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# 角膜上皮細胞の生体外での未分化能維持の研究

平成20年度　総括・分担研報告書

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総括研究報告書

# 角膜上皮細胞の生体外での未分化能維持の研究

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**研究要旨**

マウス角膜上皮未分化細胞の培養では、低細胞外カルシウムイオン濃度と無血清の培地で、200継代以上、また3年以上、細胞サイズ、形態、未分化マーカーを維持して培養が可能であり、なおかつ継代培養によるコロニー形成能力の低下も認めなかった。

この細胞は、角膜上皮特異的タンパクであるケラチン12の発現をタンパク、RNAレベルで失っており、Pax6導入などによってもケラチン12の発現を誘導することはできなかった。 また100継代を過ぎた細胞の中にも自然発生的にケラチン12を発現する細胞集団が存在することがわかった。1%未満の細胞集団ではあるが、免疫染色では明らかに陽性を示したので、これらの細胞はケラチン12を発現する能力は失っていないと考えた。最近、こういった遺伝子発現にエピジェネティックな制御が関わっているという報告があり、我々もトリコスタチンAを用いて、実験を行った。その結果、容量依存的にRNAレベルでケラチン12の発現レベルの上昇を認めた。

異常分化の研究では、前年度、継代培養の際、細胞播種密度を高くすると、ParacrineなTGF-βの刺激により、Smadを介したシグナルの活性化により、細胞がEMTを起こし、αSMAとp63を同時発現する細胞が出現することがわかった。またこれにともない、ベータカテニンの細胞間から細胞質への移動、snailの発現上昇を認めた。 今後、Smadシグナリング、Wntシグナリングの阻害剤を用いることにより、各パスウェイがどれだけ関わっているのかを調べていく予定である。

また、海外アイバンクのドナー眼の角膜輪部組織を用い、ヒトでの角膜上皮細胞の生体外での未分化能維持に輪部の構造が影響しているかどうかを調べた。角膜上皮シートとして一塊に分離した輪部シート構造の中にはp63陽性の細胞塊が局在し、そのp63陽性の細胞塊を多く含む領域は、少ない領域と比較し、コロニー形成率が有意に高かった。

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**A. 研究目的**

現在、このマウス角膜上皮未分化細胞は150以上の継代を経てもそのクローナルに増殖し、p63とK14を強発現しコンパクトで敷石状の細胞形態は変化していない。p63とK14という重層上皮の基底細胞のマーカーが強陽性ということが示す用に、この細胞をヒトに臨床応用されている培養上皮シート作成の条件（羊膜上培養、SHEM、3T3をフィーダー細胞に使用、エアーリフティング）を適用すると、細胞は重層化し、マウス角膜培養上皮シートを作成することができた。ただ眼のマスター遺伝子であるpax6とケラチン12といった角膜上皮特異的ケラチンの発現を認めず、昨年度pax6を遺伝子導入した細胞もケラチン12を発現することはなかった。

しかし、自然発生的にケラチン12を発現する細胞群を認めることが免疫染色で確認された。そこで我々は、他にも報告があるように、この細胞においてエピジェネティックな修飾によりケラチン12の発現が制御されているのではないかと考え実験に至った。

また、海外アイバンクのドナー眼の角膜輪部組織を用い、ヒトでの角膜上皮細胞の生体外での未分化能維持に輪部の構造自体が影響しているかどうかを調べた。

**B. 研究方法**

1. 長期継代培養細胞のケラチン12染色
通常継代した200継代前後の培養細胞、ストックから起こした40、及び80継代前後の細胞とケラチン12染色を比較する。

2. TSA刺激実験
①. マウス角膜上皮未分化細胞の通常培養(100継代以上のもの)を通常培養する。
②. TSAを0, 10, 100, 300, 1000 nMの濃度で24時間培養液に添加し刺激する
③. RT-PCRでケラチン12の発現をみる
④. タンパクレベルでの発現を確認する

3. 異常分化の研究
①. マウス角膜上皮未分化細胞の通常培養(100継代以上のもの)を通常培養
②. TGF-β刺激(5ng/ml)48時間刺激する
③. 細胞を固定し、Smad3、βカテニンの免疫染色で核内移行があるか観察する
④. 時間経過でsnailなど、そのほかのEMT関連分子の発現を確認する

4. 角膜輪部上皮組織構造のニッチとしての役割の研究
①. アイバンク(海外)の輪部組織をDispase処理（14h、4度
②. 輪部組織から上皮シートを分離する
③. シートをそのまま培養皿に静置して48時間培養する。（コントロールとしてシートをcollagenase処理したシングルセルを用いる)
④. p63 (4A4) の免疫染色を行い、p63陽性細胞塊を観察する
⑤. p63陽性細胞塊の多い部分、少ない部分をレーザーマイクロダイセクションで切り出し、コロニー形成率を比較する。

**C. 研究結果**

1. 長期継代培養細胞のケラチン12染色
継代培養した細胞においても、細胞播種密度、培地を管理すれば、コロニー形成率（3-5%）、小細胞サイズ、敷石状形態を保つマウス角膜上皮由来の未分化細胞を維持可能であった。40、80、200継代前後の各培養細胞ではケラチン12の発現は1%未満の細胞で認めた。し

2. TSA刺激実験
上記でケラチン12陽性細胞の存在を確認した。ケラチン12陰性の細胞もケラチン12を誘導発現することができるか、をみるため、TSAで刺激した。300, 1000 nMの濃度でRNAレベルのケラチン12発現上昇を認めた。

3. 異常分化の研究

マウス角膜上皮未分化細胞をTGF-βで刺激(5ng/ml)48時間刺激することにより、Smad3、βカテニンの細胞間から細胞質への移行、また核内にも移行する染色像が観察された。またsnailといったEMT時に上昇する分子も上昇が確認された。

4. 角膜輪部上皮組織構造のニッチとしての役割の研究

海外アイバンクのドナー眼の角膜輪部組織を用い、ヒトでの角膜上皮細胞の生体外での未分化能維持に輪部の構造が影響しているかどうかを調べた。角膜上皮シートとして一塊に分離した輪部シート構造の中にはp63陽性の細胞塊が局在し、そのp63陽性の細胞塊を多く含む領域は、少ない領域と比較しコロニー形成率が有意に高かった。

## D. 考察

他の角膜上皮細胞株でもケラチン12の発現はなく、Primary Cultureでは数代の継代後、ケラチン12の発現がなくなること、それらにはエピジェネティックスが関与しているとの報告もあり、この細胞にも培養の環境変化によるDNAのメチル化やヒストンのアセチル化による影響が働いていると考え、トリコスタチンAなどの薬剤を用いて、異常なメチル化を抑制することにより、ケラチン12の発現を誘導できた。このことから、この細胞はケラチン12の発現を誘導できる細胞であることがわかった。

またこの細胞をTGF-βで刺激することにより、Smad3、βカテニンの細胞間から細胞質への移行、また核内にも移行する染色像が観察されたことから、角膜上皮未分化細胞が生体内で強い炎症下では、筋線維芽細胞に分化し組織繊維化の一端を担う可能性が示唆されるが、結論づけるには、さらに研究が必要である。

角膜輪部上皮組織構造のニッチとしての役割の研究から、角膜輪部上皮の特殊なしわ状構造の中にp63陽性の細胞塊が存在し、その部分がコロニー形成率の高い細胞を含む、すなわち幹細胞に近い性質をもとことがわかった。その構造を崩すことにより(細胞をシングルセル化する)、生体外での細胞増殖は早まるが、細胞老化を促進しているのかもしれない。これも、臨床応用に向けて、培養方法をまだまだ改善する余地があると考えられる。

## E. 結論

1. マウス角膜上皮未分化細胞のParacrineなTGF-βによるEMTは、Wnt, Smadの活性化を介していた。

2. マウス角膜上皮未分化細胞株のケラチン12の発現が陰性になったのは、エピジェネティックな修飾によるものであることがわかった。

3. ヒト輪部角膜にはp63陽性細胞塊が含まれており、そのp63陽性細胞塊に幹細胞が含まれている可能性がある。この構造を維持することが未分化性維持、細胞老化遅延に関係するかもしれない。

## F. 健康危険情報

なし

## G. 研究発表

1)論文発表

1. Ma X, Shimmura S, Miyashita H, Yoshida S, Kubota M, Kawakita T, Tsubota K. Long-Term Culture and Growth Kinetics of Murine Corneal Epithelial Cells Expanded from Single Corneas. Invest OphthalmolVis Sci. In print.
2. Omoto M, Miyashita H, Shimmura S, Higa K, Kawakita T, Yoshida S, McGrogan M, Shimazaki J, Tsubota K. The use of human mesenchymal stem cell-derived feeder cells for the cultivation of transplantable epithelial sheets. Invest OphthalmolVis Sci. In print.
3. Miyashita H, Shimmura S, Higa K, Yoshida S, Kawakita T, Shimazaki J, Tsubota K. A novel NIH/3T3 duplex feeder system to engineer corneal epithelial sheets with enhanced cytokeratin 15-positive progenitor populations. Tissue Eng Part A; 7. 1275

2)学会発表

なし

## H. 知的所有権の出願・取得状況(予定を含む。)

1)特許取得

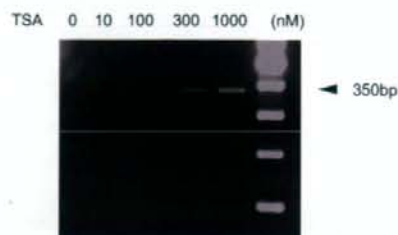
なし

2)実用新案登録

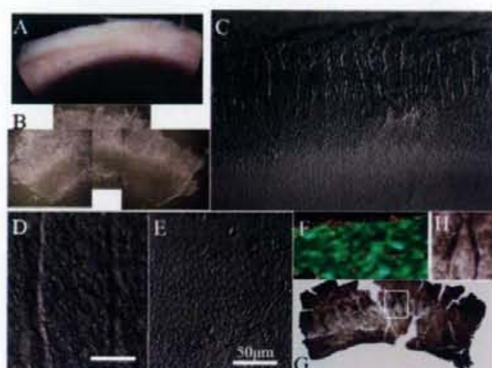
なし

3) その他

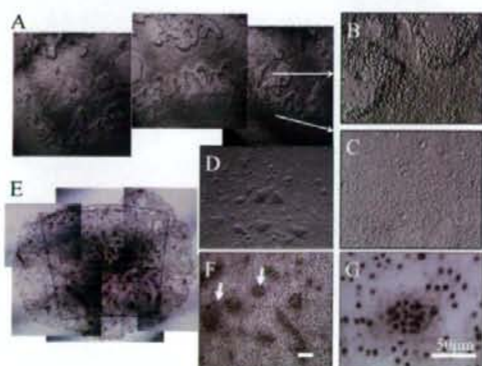
なし



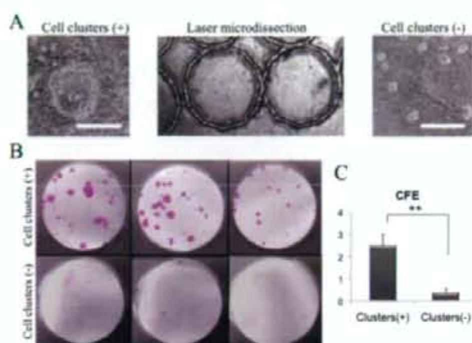

0-1000nMのトリコスタチンA刺激により、ケラチン12の発現がRT-PCRで確認された。またその発現は用量依存的に増える傾向にあった。



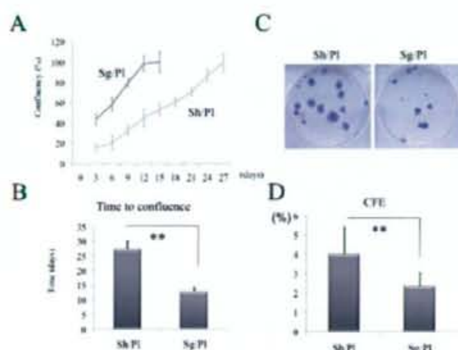

Ａ：角膜輪部組織
Ｂ，Ｃ：分離した輪部上皮シート
Ｄ：シートのしわ状構造
Ｅ：角膜周辺の上皮構造
Ｆ：このシートの細胞はViabilityもよく、シートのままｐ63の抗体で免疫染色すると、しわの部位に一致して濃染し、（Ｇ）その部位を拡大すると、ｐ63陽性細胞が密になっていた。



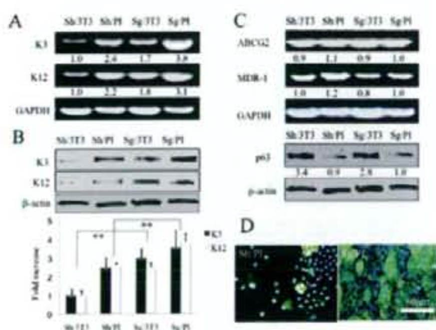

Ａ：輪部上皮シートを培養すると、しわ状構造が崩れ、Ｂ，Ｃのような構造になる。もう少し（３days）培養すると、細胞塊（Ｄ）が出現し、これらはｐ63陽性（Ｆ）の小細胞の集団であった。（Ｇ）




細胞塊が豊富な場所、近接するが細胞塊の内場補をレーザーマイクロダイセクションで切り出し（A）、コロニー形成率を比較すると、明らかに細胞塊を含むほうがコロニー形成率は高かった。（B, C）




細胞塊を崩して培養した方が、細胞塊を維持して培養するより、増殖が早い（A, B）一方、コロニー形成能が落ちていくのも早かった。（C, D）




ケラチン3, 12といった分化マーカーは細胞塊を維持して培養したほうが、発現は低かった（A, B、D）。
未分化マーカー（p63、ABCG2）は細胞塊よりも3T3フィーダー細胞の有無に左右された。（C）

厚生労働科学研究費補助金(再生医療等研究事業)
分担研究報告書

# 角膜上皮細胞培養条件の研究

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**研究要旨**

マウス角膜上皮細胞はDispaseで角膜上皮シートを分離し、Trypsinで細胞をシングルセルの状態に持って行き、低カルシウム無血清培地にて培養皿で培養する方法を我々が報告したが、また細胞数を集めるのに、多くのマウスが必要名上、成功する確率は20%程度と、決して高くはなかった。 そこで、マウスの角膜組織片培養とコレラトキシン添加による培養において、50%程度の確率で細胞を継代できるようになった。

コロニー形成率、細胞形態、および増殖曲線で細胞増殖能と未分化細胞の評価を行った。分化に対する影響は、角膜上皮未分化マーカー、角膜上皮分化マーカーで評価を行った。その結果、この培養方法での角膜上皮細胞は、未分化能を維持している可能性が示唆された。この方法で増殖させた細胞でより未分化細胞を含んだ時重層化上皮シートが作成できるならば、ヒト臨床応用へ実現できる可能性は高い。

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分担研究報告書

角膜上皮細胞の培養条件の研究

分担研究者 榛村重人
慶應義塾大学医学部眼科学教室 准教授

**A.研究目的**

より効率よくマウスより角膜上皮細胞培養を可能にするため、角膜ひとつからの培養系を確立するため、改めて培養条件の検討を行った。

**B.研究方法**

マウス摘出眼球において、Dispase処理を施行し、上皮細胞シートを単離し、低カルシウム無血清培地にて器官培養を施行した。

培地にコレラトキシンを用いる、用いないでコロニー形成率、および増殖曲線で細胞増殖能と未分化細胞の評価を行った。

また、細胞増殖が停止するまで継代を行い、細胞集団倍加数を求めた。分化に対する影響は、角膜上皮未分化マーカー(K15, p63)、角膜上皮分化マーカー(K3,Involucrin)のRT-PCR,Western blot,および免疫染色で評価を行った。

**C.研究結果**

コレラトキシン追加、角膜組織片培養から培養することにより、マウス角膜ひとつから高い効率で継代培養することが可能であった。
この培養条件での細胞における角膜上皮未分化マーカー(K15, p63)の発現を維持していた。

**D.考察**

これらの結果から、角膜組織片培養、コレラトキシン併用により、未分化細胞培養においては、より効率よく増殖能の高い細胞を維持させることができる可能性がある。
この方法で用いた細胞でより未分化細胞を含んだ時重層化上皮シートが作成できるならば、ヒト臨床応用へ実現できる可能性は高い。

**E.結論**

角膜組織片培養、コレラトキシン併用によりマウス角膜輪部上皮細胞の増殖を促進し、また分化を抑制した。低カルシウム、無血清培地と組み合わせることで、より少ない細胞数から多くの未分化細胞を継代することが可能となった。

**F.健康危険情報**

なし

**G.研究発表**

1)論文発表

4. Ma X, Shimmura S, Miyashita H, Yoshida S, Kubota M, Kawakita T, Tsubota K. Long-Term Culture and Growth Kinetics of Murine Corneal Epithelial Cells Expanded from Single Corneas. Invest OphthalmolVis Sci. In print.
5. Omoto M, Miyashita H, Shimmura S, Higa K, Kawakita T, Yoshida S, McGrogan M, Shimazaki J, Tsubota K. The use of human mesenchymal stem cell-derived feeder cells for the cultivation of transplantable epithelial sheets. Invest OphthalmolVis Sci. In print.
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2)学会発表

なし

**H. 知的所有権の出願・取得状況(予定を含む。)**

1)特許取得

なし

2)実用新案登録

なし

3) その他

なし

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分担研究報告書

# 角膜上皮シート作成条件の研究

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これまで培養上皮細胞シートの3T3フィーダー細胞は1層の細胞のみであり、上皮はその3T3フィーダーと接触するか、または隔離されるか、のいずれかで培養がなされてきた。前者では、上皮の増殖と共に3T3フィーダー細胞が減少し、後者では、上皮が3T3フィーダーと接触することによるNiche効果を享受できない。そこで3T3フィーダー層を2層用いたDuplex培養法を開発し、比較した。Duplex培養法は、上皮細胞のコロニー形成率が他の2法と比較して有意に高く、培養上皮シートの重層化、ケラチン発現パターンもより輪部に近いようなパターンをとっており、質の高い上皮シートができたと言える。3T3細胞ほどは効果がないと報告があるヒト由来各種フィーダー細胞を用いても、Duplex培養法を用いることにより、質の高いシートを作成できる可能性がある。

## A.研究目的

これまで培養上皮細胞シートのフィーダー細胞は1層の細胞のみであり、上皮はそのフィーダーと接触するか、または隔離されるか、のいずれかで培養がなされてきた。前者では、上皮の増殖と共にフィーダー細胞が減少し、後者では、上皮がフィーダーと接触することによるNiche効果を享受できない。そこでフィーダー層を2層用いたDuplex培養法を開発し、比較した。

## B.研究方法

6ウェルプレートに培養用インサートを準備し、3T3フィーダー細胞を

1. インサート内のみ
2. ウェル内のみ
3. インサート内、ウェル内両方(Duplex)

に播種した。

その後、海外アイバンク由来のヒト輪部上皮細胞を準備し、インサート内に同密度で播種した。コロニー形成率、及び上皮面積を計測した。

また、各方法にて、エアーリフト法を用いて重層上皮シートを作成し、分化マーカー(ケラチン3, 12)、未分化マーカー(ケラチン15)で免疫染色を施行した。

またこのシートからの2次コロニー形成率を測定した。

## C.研究結果

各方法におけるコロニー形成率はインサート内のみが0.2%、ウェル内のみが0.2%、Duplex培養法が1.1%であった。2次コロニー形成率も、インサート内のみが2.3%、ウェル内のみが1.4%、Duplex培養法が4.1%、といずれも、Duplex培養法が高かった。

これは培養表面に占める上皮面積の測定でもDuplex培養法が最も優れていた。

## D.考察

2次コロニー形成率がいずれも増えていることから、組織にコミットした増殖性の高い細胞が増えていると考えられた。今後、動物由来の細胞などを用いないようにしていくには、このDuplex培養法は、臨床応用での培養シート作成方法の選択肢の一つと考えることもできるかもしれない。

## E.結論

インサート内、ウェル内両方にフィーダー細胞を播種する方法(Duplex培養法)を用いることにより、より増殖能の高い細胞を維持培養できた。またこの方法で培養上皮シートを作成することにより、より質の高い培養上皮シートを作成可能であった。

## F.健康危険情報

なし

## G.研究発表

1)論文発表

1. Omoto M, Miyashita H, Shimmura S, Higa K, Kawakita T, Yoshida S, Mc Grogan M, Shimazaki J, Tsubota K.The use of human mesenchymal stem cell-derived feeder cells for the cultivation of transplantable epithelial sheets. Invest OphthalmolVis Sci. In print.
2. Miyashita H, Shimmura S, Higa K, Yoshida S, Kawakita T, Shimazaki J, Tsubota K. A novel NIH/3T3 duplex feeder system to engineer corneal epithelial sheets with enhanced cytokeratin 15-positive progenitor populations. Tissue Eng Part A; 7. 1275

2)学会発表

なし

## H. 知的所有権の出願・取得状況(予定を含む。)

1)特許取得

なし

2)実用新案登録

なし

3) その他

なし

別紙４

研究成果の刊行に関する一覧表

雑誌

| 発表者氏名 | 論文タイトル名 | 発表誌名 | 巻号 | ページ | 出版年 |
|---|---|---|---|---|---|
| Kawakita T, Shimmura S, Higa K, Espana EM, He H, Shimazaki J, Tsubota K, Tseng SC. | Greater Growth Potential of p63-positive Epithelial Cell Clusters Maintained in Human Limbal Epithelial Sheets | Invest Ophthalmol Vis Sci. | In print | | 2009 |
| Ma X, Shimmura S, Miyashita H, Yoshida S, Kubota M, Kawakita T, Tsubota K. | Long-Term Culture and Growth Kinetics of Murine Corneal Epithelial Cells Expanded from Single Corneas. | Invest Ophthalmol Vis Sci. | In print | | 2009 |
| Omoto M, Miyashita H, Shimmura S, Higa K, Kawakita T, Yoshida S, McGrogan M, Shimazaki J, Tsubota K. | The use of human mesenchymal stem cell-derived feeder cells for the cultivation of transplantable epithelial sheets. | Invest Ophthalmol Vis Sci. | In print | | 2009 |
| Miyashita H, Shimmura S, Higa K, Yoshida S, Kawakita T, Shimazaki J, Tsubota K. | A novel NIH/3T3 duplex feeder system to engineer corneal epithelial sheets with enhanced cytokeratin 15-positive progenitor populations. | Tissue Eng Part A | 7 | 1275 | 2008 |
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# Stratified epithelial sheets engineered from a single adult murine corneal/limbal progenitor cell

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## Abstract

The limbal region of the adult cornea contains stem cells which are ultimately responsible for regeneration of the corneal epithelium during wound repair. However, primarily-isolated murine corneal/limbal epithelial cells rapidly senesce on plastic in a serum-free low [$Ca^{2+}$] medium, suggesting only transit amplifying cells are promoted. We developed a novel expansion method by seeding at a low cell density (<500 cells/$cm^2$) and prolonging each culture time beyond the lifespan of transit amplifying cells (4 weeks). Expanded cells were uniformly small, negative to K12 keratin, but positive for p63 nuclear staining, and could be subcultured beyond 100 passages. After limiting dilution, one clone (TKE2) was selected that exhibited single cell clonal expansion with a doubling time of 34.2 hrs, and had normal karyotyping, but no anchorage-independent growth. A single cell could be continually expanded to a confluent monolayer on denuded amniotic membrane and became stratified by exposing to the air-medium interface. The resultant stratified epithelium expressed K14 keratin, involucrin, connexin 43 and p63, but not K12 keratin or Pax 6. However, expression of K12 could be up-regulated by increasing extracellular calcium concentration and addition of foetal bovine serum (FBS) at P12, but less so at P85. Therefore, this murine limbal/corneal epithelium-derived progenitor cell line still retained the plasticity for adopting corneal lineage differentiation, could be useful for investigating limbal niche cues that may promote corneal epithelial fate decision.

**Keywords:** cornea • epithelium • stem cell • regenerative medicine • culture • senescence and growth

## Introduction

Stem cells (SCs) with extensive proliferative potential are crucial for maintaining the homeostasis of a given tissue. Although SCs hold considerable promise for treating a number of diseases in regenerative medicine, availabilityof SCs in sufficient quantities remains a key obstacle to overcome, and until now has relied on *ex vivo* expansion, which is a task dependent on isolation, preservation and proliferation of SCs in an *in vitro* environment.

The corneal epithelium is unique in that its SCs are exclusively located in the basal layer of the limbus (between the cornea and the conjunctiva), while transit amplifying cells (TACs) are located in the basal to suprabasal layers of the limbal epithelium and the entire corneal epithelium. [1] This unique anatomic enrichment at the limbus allows one to gain an easy access to these adult somatic SCs [2], which have the smallest cell size [3] and a long cell cycle [4], do not express K3/K12 keratins [1, 5] and connexin 43 [6], but preferentially express p63 [7], Bcrp1/ABCG2 [8] or N-cadherin [9]. As a result, the SC-containing limbal epithelium has higher clonogenecity on 3T3 fibroblasts feeder layers [10, 11].

Despite a variety of transgenic mice have been available, studies of murine limbal/corneal epithelial SCs have met a greater challenge. Toward this goal, we reported a method to successfully isolate viable mouse corneal/limbal epithelial sheets, of which subsequent growth and differentiation is greatly influenced by extracellular calcium concentration ([$Ca^{2+}$]) and the presence of foetal bovine serum (FBS). [12] Even if cultured at a high density in keratinocyte serum-free defined medium (KSFM) containing 0.07 mM [$Ca^{2+}$] and supplemented with growth-promoting agents, cells reached confluence in 1 week and could only be subcultured at 1:3 splits for up to 2–3, suggesting only TACs were expanded [12].

#Proprietary Interest: SCGT and TK have filed a patent based on this work.

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**Table 1** Sources of primary antibodies

| Antigens | Category | Clone | Dilution | Method | Source |
|---|---|---|---|---|---|
| PCNA | Mouse monoclonal | PC10 | 1:50 | IHC | DAKO* |
| p63 | Mouse monoclonal | 4A4 | 1:50 | IHC | DAKO |
| Pan-cytokeratin | Mouse monoclonal | Mixed‡ | 1:100 | IF | Sigma** |
| Cytokeratin K12 | Goat polyclonal | L15 | 1:20 | IF | SantaCruz† |
| Cytokeratin K14 | Mouse monoclonal | B429 | 1:100 | IF | Abcam†† |
| Pax6 | Rabbit polyclonal | NA | 1:100 | IF | Chemicon‡‡ |

‡Mixed clone: C-11, PCK-26, CY-90, KS-1A3, M20 and A53-B/A2.
*Carpinteria, CA. **St. Louis, MO. †Santa Cruz, CA. ††Cambridgeshire, UK. ‡‡Temecula, CA.

Herein, we demonstrated that it was possible to preferentially encourage expansion of limbal epithelial progenitor cells, characterized by small cell size, negative K12 keratin expression, and strong nuclear p63 expression, when the culturing time was extended to 4 weeks (*i.e.* beyond the TAC's lifespan) and when the seeding density was lowered to minimize any paracrine influence from TACs. As a result, clonal initiation and continuous expansion was achieved for more than 100 passages. Such expanded progenitor cells exhibited single cell clonal growth, could be used to engineer a stratified epithelium, and upon increasing extracellular calcium concentration and adding FBS a small proportion of cells expressed K12 keratin. The significance of this as-yet-unrecognized culturing method to isolate and expand murine limbal/corneal progenitor cells is discussed.

# Materials and methods

## Reagents

Tissue culture plastic wares were purchased from Becton Dickinson (Lincoln Park, NJ, USA). Amphotericin B, Dulbecco's modified Eagle's medium (DMEM), F-12 nutrient mixture (F12), Defined Keratinocyte-SFM (KSFM), FBS, phosphate-buffered saline (PBS), TripLE® and 0.25% trypsin/1 mM ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco-BRL (Grand Island, NY, USA). Dispase II powder was from Roche (Indianapolis, IN, USA). Other reagents and chemicals including bovine serum albumin (BSA), cholera-toxin, dimethyl sulfoxide, hydrocortisone, insulin, mouse epidermal growth factor (EGF), sorbitol, Hoescht 33342 and fluorescein-conjugated (FITC) secondary antibodies were from Sigma (St. Louis, MO, USA). Optimal cutting temperature (OCT) compound was from Sakura Finetek (Torrance, CA, USA). Isotype mouse IgG1 and rabbit IgG were purchased from Dako Cytomation and Jackson ImmunoResearch Laboratories (West Grove, PA, USA), respectively. Rhodamine-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories and Chemicon International Inc. Live/Dead Assay® was from Molecular Probes (Eugene, OR, USA). Penicillin and streptomycin was from Wako (Osaka, Japan). The SV total RNA isolation system was from Promega (Madison, WI, USA). Avian myeloblastosis virus (AVM) reverse transcriptase XL was from Takara, Bio (Shiga, Japan). All primary antibodies used in this study are summarized in Table 1.

## Isolation of murine corneal/limbal epithelial sheets

CD-1 albino mice of more than 3 weeks old (Charles River, Boston, MA, USA) were handled according to the Association for Research in Vision and Ophthalmology (ARVO) guidelines for animal care. Mouse corneal/limbal epithelial sheets were isolated in the same manner as recently reported [12]. In brief, more than 200 eye globes were enucleated by forceps, washed profusely in PBS, stored in KSFM and then transported at 4°C within 24 hrs to the laboratory. These eyes were digested at 4°C for 18 hrs in KSFM containing 15 mg/ml dispase II and 100 mM sorbitol. KSFM contained 0.07 mM $[Ca^{2+}]$ and was supplemented with 10 ng/ml EGF and $102^{-10}$ M cholera toxin. Subsequently, each mouse eye was held in place by suction applied to the posterior pole using a transfer pipette and was gently shaken in KSFM to loosen the ocular surface epithelial sheet.

## Culture manipulation

Single cells obtained from the above corneal/limbal epithelial sheets by 0.25% trypsin/1 mM EDTA in HBSS for 10 min. followed by vigorous pipetting were seeded at a density of 20,000 cells per $cm^2$ on plastic containing KSFM. In 1 week, cells reached confluence and were subcultured by trypsin/EDTA at 1:3 split to Passage 1 (P1) cultures. At this point, cells were subcultured at 1:3 split either in 1 week as previously reported [12] or in 4 weeks, that is, 3 weeks beyond confluence. Cells subcultured in the latter manner could continually be passaged, and at P3, the average cell size ($\mu m^2$) was monitored by phase contrast micrography weekly for 100 randomly selected cells using Image J (NIH, Bethesda, MD, USA), and the total cell number was determined in triplicate by haemocytometry during the 4 week course. At P4, cells were also seeded at a density of 500, 5000 or 50,000 cells per $cm^2$ and cultured for 4 weeks ($n = 5$). Cell viability was measured by Live/Dead Assay®, and Hoescht 33,342 staining.

## Immunostaining

To determine the cornea-type epithelial differentiation, immunofluorescence staining to K12 keratin was performed as previously reported [12].



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**Table 2** Primers used for RT-PCR

| Primer | Sequence (5′ → 3′) | Product size (bp) |
|---|---|---|
| Keratin 10 | GGCTCTGGAA GAATCAAACT ATGAGC | 167 |
| | GGATGTTGGC ATTATCAGTT GTTAGG | |
| Keratin 12 | CGGGAGTGGTATGAAGCA | 188 |
| | CATTCTGAAGTCGTCGGC | |
| Keratin 14 | CCCCTCCACGTGGAGATTCA | 1417 |
| | CCTGCAGATGGATAAGAGGG | |
| Pax 6 | AGTTCTTCGC AACCTGGCTA | 500 |
| | TGAAGCTGCT GCTGATAGGA | |
| Involucrin | CAGGACATGCTAGTACCACAGG | 883 |
| | GTGTCCGGTTCTCCAATTCGTG | |
| Connexin 43 | CCTTCTTGCTGATCCAGTGGTAC | 154 |
| | ACCAAGGACACCACCAGCAT | |
| GAPDH | ACCACAGTCCATGCCATCAC | 452 |
| | TCCACCACCCTGTTGCTGTA | |

To determine the status of epithelial progenitor cells including SCs, we performed immunohistochemistry to p63 using clone 4A4, which recognizes all six p63 isotypes [13], similar to what has been reported by Pellegrini *et al.* [7] Immunostaining to detect nuclear expression of proliferating cell nuclear antigen (PCNA) was used to evaluate the proliferative potential. Immunofluorescence staining to K14 keratin and Pax 6 besides aforementioned marker p63 and K12 keratin was also performed in stratified epithelial sheets generated from a single cell. Substitution of primary antibody with PBS served as negative controls. Images were photographed with a NikonTE-2000U Eclipse epi-fluorescent microscope (Nikon, Tokyo, Japan).

## Clonal Assay in KSFM and on 3T3 feeder layers

To determine whether the newly devised cultivation method of a prolonged culturing time and a lower seeding density could maintain SCs, we seeded primary and cells subcultured to P4, P8 and P12 at a density of 40 cells per cm$^2$ in KSFM for 4 weeks. The clonal growth visualized by crystal violet staining, colony forming efficiency, colony size and cell sizes in the central and peripheral area of the colony were analysed and compared in triplicate to those established by seeding at the same density on mitomycin C-treated 3T3 feeder layer as previously described [14].

The doubling time of cells was measured by counting the asynchronously growing cells at day 7.

## Soft agar colony assay

To determine whether expanded cells were transformed, P23 cultures were trypsinized and washed to generate single cell suspensions and seeded as $1 \times 10^3$ cells/ 24-well flat-bottomed plates using a two-layer soft agar system in a volume of 1000 μl/well as previously described [15]. Clonal growth was compared to that of 3T3 fibroblasts as the negative control and that of a retinoblastoma cell line (YB67) (kindly provided by Dr. Chia-Yang Liu, Cincinnati, OH) as the positive control.

## Engineering of stratified epithelial sheets from a single cell

P20 cells were subjected to limiting dilutions in order to achieve single cell clonal growth using 96 wells in KSFM. One of these clones, designated as TKE2, was treated by TripLE® for 10 min., rendered into single cells, and cultured in KSFM on EDTA-denuded amniotic membrane fastened to a culture insert as reported [16]. The culture was submerged in KSFM until confluence, switched from KSFM for 1 day to the supplemental hormonal epithelial medium (SHEM), made of equal volumes of DMEM/F12 containing bicarbonate, 10 ng/ml human EGF, 5 μg/ml insulin, 100 ng/ml cholera toxin, 15% FBS, 70 μg/ml penicillin and 140 ng/ml, and exposed to the air-medium interface for 1 week with

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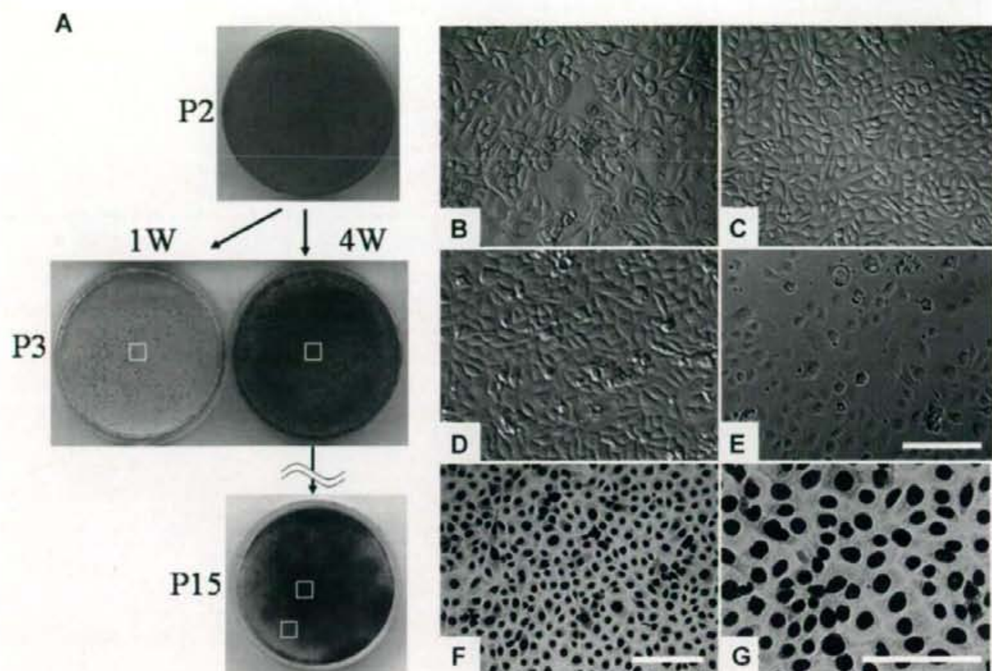


**Fig. 1** Growth potential promoted by prolonging the culturing time. Primary (P0) cells seeded at a high density of 20,000 per cm$^2$ reached confluence in 1 week in keratinocyte serum-free defined medium (KSFM). When subcultured at 1:3, cells at Passage 1 (P1) became confluent in 1 week, and similarly subcultured to P2 for 1 week (1 W) revealing a mixture of small and large cells **(B)**. In contrast, cells were predominantly small if cultured for 4 weeks (4 W) before subculturing **(C)**. P2/4 W cultures subcultured at 1:3 did not reach confluence in 1 W (**A**, P3/1 W), but reached confluence in 4 W (**A**, P3/4 W). P3/1 W cultures could not be subcultured. In contrast, P3/4 W cultures could continually be subcultured if each culture was grown for 4 weeks (**A**, 4 W). Their growth was more clonal after P5. Cells in the centre of the clone (**A**, P15, inset) were uniformly small and compact **(D)**, while cells in the periphery (**A**, P15, inset) were also small but less compact **(E)**. Nuclear staining to PCNA was uniformly positive in more than 95% of P15 cells (**F** and **G**). Bars represent 100 μm.

mitomycin C (MMC)-treated 3T3 fibroblasts feeder layers pre-seeded on the plastic to promote stratification.

## RT-PCR

Total RNA was isolated from expanded cells, stratified epithelial sheets, mouse skin and mouse corneal epithelium using the SV total RNA isolation system according to the manufacturer's recommendations, and generated cDNA using oligo(dT) priming and AVM reverse transcriptase XL by incubation of a 25 μl mixture at 41°C for 1 hr. RT-PCR was performed by containing oligonucleotide primers specific to each gene (Table 2) in 1 μl cDNA in a total reaction volume of 50 μl and amplified at 95°C for 30 sec. at 53°C for 30 sec. at 72°C for 20 sec. (20 cycles) using the Takara EX Taq DNA polymerase (Takara). Using glyceraldehyde-3-phosphate dehydrogenase (GADPH) as an internal control, PCR amplified products were separated by electrophoresis on a 1.5% agarose gel. Table of used primers.

# Results

## Prolonged culturing time preferentially preserved small epithelial cells

As reported previously [12], primary cultures (P0) seeded at 20,000 cells per cm$^2$ in KSFM reached confluence in 1 week. When subcultured at 1:3 splits, passage 1 (P1) cells reached



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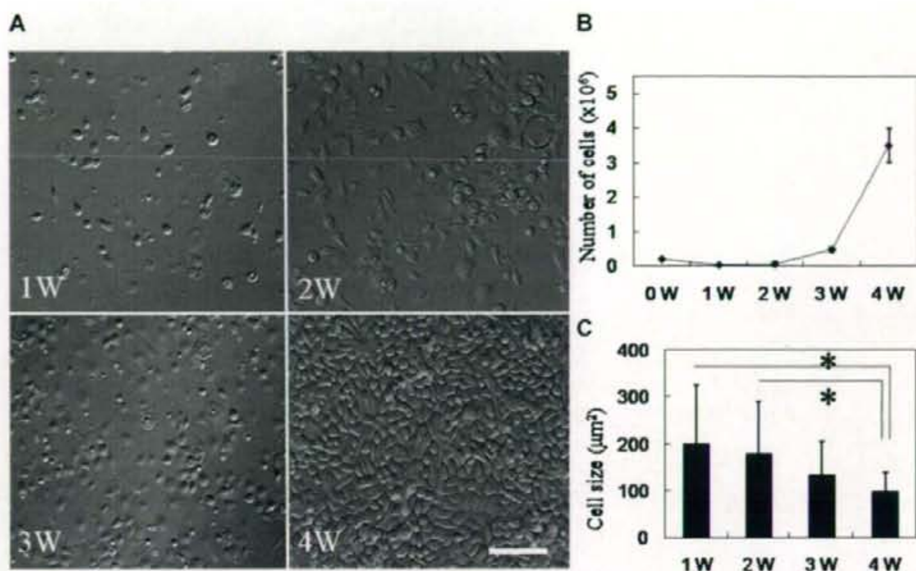


**Fig. 2** Cell morphology and size affected by prolonging the culturing time. In P3 cultures, cells were mostly of an intermediate size with large squamous cells and 30–40% confluence at one week (**A**, 1 W), and mostly large squamous cells and 40–50% confluent at 2 weeks (**A**, 2 W). However, some small cells appeared with 60–70% confluence at 3 weeks (**A**, 3 W), and were mostly uniformly small and near confluence at 4 weeks (**A**, 4 W). The total cell number increased more dramatically after 3 weeks (**B**). The average cell size decreased steadily from 1 to 4 weeks (**C**). Bar represents 100 μm.

confluence again in 1 week. P2 cells subcultured for 1 week (1 W) revealed a mixture of small and large cells, and could not be subcultured at P3. However, P2 cultures consisted of predominantly small cells if cultured for 4 weeks (4 W) (Fig. 1). Furthermore, P3/4 W cultures could be continually subcultured for at least 100 passages if each passage was maintained low-seeding density. Importantly, clonal growth was observed after P4, and nuclear staining to PCNA was uniformly positive in more than 95% of cells (Fig. 1), indicating a highproliferative activity. To determine whether such small cells were selectively preserved when the culturing time was prolonged to 4 W, we measured the total cell number and the cell size weekly during the 4 weeks of P3 cultures. After 1 week, cells were heterogeneous and consisted of large cells with a prominent cytoplasm and small cells with a scanty cytoplasm (Fig. 2A, 1 W). The proportion between small cells to large cells was increased by the second week (Fig. 2A, 2 W) and the third week (Fig. 2A, 3 W). Notably, most cells were small by the $4^{th}$ week (Fig. 1A, 4 W). The total cell number dramatically increased after the third week (Fig. 2B). The average cell size, however, decreased steadily from 1 W to 4 W as larger cells desquamated from the dish (not shown). Although most small cells remained in a monolayer at the end of 4 weeks, cells showed stratification and spontaneous desquamation in some areas. Such desquamated small cells still retained proliferative capacity when transferred to another dish in KSFM (not shown).

## Further enrichment of small epithelial cells by lowering the seeding density

Small epithelial cells were selectively promoted by prolonging the culturing time to 4 weeks (Fig. 2), at the time when large differentiated cells had desquamated *via* senescence. Therefore, we speculated that small epithelial progenitor cells could be further selected by lowering the seeding density, which decreased the proportion of large differentiated cells to small cells. In P4 cultures, cells seeded at 50,000 cells per $cm^2$ degenerated into cell debris after 1 week of culturing (Fig. 3A). Hoescht 33342 staining revealed pronounced nuclear fragmentation suggestive of apoptosis (Fig. 3D), and the Live and Dead Assay showed marked cell death (Fig. 3G). Cells seeded at 5000 cells per $cm^2$ showed some spindle cells mixed with small cells (Fig. 3B, indicated by *), which had less fragmented nuclei (Fig. 3E), and fewer dead cells (Fig. 3H). In

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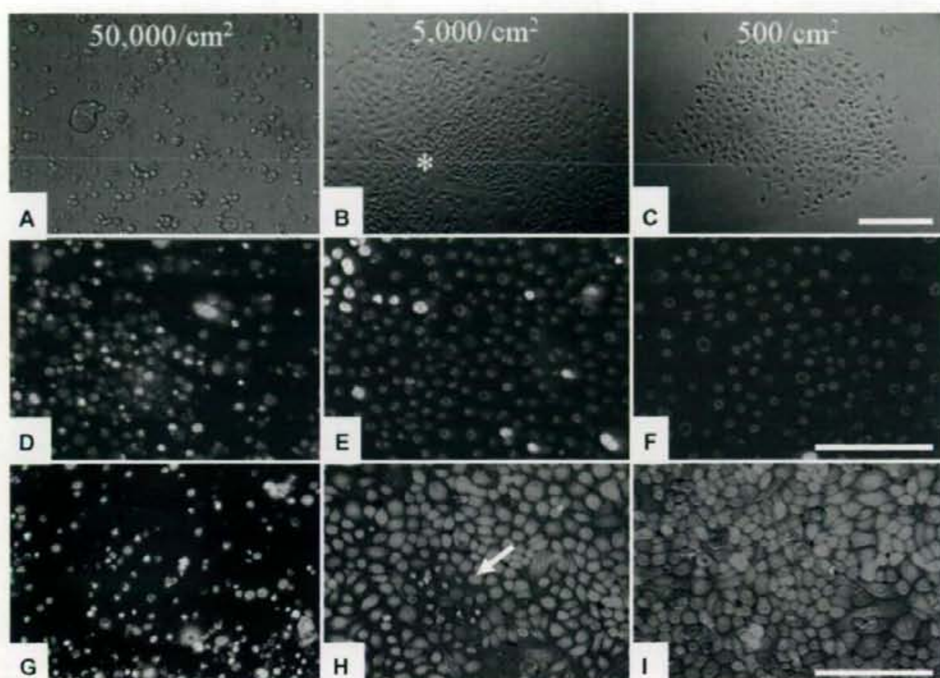


**Fig. 3** Morphology (top), nuclear fragmentation (middle) and Live and Dead Assay (bottom) at different seeding densities. In P4 cultures, cells seeded at 50,000 cells per $cm^2$ degenerated into cell debris after 1 week of culturing **(A)**. This change was associated with pronounced nuclear fragmentation shown by Hoescht 33,342 staining **(D)**, and by marked cell death **(G)**. Cells seeded at 5000 cells per $cm^2$ showed some spindle cells mixed with small cells (**B**, indicated by *). Some cells showed fragmented nuclei **(E)**, and there were patches of dead cells (**H**, indicated by arrow). Cells seeded at 500 cells per $cm^2$ showed uniform small cells **(C)** without fragmented nuclei **(F)** or dead cells **(I)**. Bars represent 50 $\mu$m.

contrast, cells seeded at 500 cells per $cm^2$ were uniformly small (Fig. 3C) without fragmented nuclei (Fig. 3F) or dead cells (Fig. 3G). These results indicated that small epithelial progenitor cells were indeed preferentially enriched by lowering the seeding density.

## Epithelial differentiation at different seeding densities

Because cells seeded at higher densities contained a heterogeneous population of small and large cells (Fig. 3), we wondered whether these large cells consisted of more differentiated cells. To resolve this issue, immunostaining was performed with antibodies against K12 keratin, a marker for corneal-type epithelial differentiation [5], and p63, a transcription factor specific for epithelial progenitor cells [7] in the above P4 culture. At the density of 50,000 cells per $cm^2$, 42.3 $\pm$ 7.8% of cells cultured for 1 week were positive for K12 keratin in the cytoplasm (Fig. 4A), and 59.3 $\pm$ 7.4% of them were positive for p63 in the nucleus (Fig. 4D). Large cells tended to be positive for K12 keratin and negative for p63. At a density of 5000 cells per $cm^2$, 34.7 $\pm$ 7.1% of cells were positive for K12 keratin (Fig. 4B), while 81.7 $\pm$ 5.0% of cells were positive for p63 (Fig. 4E). In contrast, at a density of 500 cells per $cm^2$, nearly all cells were negative for K12 expression (Fig. 4C), but uniformly positive for p63 expression (Fig. 4F) Cells at a low-seeding density (500 cells/$cm^2$) had significantly lower K12 and higher p63 expression than those at intermediate and high seeding densities (5000 and 50,000 cells/$cm^2$, respectively) (both $P < 0.01$). These results indicated that cell differentiation was promoted by at a high seeding density, which explained in part why lower seeding density further enriched epithelial progenitor cells.


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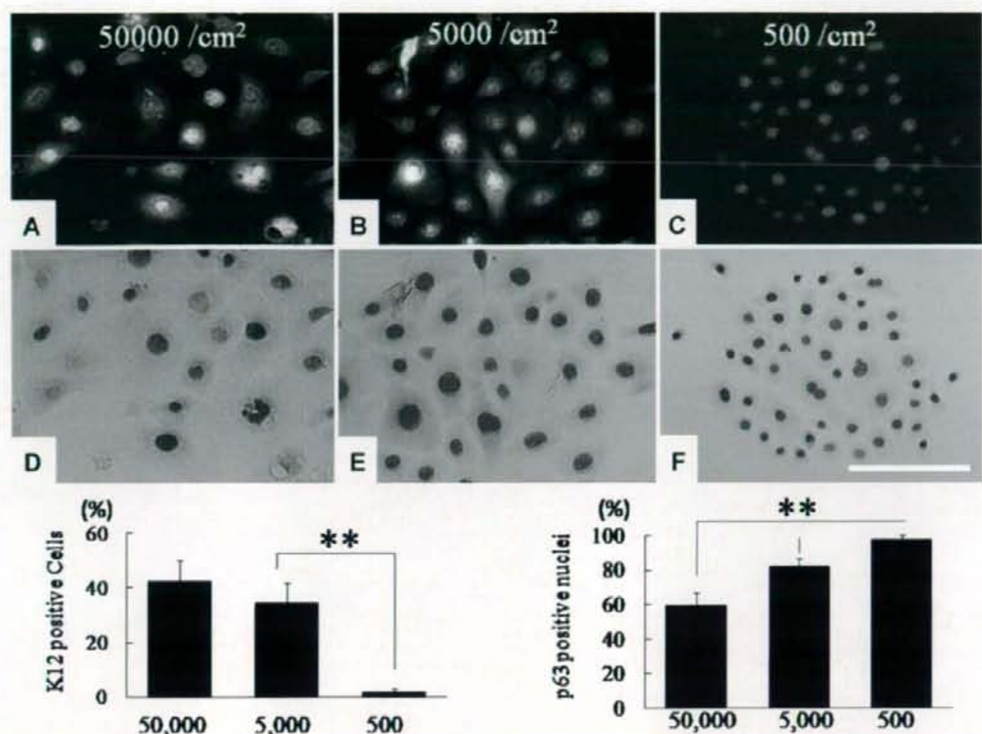


**Fig. 4** Expression of K12 Keratin (top) and p63 (bottom) at different seeding densities. In the above P4 culture (Fig. 3), immunostaining was performed with antibodies against K12 keratin and p63. At 50,000 cells/cm$^2$, ~40% of cells cultured for 1 week were positive for K12 keratin in the cytoplasm **(A)**, and ~60% of them were positive for p63 in the nucleus **(D)**. Large cells tended to be positive for K12 keratin and negative for p63. At 5000 cells/cm$^2$, less cells were positive for K12 keratin (B), while more cells were positive for p63 **(E)**. In contrast, cells seeded at 500 cells/cm$^2$ were negative for K12 expression but uniformly positive for p63 expression **(F)**. PI revealed nuclear size was not different between K12-positive and negative cells **(D)**. Bar represents 50 $\mu$m. When the seeding density was decreased from 50,000 to 500 cells/cm$^2$, K12 positive cells decreased from 42.3% to 1.7%, while p63 positive cells increased from 59.3% to 97.7% (**$P < 0.01$).

## Clonal growth by lowering the seeding density and prolonging the culturing time

To further confirm that small cells were indeed progenitor cells, we compared their clonal growth in KSFM by lowering the seeding density and prolonging the culturing time simultaneously. P0 cells after isolation were seeded at the density of 40 cells/cm$^2$ on plastic in KSFM and on mitomycin C-arrested 3T3 fibroblast feeder layer. Large clones with a smooth contour resembling holoclones [17] wereformed in both KSFM (Fig. 5A) and 3T3 fibroblast feeder layer (Fig. 5B). Cells in both the centre and the periphery of the clone formed in KSFM were uniformly smaller (Fig. 5C and E, respectively). In contrast, cells in the centre were large and squamous but in the periphery were small in the clone formed on 3T3 fibroblast feeder layer (Fig. 5D and F, respectively). The colony-forming efficiency was 0.27 ± 0.25% in KSFM, which was significantly fewer than 2.4 ± 0.5% in 3T3 fibroblast feeder layer (Fig. 5G, $P < 0.001$). In contrast, the average diameter of colonies formed in KSFM was 7.0 ± 4.2 mm, which was significantly larger than 1.3 ± 1.4 mm in 3T3 fibroblast feeder layer (Fig. 5H, $P < 0.05$).

To further determine whether small cells expanded during continuous passages in KSFM still possessed progenitor cell status, cells subcultured to P4, P8 and P12 were seeded at a density of 40 cells/cm$^2$ on plastic in KSFM, and compared to those seeded in

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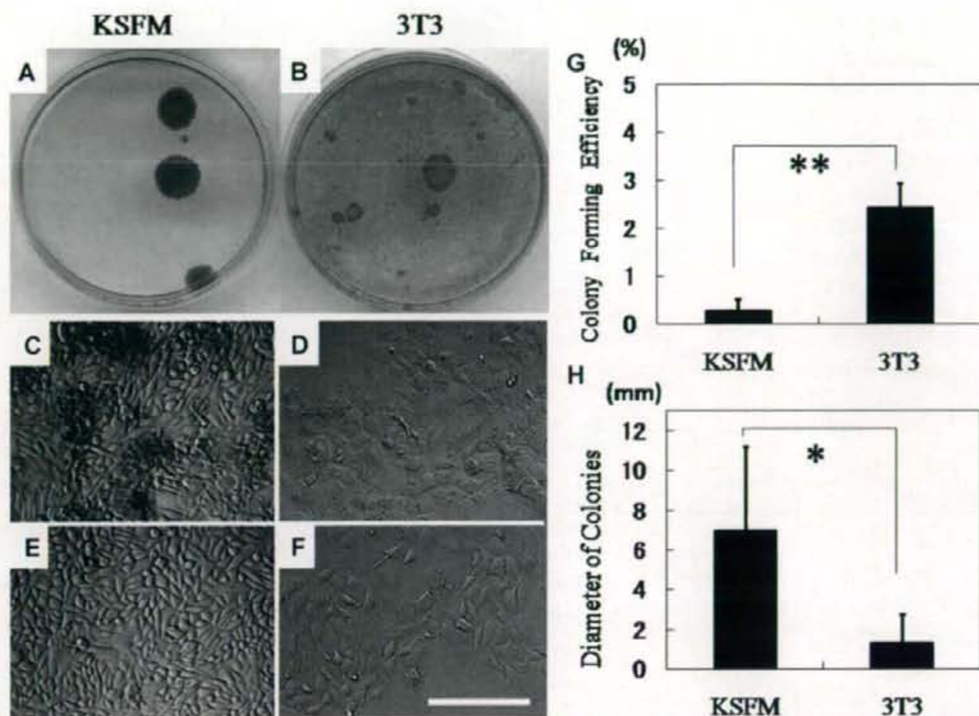


**Fig. 5** Clonal growth of primary murine corneal/limbal epithelial cells. P0 clonal cultures were established by seeding 40 cells/cm$^2$ on plastic in KSFM and in supplemental hormonal epithelial medium (SHEM) with mitomycin C-treated 3T3 fibroblast feeder layers, and cultured for 4 weeks. Clones in KSFM **(A)** visualized by crystal violet staining were fewer but larger than those in 3T3 feeder layers **(B)**. Cells in KSFM were uniformly smaller than those in 3T3 feeder layer at central (**C** and **D**, respectively) and peripheral areas (**E** and **F**, respectively). Clones formed in KSFM cultures were fewer (**G**, $^{**}P < 0.001$) but larger (**H**, $^{*}P < 0.05$). Bar represents 100 μm.

SHEM containing 3T3 fibroblast feeder layer. After 4 weeks of culturing, colonies visualized by crystal violet were found in both culturing systems. However, fewer but larger round colonies were consistently observed in KSFM than in 3T3 fibroblast feeder layers for these three subpassages (Fig. 6A). In KSFM, cells remained uniformly small (Fig. 6B), while cells on 3T3 fibroblast feeder layer were initially small but rapidly enlarged to squamous and elongated cells (Fig. 6C and D). Large P12 squamous cells on 3T3 fibroblast feeder layer expressed more K12 keratin (Fig. 6E), contained a lower percentage of p63 nuclear positive cells (Fig. 6F), and had larger irregular nuclei (counterstained with Hoescht 33342) (Fig. 6G) than cells in colonies formed in KSFM (see Fig. 5 for comparison). These results suggested that clonal growth of expanded epithelial progenitor cells were supported better by KSFM than by 3T3 fibroblast feeder layers.

## Normal differentiation induced by increasing [$Ca^{2+}$] and adding serum at P12

Previously, we noted that an increase of [$Ca^{2+}$] to 0.9 mM and addition of 5% FBS in KSFM restored expression of K12 keratin by large squamous epithelial cells in P2 cultures [12]. To make sure that the aforementioned expansion of small cells still retained the capability of adopting normal epithelial differentiation, we raised [$Ca^{2+}$] to 0.9 mM and/or added 5% FBS for 2 days in P12 cultures. In the control culture containing KSFM alone, cells expanded up to P12 remained uniformly small (Fig. 7A), did not express K12 keratin (Fig. 7E) and uniformly expressed p63 in the nucleus (Fig. 7I). An increase of [$Ca^{2+}$] to 0.9 mM rendered them into large squamous cells (Fig. 7B), of which some expressed K12 keratin (Fig. 7F), and lost p63 nuclear


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