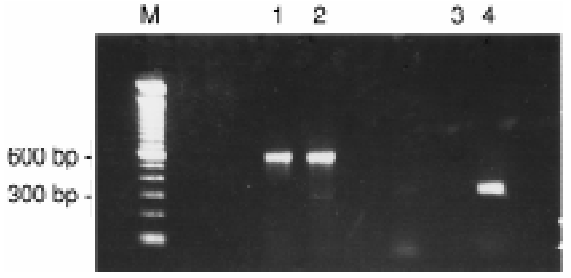

Fig. 3. Detection of DSC1 in adult mouse skin. *DSC1 was detected in mouse skin (lane 4), but not in palate (lane 3). DP was used as a positive control. DP was detected both in adult palate and skin (lanes 1 and 2 respectively).*

limited to the medial edge epithelium at the time of initial adherence of the opposing palatal shelves (Fig. 1B). The desmosome concentration remained high as the midline seam thinned (Fig. 1C) and was elevated in all islands of MEE as the midline seam degenerated (1E). Following epithelial–mesenchymal transformation of the MEE, there was no significant concentration of desmosomes in the mesenchyme. The temporal increase of desmosome quantity between E14 and E15 of mouse development is coincident with critical timepoints in the process of palatogenesis.

Gene expression of desmosome components

The increased desmosome quantity in the MEE at critical stages of palatal fusion was further analyzed for the expression pattern of specific genes critical to desmosome assembly. Six desmosome component genes were expressed between E13 and E16 in the developing palate (Fig. 2). Desmoplakin gene expression was found in all the examined developmental stages (Fig. 2 A-D, lane 1). All three different desmoglein isoforms were identified at all stages examined (Fig. 2, A-D, lanes 5-7). Only two of the three desmocollin isoforms were identified (Fig. 2, A-D lanes 2-4). Both Desmocollin 2 and 3 were identified at all stages of palate examined, however, desmocollin 1 was not found in the palatal tissues. When we examined the expression of DSC1 message in tissues known to express DSC1 such as adult skin or tongue, we detected the DSC1 message both in the skin and tongue (Fig. 3, lane 4). At least one DSG and DSC are required to form desmosomes in epithelial tissues (King *et al.,* 1996), but our data suggested that the pairing of DSC1 and DSG1 for palatogenesis is not required. The type 1 DSG may pair with other DSCs such as DSC2 or DSC3, which are expressed in the palate. There are other examples where DSC1 and DSG1 do not have paired expression (Chitaeve and Troyanosky, 1997; Marcozzi et al., 1998; North *et al.,* 1996). We established the expression of two DSC2 variants, DSC2a and DSC2b (Fig. 4). The two variants are expressed at the same level.

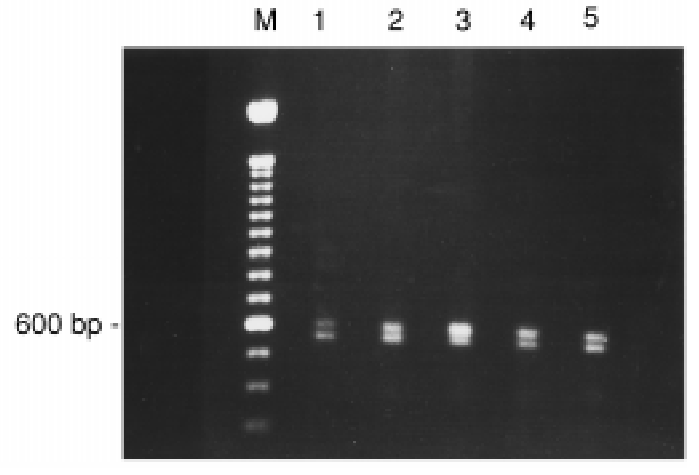

Fig. 4. The expression pattern of the two DSC2 variants during palatogenesis. *The level of expression of the two variants remained the same during palatogenesis. The two variants were coordinatly expressed at the same level. Lanes (M) DNA size marker, (1) E13, (2) E14, (3) E14.5, (4) E15, and (5) E16.*

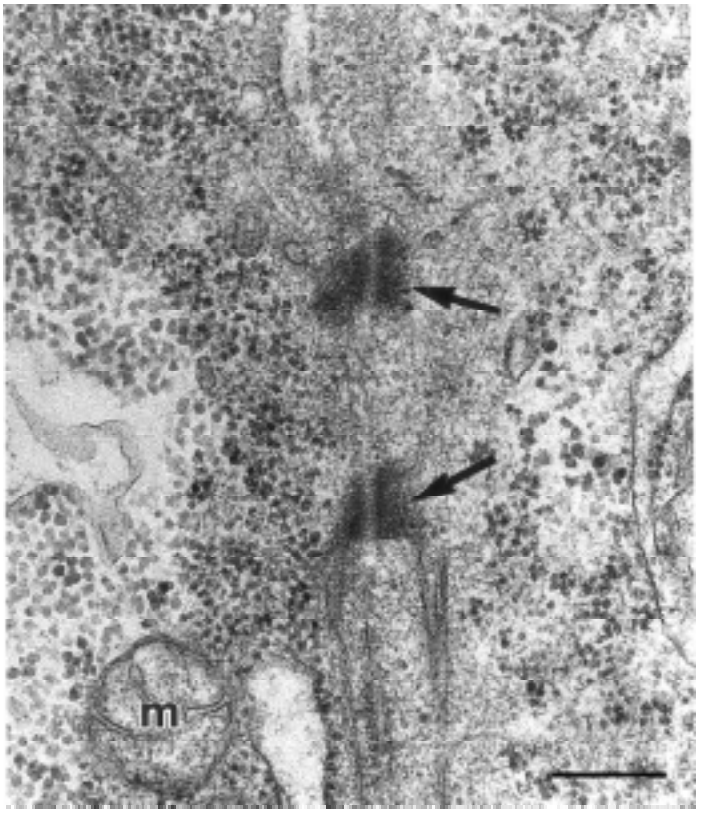

Fig. 5. Electron micrograph of fused palatal shelves. *Electron microscopy localized desmosome between two MEE cells (arrows). Desmosomes are well organized and look mature in fused palatal shelves. m, mitochondria; scale bar represents 200 nm.*

Electron microscopy

Desmosomes are organized between the MEE of each palatal shelf. We found that desmosomes are well organized and matured in touching palatal shelves, between E14 and E15 (Fig. 5). Electron microscope examination can show an ultrastructurally recognizable desmosome. Antibody staining alone indicates only accumulation of desmosomal antigen at a cellular interface and does not necessarily indicate that these are assembled into ultrastructurally recognizable desmosomes (Mattey and Garrod, 1985). However, when antigen accumulation has been found between E14 and E15, electron microscope examination has shown that desmosomes are well organized and look mature in touching palatal shelves.

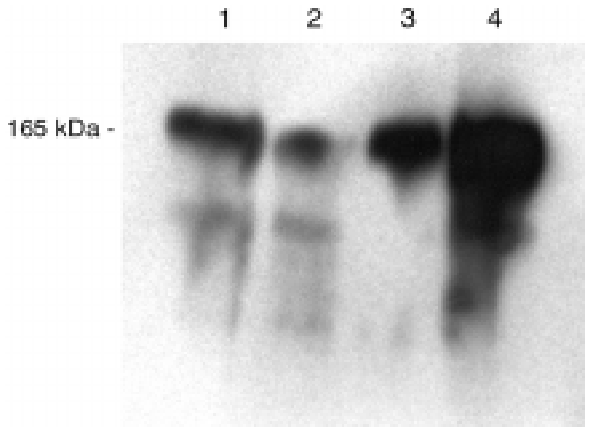

Fig. 6. Western analysis of desmosomal protein from the palate. *Palatal tissues were homogenized and analyzed following electrophoretic separation and immunoblotting. The blots were probed with anti-desmoglein 3.10 antibody. Lanes 1-4 (protein from E13-E15 palates); lanes (1) E13, (2) E14, (3) E14.5 and (4) E15.*

Western analysis

As a further confirmation that the desmosomal gene expression resulted in the production of desmosomal protein components, the tissues were homogenized and analyzed following electrophoretic separation by immunoblots (Fig. 6). Desmoglein was used as an example of the production of proteins from desmosomal component genes that were identified at all stages of palatal development. The result means that the MEE are actively engaged in new gene expression and new protein synthesis throughout the process of palatal fusion. Thus, the MEE are metabolically active with differential patterns of gene expression compared to the adjacent and continuos oral and nasal epithelia.

Discussion

The adhesion of the opposing palatal shelves is critical to initiating the process of palatal fusion. If the adhesion does not occur appropriately, the palatal shelves will be pulled apart as the head grows and a cleft palate will result. Palatal shelf adhesion could be the result of multiple types of cell-cell adhesion molecules. In the present study we have characterized the pattern of expression of one cell-cell adhesion molecule, the desmosome. It was shown that the MEE have a marked increase in the quantity of desmosomes at the critical stages of palatal adhesion. These increased desmosomes are the result of new desmosomal gene expression and new protein production and not the result of reorientation of desmosomes previously present in the MEE. This synthetic capability of the MEE is a critical element supporting the vitality of the MEE during palatal fusion and not the onset of programmed cell death. The mechanism regulating desmosome production in the MEE will be the goal of future experiments that may link the expression of specific growth factors in the MEE, such as TGF beta 3, and the alterations in desmosomal gene expression. The defect in palatal fusion in TGF-_3 knock-out mice has been hypothesized to be due to abnormal adhesive interaction following palatal shelf contact (Proetzel *et al.,* 1995). Importantly, the analysis of desmosomal production in the MEE has demonstrated some specific patterns of gene expression that may be related to the role of the desmosome in palatal fusion.

The pattern of specific desmosome gene expression provided new insight into the variations in the structure that can occur. We established the expression of two DSC2 variants, DSC2a and DSC2b (Fig. 4). The two variants were coordinately expressed at the same level in E13-16 palate. The a-form is approximately 55aa longer than the b-form (Collins *et al.,* 1991). It was demonstrated that a chimeric molecule with the connexin extracellular domain and desmocollin "a" cytoplasmic domain, when expressed in A431 cells, supported plaque assembly and the attachment of intermediate filaments. However, a similar chimera made using the "b" form did not (Troyanovsky *et al.,* 1993). Further investigation is required in order to determine the functional relevance of DSC2b in palatogenesis. DSC1 and DSG1 did not have coordinated expression in the palate as had been reported by others (Schafer *et al.,* 1994; Nuber *et al.,* 1995). In the palate DSG1 was expressed alone, which is a result similar to reports of DSG1 expression during examples of epithelial morphogenesis (King *et al.,* 1996). Thus, our data suggested that DSC1 and DSG1 expression is not necessarily paired. Similar results have been obtained by others. Expression of full length DSC1a in human fibroblast HT-1090 cells that lack mature desmosomes and express a single known desmosomal protein DSG 2 results in a redistribution of endogenous DSG 2 (Chitaeve and Troyanovsky, 1997). Expression of DSC2a and DSG1 by transfection in mouse L cells resulted in potentiation of cell-cell aggregation and the accumulation of desmosomal proteins at points of cell-cell contact (Marcozzi *et at.,* 1998). However, different desmocollin isoforms can mix in the same desmosomes as seen in bovine nasal epidermis, the three DSC isoforms are expressed in overlapping domain, DSC3 expression is strongest in the basal layer while DSC2 and DSC1 are strongly expressed in the suprabasal layers (North *et al.,* 1996). Therefore, paired expression of specific DSC and DSG gene pairs overlap is far from clear.

TABLE 1

THE SYNTHETIC OLIGONUCLEOTIDES USED FOR PCR AMPLIFICATION

Name	Sequence (forward, 5'-3')	Sequence (reverse, 5'-3')	Annealing temp	Product size (bp)
Desmoplakin	CATGGTCAAGAGAGAGCAGAGGGAG	CTGGATGAGGCTGAGCTGAGAG	62°C	566
Desmocollin1	GGTTCTCCATCTTGCTCTCA	CAATAGATGTCGCCGGTGTC	55°C	296
Desmocollin3	GATGGTGGTTCCTGAGTTCC	CTCATCTTTATCCGTGGCAC	55°C	762
Desmoglein1	GTCTTTAGGGCCTGGCTTTGCTTGCTTG	GCTGCGCCAGTCAACATGACTTTCCTAG	55°C	285
Desmoglein2	CCTGTGTGGCTCTGTAATATAAGGGGTGG	CCTCCATCACGGCTAGGAACAATGTGC	55°C	542
Desmoglein3	GGACAGCACAGAGAAGATGG	GTTTGGTTCATCTGCATCCG	55°C	454

Materials and Methods

Mouse embryos

Female and male Swiss-Webster mice obtained from Simonsen Labs (Gilroy, CA) were housed in a controlled light and temperature environment with ad libitum food and water. By mating the females with the male mice overnight, timed pregnancies were obtained. The following morning being designated day 0 of gestation. The presence of a vaginal plug was used as an indication of pregnancy. At selected developmental stages, the pregnant mice were sacrificed. Palatal shelves were removed from embryos by microdissection. The palates were used immediately or stored in -80 ºC for RT-PCR and Western analysis.

RNA extraction

Palates were isolated from embryonic mouse heads and then pooled for RNA extraction. Total RNA was extracted using Trizol reagent (GIBCO-BRL). The total RNA was quantitated by a 260nm spectrophotometer.

cDNA synthesis

2ug of total RNA was incubated with DEPC treated water at 70-80 ºC for 3 min to denature secondary structure. Then the RNA was placed on ice and the following components were added to it: 45 uM oligo (dT), 5x transcription buffer, 0.5 mM dNTP, 1 U/ul RNase inhibitor, 13.3 U/ul MMLV reverse transcriptase (GIBCO-BRL). Subsequently the mixture was incubated at 42 ºC for 1 hr. Heating the cDNA at 70-80 ºC for 10 min terminated the reaction. Some of the cDNA was removed for PCR amplification and the remainder was stored at -80^{0}C.

PCR amplification

PCR amplification was done in a thermal cycler (MJ Research, Inc. Watertown, MA). The PCR amplification mixture contained PCR buffer with 1.5mM $MgCl_2$ (Boehringer Mannheim, Germany), 0.2 mM dNTP, 2-5 U *Taq* DNA polymerase (Boehringer Mannheim, Germany), 0.2mM of each amplimer and 0.3 ug of the reverse transcription product. The amplimers used for DSC2, DSC2a and DSC2b were as described by Lorimer and others (Lorimer *et al.* 1994). The amplimers used for the remainder were designed based on the published and Gene Bank cDNA sequences of desmosomal components (Chidgey *et al.,* 1997; Collins *et al.,* 1995; Geisel *et al.,* 1996, see Table 1). The PCR product was run on a 1 % agarose gel (GIBCO-BRL) and then stained with 1 ug/ml ethidium bromide. The size of the amplified product was evaluated using 100 bp DNA marker (GIBCO-BRL). Mouse G3PDH was used as internal positive control (ClonTech). The amplification of G3PDH confirmed that the RNA in each sample was not degraded and differences in amplifications were not due to differences in the amount of RNA amplified.

Immunohistochemistry

Heads were fixed in Carnoy's fixative solution overnight at 4°C, dehydrated in 100% ethanol, cleared in xylene and embedded in paraffin. Serial sections of 5 um were cut and mounted onto L-polylysine (Sigma St. Louis, MO) coated slides. The slides were dried overnight at 58°C, deparaffinized, dewaxed in xylene, dehydrated in decreasing grades of ethanol: absolute, 95%, 70%, 50% and rinsed in distilled water.

Microwave treatment of tissue sections was done in prewarmed 10 mM Na-citrate buffer, pH 6.0 at 600 watt in a microwave oven, according to the manufacture's protocol (Boehringer Mannheim, Germany). It was repeated three times for 5 minutes each. The slides were cooled between the repeated steps. The slides were rinsed in distilled water and then in PBS Then Desmoplakin I and II multiepitope cocktail (Boehringer Mannheim, Germany) was used as a primary antibody. The primary antibody was incubated overnight at 4 ºC in a humidified chamber. The tissues were washed with PBS and then incubated with secondary antibody following the Zymed Streptavidin Biotin system protocol (Zymed Laboratories, Inc., San Francisco, CA). The slides were counterstained with hematoxylin and examined by light microscopy to identify desmosomes in the MEE.

Electron microscopy

Palates were immersed immediately after dissection from the embryos in glutaraldehyde-paraformaldehyde, buffered with 0.1 M phosphate buffer at pH 7.4 for 10 min at 37°C, followed by fixation at room temperature for 4 hr. After primary fixation, specimens were postfixed overnight at 4 ºC in 1.5% osmium tetroxide followed by *en block* staining in uranyl acetate. Specimens were then rapidly dehydrated through a series of graded acetones and embedded in Epon. Thick sections (1 um) were cut, stained with 1% toluidine blue and examined by light microscopy to obtain the desired orientation for subsequent thin sections. Thin sections with interference colors of silver to pale gold were cut with diamond knives on a Sorvall MT-2 ultramicrotome, stained with lead citrate and examined using a JEOL-1200 transmission electron microscope.

Western analysis

To confirm PCR results as well as to examine any change in level of desmosomal components due to post-transcriptional regulation we did Western immunoblots. Crude extracts of palatal shelves from various stages of development were prepared by adding 1x loading buffer (from 10x stock) (Sambrook *et al.,* 1989) to palatal shelves. Then boiled for 5 min at 85°C. The tissue was homogenized by passing through 26-gauge needle in 1cc syringe 3-4 times. Then the samples were centrifuged for 10 min at 1600 rpm and the supernatant removed. The protein concentration of the supernatant was determined using a BioRad protein assay reagent (BioRad Laboratories, Hercules, CA). Following protein quantification, 100 ug of total protein homogenate from each stage was separated by 12% SDS polyacrylamide gel electrophoresis. A broad range molecular weight marker (BioRad Laboratories) was used as the molecular weight standard. The proteins were transferred overnight to PVDF western blotting membrane (Boehringer Mannheim, Germany) using a mini transfer blot cell (BioRad Lab). The membrane was washed for 20 min in TBST pH 7.5 (6.069 g Tris, 8.76 g NaCl, and 1 ml Tween20) per liter. Then the membrane was incubated for an hour in Boehringer Mannheim blocking solution (Boehringer Mannheim) to reduce nonspecific protein background and then in primary antibody DSG 3.10 (Transduction Laboratory San Diego, CA), (1:2400 dilution) overnight at 4°C. Incubation with enzyme coupled secondary antibody (horseradish peroxidase linked anti mouse IgG, 1:2400) was done according to the manufactures' instructions (Boehringer Mannheim). The immunoreactive bands were detected after incubation of the membrane in peroxidase substrate (Boehringer Mannheim).

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