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Isolation and characterization of an immortalized oral keratinocyte cell line of mouse origin

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Abstract

Objective—To establish an oral epithelial cell line of mouse origin for molecular and biochemical assays.

Design—Epithelial cells were isolated from the oral cavity of adult mice and established as a spontaneously immortalized cell line in culture, designated immortalized oral keratinocyte cells (IMOK cells). The cells were then characterized for growth characteristics, differentiation potential, karyotype, transfectability, susceptibility to viral infection and responses to siRNA.

Results—The IMOK cells exhibit robust growth in both serum-containing and serum-free medium for at least 100 population doublings. IMOK cells have a near diploid karyotype, express keratinocyte marker proteins and can be induced to undergo differentiation by addition of high levels of calcium to the medium. The differentiation process is characterized by morphological changes and by the induction of oral epithelium specific differentiation marker proteins such as K4 and K13. Transient transfection analyses reveal that IMOK cells are highly transfectable and that several promoters of epithelial cells are active in these cells. Moreover, upon differentiation with calcium, there is an up-regulation of differentiation-specific K4 and Elf5 promoter activity. Finally, we show that the oral keratinocytes are also amenable to infection with retroviruses and to siRNA based knock-down of gene expression.

Conclusions—Our study is the first to establish an immortalized oral keratinocyte cell line of murine origin that can recapitulate the oral epithelium differentiation program and thus could serve as a useful tool for toxicological and molecular analyses of the oral tissue.

Keywords

Keratinocyte; oral epithelium; differentiation; calcium; transfection; viral infection; siRNA

Introduction

The stratified oral mucosal epithelium is composed mainly of keratinocytes that provide a protective barrier to pathogenic agents, mechanical and chemical abrasion. The epithelial layer of the oral mucosa exhibits distinct and variable degrees of stratification depending on its

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anatomical location within the oral cavity and the specialized function of that area (1–5). For example, the masticatory mucosa of the hard palate and gingiva consists of keratinized stratified squamous epithelium. The lining mucosa that covers the buccal cavity on the other hand is primarily non-keratinized stratified squamous epithelium. Despite these regional differences, the generation of the multi-layered epithelium follows a common developmental paradigm, whereby proliferating keratinocytes in the basal layer undergo a program of differentiation to give rise to the mature surface barrier consisting primarily of dead cells.

Defects in oral epithelial organization or function can lead to severe pathology such as an increased rate of infection. Indeed, oral keratinocytes secrete a number of proteins with antimicrobial activity such as the β defensins (6,7). In addition, defects in oral mucosal differentiation can lead to the development of oral squamous cell cancers (8,9). Mice have frequently been used as models for studying the underlying molecular mechanisms of oral diseases including infectious diseases (e.g. periodontitis, candidiasis, etc.), developmental defects and cancer (squamous cell cancers). This is in part due to the availability of a large number of gene-targeted and transgenic mouse strains that exhibit altered physiology of the oral epithelium. While *in vivo* animal models provide valuable insights to overall understanding of epithelial biology, *in vitro* cell culture based models are useful for dissecting the various molecular signaling pathways in epithelial cells cultured under different conditions. Because of the ease of culture of epithelial cells and the ability to manipulate gene expression in these cultures, much of our understanding of molecular signaling associated with the epithelial proliferation, differentiation and programmed cell death is derived from studies carried out with keratinocytes grown in culture.

The most frequently cultured keratinocytes are from epidermal origin and established protocols are available to isolate and expand epidermal keratinocytes and to induce their differentiation in culture (10,11). For example, elevated extra-cellular calcium or the addition of phorbol esters or retinoic acid are able to induce epidermal keratinocyte differentiation in culture (12–14). Upon induction of differentiation, keratinocytes show distinct morphological features such as flattening of the cells, re-organization of sub-cellular cytoskeletal components and increased cell-cell contacts leading to epithelial sheet formation (15). These features are accompanied by differential induction of well-characterized markers such as keratins and other structural proteins. Over time, considerable improvements in cell culture techniques have facilitated the establishment of keratinocytes including growth with and without fibroblast feeders and growth in serum-free medium (16–18). The valuable lessons learned from growing epidermal keratinocytes in culture have been instrumental in the isolation, maintenance and characterization of other keratinocytes, including those of oral origin.

Although a large number of different studies have reported the establishment of human or rat oral epithelial cells (both primary and transformed cells), few reports have described establishment of oral epithelial cells from mice. This is likely due to the small size of the oral cavity in mice and the limited amount of tissue available. However, one study has reported the development of a lingual epithelial cell line of mouse origin (KT-1 cells) (19). The KT-1 cells express markers consistent with a differentiation into gustatory epithelium rather than conventional oral epithelium. Another study described the isolation of gingival epithelial cell lines from mice (GE1 and GE6 cell lines) using a temperature-sensitive SV40 T antigen gene (20). These gingival epithelial cells express markers of oral keratinocytes and undergo stratification when cultured for extended periods of time. We describe the isolation of a new line of spontaneously immortalized mouse oral keratinocytes that recapitulates many of the features of normal mouse oral epithelium. This cell line may prove useful in a number of studies to dissect molecular mechanisms that control oral epithelial growth and functional properties.

Materials and Methods

Isolation of oral epithelial cells

Seven six-month old female 129Sv mice obtained from Taconic Farms were euthanized by CO₂ inhalation and their heads removed. Heads were cut open to expose the oral cavity. Blocks of tissue containing oral epithelium (buccal and palatal regions) were removed and sterilized by brief incubations in penicillin/streptomycin and gentamycin solutions. Tissue pieces were then washed in PBS several times and transferred into a tube containing a solution of dispase (neutral protease grade II from *Bacillus polymixa* in PBS, Roche Applied Sciences, Indianapolis, IN). Tissues were digested with dispase at 4°C overnight. After dispase treatment, the tissues were rinsed again in PBS and the oral epithelium from the buccal and palatal regions was carefully removed from the underlying substrate. Isolated oral epithelium was cut into small pieces with a razor blade and digested in TrypLE Express cell dissociation reagent (Invitrogen, Carlsbad, CA). Released buccal and palatal keratinocytes were filtered through a 100 micron Cell-Strainer (BD Biosciences, San Jose, CA), resuspended in 5 ml of low calcium medium (described below) and transferred to a tissue culture incubator. After 24 hours, the medium was changed to remove non-adherent cells. Keratinocytes were cultured in low calcium media (changing media every two days) and subcultured using TrypLE when they became confluent. After 2–3 passages, the keratinocytes entered a crisis phase in which a majority of the cells underwent apoptosis. The few surviving keratinocytes became spontaneously immortalized and were designated immortalized mouse oral keratinocyte (IMOK) cell line. The Institutional Animal Care and Use Committee of the State University of New York at Buffalo approved all experimental procedures involving mice.

Cell culture and reagents

IMOK cells were cultured in a custom-formulated low calcium medium. This medium consisted of a 1:3 mixture of calcium-free Dulbecco's Modified Eagle's Medium (DMEM) and calcium-free F12 medium supplemented with 15% fetal bovine serum pre-chelated with Chelex-100 resin (BioRad Laboratories, Hercules, CA) to remove the majority of free calcium. Additional media supplements included 26.8 mM sodium bicarbonate, 50 pM tri-iodothyronine, 127 pM cholera toxin, 1.9 pg/ml hydrocortisone, 12.7 µg/ml insulin, 12.7 µg/ml transferrin, 100 U/ml penicillin and 100 µg/ml streptomycin. The resulting media had a free calcium concentration of 0.05 mM as determined by atomic absorption spectroscopy (21). Oral keratinocytes were also cultured in serum-free keratinocyte growth medium CnT-02 medium (Millipore, Billerica, MA). Keratinocyte differentiation was induced by the addition of 0.5 to 1 mM calcium chloride for various periods of time (24 or 48 hrs).

Karyotype analysis

The karyotype of the IMOK cell line was analyzed using Spectral Karyotyping (SKY) analysis through the Roswell Park Cancer Institute SKY/FISH Analysis Resource. Cell cultures were treated with colcemid (0.03–0.06 µg/ml). Chromosome spreads were prepared using an air-drying method. After sequential digestion with RNase and pepsin according to the procedure recommended by Applied Spectral Imaging, Inc. (Vista, CA), the chromosomal DNA on slides was denatured in 70% formamide and then hybridized with a cocktail of mouse SKY paint probes tagged with various nucleotide analogues (i.e., a mixture of individual chromosome DNAs prepared by flow-sorting and PCR amplification). Thirty to fifty mitoses were chosen at random and the images developed by combinations of five different fluorophores such as Rhodamine, Texas-Red, Cy5, FITC and Cy5.5 were captured with a Spectral cube and Interferometer module installed on a Nikon microscope. Spectra-Karyotypes were carried out using SKYView software (Applied Spectral Imaging, Version 1.62).

Antibodies and related reagents

Rabbit polyclonal antibodies to keratin 5 (K5), keratin 6 (K6), involucrin and filaggrin were a kind gift from Dr. Julia Segre (National Human Genome Research Institute, Bethesda). Other primary antibodies were obtained from the following commercial sources: anti-K13 (AE18, AbCam, Cambridge, MA), anti- β -tubulin (KMX-1, Chemicon Inc., Temecula, CA), anti-GAPDH (6C5, Chemicon), anti-histone H1 (AE-4, Santa Cruz Biotechnology, Santa Cruz, CA), anti- Δ Np63 (H-129, Santa Cruz Biotechnology) and anti-E-cadherin (DECMA-1, Santa Cruz Biotechnology). Alexa-488 and Alexa-568 conjugated secondary antibodies were obtained from Invitrogen. Rhodamine-conjugated phalloidin was obtained from Cytoskeleton, Inc. (Denver, CO).

Construction of K4 promoter luciferase plasmid

The human K4 promoter (−1040 to +59 bp), which has previously been shown to express at high levels in oral mucosal cells (22), was obtained from Dr. Anil Rustgi (Massachusetts General Hospital, MA). The K4 promoter spanning the region from −790 to +59 bp was subcloned into the pGL3-Basic vector using Kpn-I (5' end) and Bgl-II (3' end) restriction enzymes. The Elf5, K14 and Δ Np63 luciferase promoter constructs have been described previously (23–25). The pCMV-*LacZ* plasmid encoding a β -galactosidase reporter gene was used to determine the percentage of cells transfected. The pEF1 α -RLuc plasmid, encoding a Renilla luciferase reporter gene, was used to normalize transfection efficiency.

Transient transfections and promoter assay

IMOK cells were plated the day before transfection at 70,000–80,000 cells per well in 12-well plates. Transfections were performed at 30–40% confluency using Fugene 6 reagent (Roche Applied Sciences) according to the manufacturer's instructions. Routinely, 0.5 μ g of each luciferase reporter construct was transfected per well along with 0.1 μ g of internal control plasmid pEF1 α -RLuc. For inducing differentiation, calcium levels in the medium were increased to 1 mM calcium 24 h after transfection and cells were allowed to undergo differentiation for additional 24–30 h. For reporter assays, transfected cells were lysed in reporter lysis reagent and the extracts were assayed for luciferase activity using the dual luciferase assay system as per manufacturer's instructions (Promega Biosciences, Madison, WI). Transfection studies were performed in duplicates and minimum of three independent experiments were performed for each construct. Firefly luciferase activity was normalized to Renilla luciferase activity and average value was determined for each promoter with respect to empty pGL3 vector.

RNA isolation, reverse transcription and real time PCR

IMOK cells were plated in 100 mm dishes and cultured in the absence or presence of 1 mM calcium for 24 or 48 hrs. Total RNA was isolated from cells lysed with Trizol (Invitrogen). cDNA synthesis was carried out using Superscript reverse transcriptase II, random hexamers and oligo dT primers according to the manufacturer's protocol (Invitrogen). Quantitative PCR analysis was performed using SYBR Green mix (BioRad Biosciences) and primer sets that typically gave a Ct value of 22–34 using the iCycler real-time PCR machine (BioRad Biosciences). β 2-microglobulin was used as an internal control and all the reactions were performed in triplicate for each experiment. The primer sequences used were: K13 sense, TCAGCTGGAGGCTATGGA, K13 anti-sense, ATCCGGTTGTTGTCCGTGCTGG, K4 sense, CAGGCTCTCATGAACGTCAA, K4 antisense, AAGCCACTTCCAGAACCAGA, β 2 Microglobulin sense, AGACTGATACATACGCCTGCAG and β 2 Microglobulin anti-sense, GCAGGTTCAAATGAATCTTCAG.

Western blot analysis

Whole cell extracts were prepared using Laemmli sample buffer from IMOK cells (BioRad Laboratories). Equal amounts of lysates were loaded on a 10% SDS-polyacrylamide gel and transferred on to Immuno-Blot PVDF membrane (BioRad Laboratories). After blocking in 5% non-fat dry milk in TBST (Tris-buffered Saline Tween-20), the membrane was incubated with a 1:5,000 dilution of the primary antibodies overnight at 4°C. After washing in TBST, the membrane was incubated in horseradish peroxidase conjugated goat secondary antibodies (Kirkgaard Perry Laboratories (KPL), Gaithersburg, Maryland) at 1:20,000 dilution for 45 minutes at room temperature. Specific bands were detected by chemiluminescence (KPL).

β -galactosidase staining

IMOK cells transfected with 1 μ g of pCMV-*LacZ* were harvested after 48 hrs, fixed with 0.2% glutaraldehyde for 2 minutes at room temperature followed by two brief washes with PBS. Cells were subsequently stained with Beta-gal stain base solution (Invitrogen) containing 1 mg/ml X-gal for 1 hour at 37°C.

Gene knockdown assay

IMOK cells were transfected at 30–40% confluency with Ambion Corp. (Austin, TX) *Silencer* GAPDH siRNA specific for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or with a scrambled siRNA that served as negative control using Lipofectamine 2000 (Invitrogen) following manufacturer's instructions. Cells were harvested after 48h and assessed for GAPDH expression using Western blotting.

Indirect immunofluorescence

IMOK cells were cultured to 60–80% confluence on glass coverslips and differentiation was induced by the addition of calcium chloride to the culture medium to a final concentration of 1.0 mM for 6–24 hours. The cells were then fixed with 4% paraformaldehyde in 1X PBS on ice for 20 minutes, followed by three washes in 1X PBS at room temperature. Cells were permeabilized with 0.1% Triton X-100 and blocked in 5% BSA in 1X PBS for 1 hour at RT, and then incubated with the primary antibodies at a dilution of 1:50 to 1:500 at 4°C overnight. After three 1X PBS washes, the cells were incubated in 1:500 dilution of Alexa-488 conjugated goat anti-mouse or anti-rat antibodies and/or a 1:750 dilution of Alexa-568 conjugated goat anti-rabbit antibody. For detection of filamentous actin, the cells were incubated in 100 nM rhodamine-conjugated phalloidin in 1X PBS for 30 minutes at RT. The cells were counterstained with DAPI (4', 6-diamidino-2-phenylindole) to mark the nuclei and coverslips were mounted with 80% glycerol in 1X PBS. The cells were viewed and photographed with Zeiss LSM 510 Meta Axiovert 200M confocal microscope and images were analyzed using Image Examiner software.

Viral infection

The MIGR1 retroviral vector (26) was used for virus production. MIGR1 harbors a multi-cloning site followed and internal ribosomal entry site (IRES) and green fluorescent protein (GFP). Viral supernatants were prepared using the Plat-E packaging cell line by the protocol described previously (27). Retroviral supernatants were used to infect IMOK cells using a spin-infection protocol with 10 μ g/ml of polybrene. Two days after infection, the GFP positive population was sorted out using FACS Aria Cell Sorter (BD Biosciences). Histogram profiles were analyzed using FlowJo software (TreeStar Inc, Ashland, OR).

Results

Establishment and initial characterization of IMOK cells

We isolated epithelial cells from the buccal and palatal regions of the oral cavity of adult mice and established them in culture in a custom-formulated medium containing low concentrations of calcium. We chose this medium since increased levels of calcium triggers cessation of the cell cycle and leads to terminal differentiation of the keratinocytes. The oral keratinocytes demonstrated a cobblestone morphology, which is characteristic of epithelial cells in the undifferentiated stage. After a few passages, most of the oral keratinocytes entered a crisis phase as is typical of mouse keratinocytes and underwent apoptosis. However, a few surviving keratinocytes retained their cobblestone morphology, became spontaneously immortalized and could be easily passaged for many generations in low calcium medium (Figure 1A). This cell line, designated IMOK (immortalized mouse oral keratinocytes), has undergone more than 100 population doublings, confirming its immortalized state.

Upon addition of calcium to the extracellular medium (0.5–1 mM), within 6–8 hours IMOK cells became more flattened and spread out with cell-cell borders becoming less distinct (Figure 1B). This distinct transformation in cellular morphology in the presence of high calcium is in accordance with many studies with keratinocytes where calcium addition has been shown to result in increased cell-cell contacts and alterations in the attachment to extracellular matrix components (12, 14, 28). We next determined whether IMOK cells could be propagated in commercially available keratinocyte growth media in the absence of serum. For this purpose, we plated out IMOK cells in CnT-02 keratinocyte growth media. Like our custom-formulated medium, CnT-02 medium contains a low concentration of free calcium allowing robust keratinocyte proliferation. IMOK cells cultured in CnT-02 medium proliferated and displayed similar morphological features as IMOK cells cultured in low calcium medium (data not shown). Furthermore, addition of calcium to IMOK cells in CnT-02 medium resulted in typical changes characteristic of cellular differentiation (flattening and loss of distinct cell-cell borders).

Next we karyotyped the IMOK cells using SKY analysis to examine the overall genomic integrity of these cells. This assay demonstrated that unlike most established keratinocytes, which show profound changes in chromosomal numbers and are often virtually tetraploid (29–31), the IMOK cells maintained a near diploid chromosome number (Figure 2). The modal chromosome number in IMOK cells was 44 chromosomes (normal diploid mouse chromosome number is 40). However, several chromosomal aberrations were present in the IMOK cell line including trisomies of chromosomes 2, 5, 8, 18 and 19 and partial trisomy of chromosome 16 (translocated onto chromosome 12). In addition, there was monosomy of chromosome 14, an unbalanced translocation of chromosomes 1 and 3 and an interstitial deletion on one copy of chromosome 4. These chromosomal aberrations probably arose during the immortalization of the cell line and may contribute to its long-term proliferative potential in culture. IMOK cells exhibited an XX karyotype, which was expected since the cells were derived from female mice. The fact that all of the mitoses examined showed the same chromosomal pattern or slight variations from it indicates that the immortalization event probably occurred in a single cell, which then proliferated to form the IMOK cell line.

Differential expression of marker proteins in IMOK cells upon differentiation with elevated calcium

Next we wanted to examine if the visible morphological changes associated with differentiated IMOK cells are also accompanied by expression of specific markers. The non-keratinized stratified epithelia lining the oral cavity specifically express keratins K4 and K13 (1), which are associated with the onset of differentiation as the cells migrate from the basal layer into the

spinous layer. IMOK cells cultured in the presence of elevated calcium begin to express keratin proteins normally present in the differentiated suprabasal cell layers of stratified oral epithelium. Quantitative reverse transcription PCR analysis confirmed that the mRNA levels for both the early differentiation markers of oral keratinocytes K4 and K13, were up-regulated in IMOK cells treated with calcium (Figure 3A). The results of the mRNA expression were confirmed and complemented using Western blot analysis. Western blot revealed that IMOK cells express undetectable levels of K13 in low calcium media (Figure 3B). However when cultured in the presence of elevated calcium for 24 to 48 hours, there is a marked increase in the levels of K13 in IMOK cells.

To further confirm the differentiation program of IMOK cells, we performed confocal microscopy to detect expression of particular differentiation markers. As shown in Figure 4A, an antibody that recognizes the ΔN isoforms of the keratinocyte-specific transcription factor p63 revealed localization of p63 in nuclei of the proliferating IMOK cells (Figure 4A). p63 is known to mark the proliferative compartment of stratified squamous epithelial cells (32). Undifferentiated IMOK cells also expressed high levels of cytoplasmic keratin 14 (K14), which is organized in a typical filamentous pattern (Figure 4B), and keratin 6 (K6), which exhibited a more diffuse staining pattern with some clumping (Figure 4D). In addition, IMOK cells had a well-organized actin cytoskeleton, with the presence of typical stress fibers (Figure 4A and C). The stress fibers extended to the plasma membrane where they co-localized with integrin-mediated adhesions as revealed by staining for $\beta 4$ integrin (Figure 4C).

Next, we wanted to determine the differentiation profile IMOK cells in the presence of added calcium. Upon differentiation with calcium, a small number of IMOK cells expressed K10 (Figure 4E). This is in accordance with the fact that keratinized oral epithelia found on the palate and gingiva express keratin proteins K1 and K10. We were unable to monitor expression of K13 by immunofluorescent staining since the antibody against K13 did not function in this assay. However, the results of the Western blotting (Figure 3B) suggest that K13 is highly expressed in IMOK cells induced to undergo differentiation. Thus, it is likely that the majority of the IMOK cells express a differentiation program typical of non-keratinized oral epithelium. Furthermore, upon differentiation, some IMOK cells began to express involucrin (Figure 4F) and filaggrin (Figure 4G), which are markers of terminal differentiation in keratinocytes. The staining pattern with antibodies to involucrin and filaggrin was punctate, consistent with these proteins being incorporated into keratohyalin granules.

IMOK cells can be transiently transfected, infected with virus and are amenable to molecular probing

Our goal in establishing the IMOK cell line was to develop a line of murine oral epithelial cells that could be used in a variety of biochemical and molecular assays to investigate the properties of mouse oral epithelium. For this purpose, we wanted to determine whether these cells were transfectable by commonly used reagents and whether they were amenable to infection by viruses, both common means to introduce foreign genes into cells in culture. We began by transfecting IMOK cells with an expression vector encoding *E. coli* lacZ under the control of the CMV promoter (pCMV-lacZ) using a lipid-based transfection reagent (Fugene-6, Roche Applied Sciences). Beta-galactosidase activity in transfected cells was measured by staining with X-gal, which revealed that 10–20% of the cells were transfected (Figure 5A). This level of transfection efficiency is sufficient to perform many biochemical assays such as analysis of promoter-reporter gene constructs.

Although transient transfection assays indicated that IMOK cells were transfectable, only 10–20% of the cells expressed the transfected plasmids. In some situations, it might be useful to express genes in a higher percentage of cells. Retroviruses are ideal for this type of assay as it is possible to infect a larger percentage of cells. Moreover, the viral genome becomes

permanently integrated into the host cells, thus expression of the gene product is stable over an indefinite period of time. To determine whether IMOK cells could be infected with virus, we exposed them to a live murine retrovirus (MIGR1) encoding GFP downstream of a multiple cloning site (26). Forty-eight hours after infection, IMOK cells were trypsinized and the single cell suspensions were assayed using flow cytometry. As shown in Figure 5B, ~35–40% of the cells expressed the GFP marker. We sorted out these GFP positive cells and returned them to culture. After 10 days, the cells were again examined by flow cytometry, which demonstrated that ~90% of the cells were now GFP positive (not shown). Thus, IMOK cells can be infected by retroviruses leading to long-term stable expression of genes encoded in the retroviral genome.

Over the last few years, siRNA has emerged as a very useful tool to inhibit gene expression in a variety of cell types. We determined whether IMOK cells were susceptible to transfection with siRNA leading to gene knockdown. For this assay, we chose to knockdown the ubiquitously expressed gene glyceraldehyde 3-phosphate dehydrogenase, GAPDH. siRNA targeted to the GAPDH mRNA sequence or a control scrambled siRNA were transfected into IMOK cells using a lipid-based transfection procedure (Lipofectamine 2000, Invitrogen). Expression of GAPDH was assessed 48 hours later using Western blot analysis, which revealed an ~50% decrease in the levels of GAPDH protein (Figure 5C). The actual level of GAPDH knockdown is probably higher than 50% given that not all cells in the culture will be transfected using the lipid-based approach. Thus, IMOK cells can be transfected by siRNAs leading to efficient suppression of endogenous gene expression.

Cell lines are valuable tools to study transcriptional activity of genes by examining regulatory regions such as promoters. Often, these regulatory regions are active only in a specific cell type and in specific growth and differentiation conditions. We examined the promoter activity of two markers of undifferentiated, proliferative keratinocytes, p63 and K14. Similar to our published data with epidermal keratinocytes (24,25), both promoters were highly active in IMOK cells (Figure 5D). To further validate the utility of the IMOK cells in transient transfection analyses, we transfected these cells with a construct containing ~800 bp of the human K4 promoter linked to a luciferase reporter. The K4 promoter was minimally active in IMOK cells cultured in low calcium medium (Figure 5D). With increased calcium in the extracellular medium, there was a 20-fold increase in K4 promoter activity (Figure 5D). We also tested the activity of the mouse Elf5 promoter in IMOK cells. Elf5 is an epithelial cell specific transcription factor and we have previously shown that the mouse Elf5 promoter is active in skin keratinocytes and that its activity is further stimulated (20–30 fold) by calcium-induced differentiation (23). We observed a similar enhancement of Elf5 promoter activity in oral keratinocytes within 24 hours of differentiation with elevated calcium in the culture medium (Figure 5D). Thus, IMOK cells are highly transfectable using lipid-based transfection protocols and moreover the cells exhibit appropriate activity of differentiation-specific epithelial promoters under low and high calcium conditions.

Discussion

Our knowledge of the molecular pathways that govern development and differentiation of the oral epithelium has benefited tremendously from knockout and transgenic mice models. However, it would be useful to complement these studies with biochemical assays using murine oral epithelial cell lines. Yet only a few reports describe the establishment of mouse oral epithelial cells and these cell lines are not in widespread use. The small size of the mouse oral cavity imposes a limitation on the numbers of oral keratinocytes that can be easily isolated for primary cultures. In addition, the primary cultures undergo fairly rapid replicative crisis and hence can be propagated for only a few passages. These limitations have restricted the use of primary murine oral keratinocytes.

In contrast to the relatively few studies describing primary mouse oral keratinocytes or oral keratinocyte cell lines, a large number of studies have been published using primary human or rat oral keratinocytes or established human or rat oral keratinocyte cell lines derived from oral squamous cell tumors. Moreover, recently human oral keratinocytes harboring the hTERT gene have been shown to have an extended replicative life-span without disruption of their normal differentiation program (33). Although these studies have been very valuable in determining the biological responses of oral keratinocytes, there may be species-specific differences in responses of mouse, rat or human oral keratinocytes to particular experimental manipulations or culture conditions. Thus, it would be useful to have additional mouse oral keratinocyte cell lines for experimental analysis.

We report the derivation of a long-term oral keratinocyte culture of mouse origin (designated IMOK cells). IMOK cells are not derived from an oral tumor and have not been transformed by any viral oncogenes. However, they have the ability to proliferate continuously in culture for many generations and exhibit many features of normal oral epithelium *in vivo*. Karyotype analysis of IMOK cells confirms that they are of mouse origin and that they have a nearly diploid XX karyotype. The fact that the IMOK cell line is near diploid is a surprising finding since most established keratinocyte cell lines are reported to be tetraploid or nearly so (29–31). It is thought that this increase in chromosome number may provide an underlying gene dosage effect necessary for immortalization. This need may have been obviated in the IMOK cells by the modest aberrations and translocations that we observe for some of the chromosomes.

We have grown and propagated IMOK cells in a custom-formulated low calcium medium, which is identical to media widely used to grow mouse epidermal keratinocytes (11). Under these culture conditions, the proliferative capacity and the differentiation potential of the IMOK cells do not seem to vary over a wide range of passage numbers (our unpublished data). Interestingly, IMOK cells can also be grown in commercially available serum-free media, a useful feature since it will allow other investigators to grow these cells with ease and provide an opportunity to investigate biological processes without complicating effects associated with serum-derived growth factors.

Our analysis of IMOK cells has revealed that under proliferative conditions, these cells exhibit characteristic regular, tightly packed, polygonal cobblestone appearance. This is accompanied by high expression levels of basal markers such as transcription factor p63 in the nucleus and intermediate filament protein K14 in the cytoplasm. Upon increase in the calcium concentration in the medium, IMOK cells undergo differentiation. This is characterized by the establishment of cell–cell contacts due to assembly of adherens junctions as judged by Ecadherin staining (data not shown) and upregulation of a variety of markers that are associated with the spinous and granular layer of the oral epithelium, such as K4, K13, involucrin and filaggrin. Importantly these changes associated with calcium elevation in the medium induced differentiation in our mouse oral keratinocytes are consistently observed both in early as well as late passages.

We also demonstrate that IMOK cells are amenable to a variety of biochemical and molecular manipulations including transient transfection, siRNA-based gene knockdown and retroviral infection. The high level of activity of both the basal and differentiation-specific promoters suggest that this cell line can be utilized effectively for testing keratinocyte specific regulatory elements and for identifying and characterizing nuclear factors that interact with such elements. In summary, the establishment of the IMOK cell line will serve as a valuable *in vitro* cell culture based tool to test several hypotheses in the context of oral biology.

Abbreviations used

K1, keratin 1
 K4, keratin 4
 K6, keratin 6
 K10, keratin 10
 K13, keratin 13
 PBS, phosphate-buffered saline
 siRNA, small interfering RNA
 SKY, spectral karyotyping
 GAPDH, glyceraldehyde-3 phosphate dehydrogenase
 BSA, bovine serum albumin
 PVDF, polyvinylidene fluoride
 TBST, Tris-buffered saline with Tween
 DAPI, 4',6-diamidino-2-phenylindole
 IRES, internal ribosomal entry site
 GFP, green fluorescent protein

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Figure 1. IMOK cells exhibit a typical epithelial cobblestone morphology and undergo morphological changes in response to calcium
(A) Cobblestone morphology of IMOK cells cultured in low calcium medium. (B) Upon addition of calcium to the medium, IMOK cells become more flattened and the cell-cell borders become less distinct. Low and high (boxed region) magnification is shown for each condition.

Figure 2. Karyotype Analysis of IMOK Cells

IMOK cells were subjected to spectral karyotyping (SKY analysis). Shown are the staining patterns of typical cells from the culture. Note trisomies of chromosomes 2, 5, 8, 18 and 19, monosomy of chromosome 14, an unbalanced translocation of chromosomes 1 and 3, a Robertsonian translocation of chromosomes 12 and 16 and an interstitial deletion of one copy of chromosome 4. The karyotype is XX (female).

Figure 3. IMOK cells express keratins present in normal oral epithelium

(A) Real-time RT-PCR analysis of expression of the keratin 4 (K4) and keratin 13 (K13) genes in undifferentiated and differentiated IMOK cells. The values obtained in undifferentiated cells were arbitrarily set to 1. Data were normalized to the expression of the house-keeping gene $\beta 2$ microglobulin. (B) Western blot analysis of the expression pattern of keratin 13 (K13) during the differentiation process. IMOK cells were cultured in low calcium medium or in medium supplemented with 0.5 mM or 1 mM calcium for 24 or 48 hours. Western blot was then carried out on extracts of the cells using antibodies to K13 or β -tubulin as a loading control.

Figure 4. IMOK cells exhibit appropriate differentiation-specific expression of marker proteins
Undifferentiated IMOK cells cultured in low calcium medium were stained to detect markers associated with the undifferentiated state including (A) Δ Np63, (B) keratin 14 (K14), (C) β 4 integrin (β 4-Int) and (D) keratin 6 (K6). In addition, the cells were counterstained with fluorescent phalloidin to detect the polymerized actin cytoskeleton (Act) (A and C) or with antibody to histone H1 (H1) (B and D) to detect the nuclei. (E) Differentiated IMOK cells cultured in the presence of 1 mM extracellular calcium for 24 hours were stained with fluorescent antibody to keratin 10 (K10) (E), involucrin (F) or filaggrin (G). The cells were counterstained with antibody to histone H1 (H1) to detect nuclei.

Figure 5. IMOK cells can be transiently-transfected and are amenable to viral infection and to gene-knockdown by siRNA

(A) IMOK cells were transiently transfected with the pCMVlacZ plasmid encoding *E. coli* β galactosidase. Thirty-six hours later, cells were stained with X-gal (blue) to detect transfected cells. (B) IMOK cells were infected with retrovirus (MIGR1) and analyzed by flow cytometry 48 hours later. GFP fluorescence in uninfected IMOK cells (shaded histogram) and IMOK cells infected with retrovirus (open histogram) is shown. (C) Western blot analysis of GAPDH levels in IMOK cells transiently transfected with siRNA against mouse GAPDH or with a control scrambled siRNA. The blot was stripped and reprobed with antibody against β -tubulin as an internal control. (D) IMOK cells were transiently transfected with reporter gene constructs containing the mouse Δ Np63 promoter, the mouse K14 promoter, the mouse Elf5 promoter or the human keratin 4 (K4) promoter linked to firefly luciferase (luc) or with the empty luciferase

vector (pGL3 Basic). As a control, the cells were co-transfected with a plasmid harboring the constitutively-active EF1 α promoter linked to Renilla luciferase (RLuc). Twenty-four hours after transfection, half of the cells were induced to differentiate by addition of 1 mM of calcium to the medium with the other remaining in low calcium medium. After an additional 24–30 hours, cells were harvested and assayed for Luc and RLuc activity. Results represent relative luciferase values where the activity of the empty vector (pGL3 Basic) has been arbitrarily set to 1.