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Characterization of a Novel Mouse Model to Examine Oral Epithelial Stem/Progenitor Cell Functions

Section 1: Specific Aims

Keratinocytes of the oral mucosa are organized into stratified layers, where they serve crucial functions necessary for the survival of an organism by acting as a barrier against water loss, mechanical forces associated with mastication, and microbial insults. In order to combat the daily onslaught of attacks, basal progenitor cells of the oral epithelium undergo a tightly regulated program of proliferation and differentiation. This delicate balance is critical to ensure proper repair to damaged tissues and to maintain tissue homeostasis and normal tissue integrity. The initiation of stratification and differentiation of basal progenitor keratinocytes is largely controlled by the transcription factor p63 (1). We and others have shown that p63, specifically the ΔNp63 isoform, is highly expressed in basal keratinocytes and is important for both progenitor cell function and epithelial commitment primarily during embryonic oral epithelial development (2). Despite the critical role progenitor cells play in development and maintaining tissue homeostasis, very little is known regarding the molecular properties of these cells. Therefore, the generation of *in vivo* mouse models to isolate and characterize this important cell population is critical.

The main objective of this proposal is to characterize a novel red fluorescent protein (RFP) reporter transgenic mouse model, in which RFP expression is driven by endogenous ΔNp63 regulatory elements. These goals will be addressed by means of two specific aims:

Specific Aim 1: Characterize a novel ΔNp63-RFP transgenic mouse model to determine if RFP expression mimics endogenous ΔNp63 expression in tissues and organs of the oral cavity.

Specific Aim 2: Determine if ΔNp63-RFP^{+} cell population is enriched with the progenitor cell population of the oral epithelium by performing Fluorescence-Activated Cell Sorting (FACS) analysis.

Section 2: Research Strategy

Significance

The oral mucosa consists of stratified, squamous epithelium, which can be subdivided into three categories: masticatory, specialized, and lining. Masticatory and specialized mucosa in humans is keratinized,

acting as a protective barrier against mechanical, chemical, and physical forces (4). The stratified epithelium of the oral mucosa consists of the basal, spinous, granular, and cornified layers. Epithelial keratinocytes undergo a regimented differentiation program where progenitor cells of the basal layer divide to replace squames that are shed from the cornified layer (3).

The transcription factor p63 is a homologue of p53. While p53 is not important for normal development, p63 is essential for the development of stratified epithelial tissues, including the oral mucosa (5, 6). A specific phenotype is observed in null mice that have a deletion of the p63 gene. p63 null mice die shortly after birth and present with defects in limb formation, craniofacial abnormalities, and fail to develop mature stratified epithelial tissues including the skin and oral cavity (2,5,7). These phenotypic malformations observed in the epithelial tissues are believed to be the result of decreased epithelial progenitor cell proliferation and differentiation (5,7).

The p63 gene is transcribed into two main isoforms: ΔNp63 and TAp63. Both TA and ΔN isoforms are differently spliced at the carboxyl terminals, creating alpha, beta, and gamma variants (Fig.1) (2, 8,9). Studies using various mouse models have demonstrated that ΔNp63 is highly expressed in the basal progenitor cell populations of the epidermis and oral epithelium and is critical for the development of these tissues (2,8). Indeed, using ΔNp63 Knockout animals (2) and ΔNp63 overexpressing transgenic mouse models (10,11), we have shown that this isoform plays a critical role in directing epithelial cell differentiation. Furthermore, ΔNp63 plays a critical role in the development of structures of ectodermal origin, including the oral (2), olfactory (12), urogenital (13), and skin epithelium (10,11,14) and mammary gland (15,16). Conversely, TAp63 is not critical for the development and differentiation of epithelial tissues (2,8).

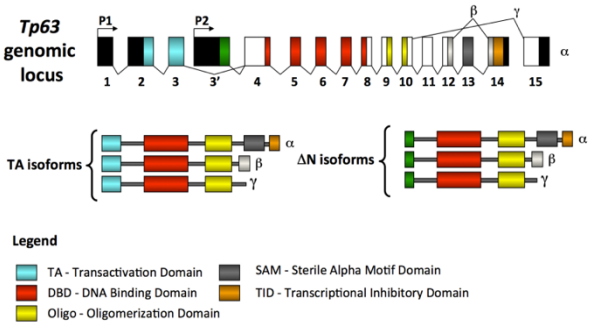

Figure 1: Upper panel shows the genomic locus of Trp63 and lower panel shows the differential splicing of Trp63 into 6 isoforms.

Innovation

Given the critical importance of $\Delta Np63^{+}$ oral basal progenitor cells in epithelial development, lineage specification and cell differentiation, it would be beneficial to isolate a pure population of oral progenitor cells

and selectively enrich for ΔNp63^{+} cells. In order to do this, we have generated a ΔNp63 red fluorescent protein (RFP) reporter transgenic mouse model, in which RFP expression is driven by endogenous ΔNp63 regulatory elements (ΔNp63-RFP) (Fig. 2).

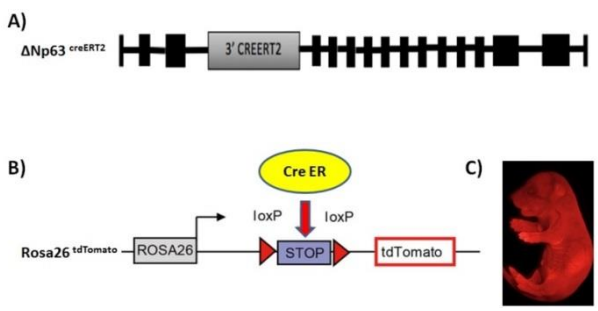

Figure 2: A) Schematic of the ΔNp63CREErt2 mice. B) Schematic of the Rosa Tdt mice. C) ΔNp63-RFP Transgenic mouse.

Approach

Immunofluorescence Staining:

Immunofluorescence staining will be performed to determine if RFP expression mimics that of endogenous ΔNp63 expression in tissues and organs of the oral epithelium of ΔNp63-RFP transgenic mice. We will use paraffin embedded tissue sections of buccal mucosa, tongue, palate, and salivary gland from adult ΔNp63-RFP mice. The paraffin embedded tissue slides will be rehydrated through immersion in a graded alcohol series. Rehydrated slides will be heated in antigen retrieval buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0) by boiling slides in a microwave for 20 minutes. Slides will be boiled for 20 minutes, and then washed briefly in Phosphate Buffered Saline (PBS). A PAP pen will be used to circle the tissue, providing a hydrophobic barrier surrounding the tissue. Slides will be blocked in 5% BSA, 0.1% TritonX-100 in PBS for one hour. Sections will be incubated with primary antibody overnight at 4°C. Slides will be washed in PBS 3 times for 5 minutes each and secondary antibodies will be added and incubated for 45 minutes in the dark. Sections will be washed in PBS 3 times for 5 minutes each and then mounted using Vectashield Mounting Medium with DAPI (Vector Labs). ΔNp63, RFP, Keratin5 and Keratin14, primary antibodies will be used for immunofluorescence staining. Staining procedures will be performed by using the reagents and protocol from the MOM Basic Kit (Vector Labs) when necessary. Leica confocal microscopy will be used to capture the images. ImageJ, Adobe Photoshop, and Adobe Illustrator software will be used to analyze the images. We do not anticipate any problems with these experiments as our lab has extensive experience using the proposed antibodies, and we already have a large collection of paraffin embedded tissue sections/slides of buccal mucosa, tongue, palate, and salivary gland from ΔNp63-RFP mice. In the event that some antibodies do not work well

with paraffin embedded tissues, we will use frozen tissue sections, which have already been collected and sectioned and are currently stored in the lab.

FACS Analysis:

The buccal mucosa will be dissected out from approximately 10-20 ΔNp63-RFP mice, providing roughly 10^7 ΔNp63-RFP$^+$ basal progenitor cells. Isolated tissues will be separated from the underlying substrate by treatment with dispase solution and CnT-PR media overnight at 4°C (Zen-Bio, NC). Next, the separated oral epithelium will be washed in CnT-PR media and then placed in a 35mm petri dish containing 500 microliters of TrypLE (Gibco, Life Technologies) cell dissociation reagent and chopped with a razor blade. CnT-PR media (2ml) will be added to inactivate the TrypLE and tissues to will be chopped again. This causes the oral keratinocytes to be released into the suspension, which will be collected in 4ml of CnT-PR media and passed through a 40 micrometer cell strainer. Single cell suspensions will be washed in CnT-PR media and centrifuged at 200xg for five minutes and then counted. Approximately 1-2 million cells will be added to each FACS tube and washed with FACS buffer (1XPBS, 0.5 BSA, 0.1% sodium azide) on ice. Cells will be re-suspended in 500 microliters of FACS buffer and blocked with one microliter of CD16/Fc receptor (BD Bioscience) for 10 minutes on ice. Flourochrome-conjugated antibodies will be added to appropriate tubes and incubated for 20 minutes in the dark. Cells will be washed in 2ml of PBS 3 times at 200xg for 5 minutes. Cells will be re-suspended in 500 microliters of PBS and subjected to FACS. 50,000 events will be collected for the analysis and FlowJo software will be used to determine if the basal progenitor population is enriched for ΔNp63-RFP$^+$ expressing cells.

Section 3: Resource Information
Experiments will be performed in the laboratory of Dr. Romano located in 210 Foster Hall, Department of Oral Biology, School of Dental Medicine, SUNY at Buffalo. The laboratory is equipped with a separate tissue culture facility, general biochemical laboratory equipment and there is ready access to a core research facility which includes a confocal microscope and FACS and Cell Sorter.

Section 4: Other Support for Applicant and Sponsor
Work for this project is supported from SDM start-up funds, R03 DE025889-01A1 and R03 AI115407-01A1

Section 5: Recombinant DNA
Not Applicable

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