Manuscript clean

Human embryonic stem cells derivatives enable full reconstruction of the pluristratified epidermis.

Hind Guenou[1], Xavier Nissan[1], Fernando Larcher[2], Jessica Feteira[1], Gilles Lemaitre[1], Manoubia Saidani[1], Marcela Del Rio[2] , Christine C Barrault[3], François-Xavier Bernard[3], Marc Peschanski[1], Christine Baldeschi[1*] & Gilles Waksman[1†]

[1] INSERM/UEVE U-861, I-STEM, AFM, Institute for Stem cell Therapy and Exploration of Monogenic diseases, 5 rue Henri Desbruères, 91030 Evry cedex, France

[2] CIEMAT –CIBER-ER, Epithelial Biomedicine Division, Avenida Complutense, 22 E-28040, Madrid, Spain.

[3] BIOalternatives SAS, 1 bis rue des Plantes, 86160 Gencay, France

[†] The authors are sorry to announce that Professor G. Waksman has died on September 22nd, 2007, during the completion of the study he had initiated.

[*] To whom correspondence should be addressed. Email: cbaldeschi@istem.genethon.fr

hal-00417656, version 1 - 16 Sep 2009

hal-00417656, version 1 - 16 Sep 2009

Summary (248 words)

Background: Cell therapy for large burns has successfully made use of autologous epidermis reconstructed *in vitro* for more than two decades. One limit to the efficacy of the current procedures is the need for a long lasting expansion of the patient's own keratinocytes. Immediate access to unlimited amounts of human epidermis may contribute to protect patients awaiting autologous grafts. The keratinocyte progeny of human embryonic stem cells is an interesting candidate for such a role, provided they are fully capable of reconstructing a pluristratified epidermis.

Methods: Human embryonic stem cells were seeded on fibroblasts feeder cells during forty days in a medium supplemented with BMP4 (0.5 nM) and ascorbic acid (0.3 mM). A molecular characterization of cell differentiation was performed all along the process by quantitative-PCR, fluorescence activated cell sorting and immunocytochemical techniques. Keratinocyte molecular differentiation and functional capacity to construct a human epidermis were assessed *in vitro* and *in vivo.*

Finding: Using a protocol that respected the chronobiology of epidermis formation during human ontogenesis, we have generated a homogenous population of cells exhibiting all phenotypic characteristics of basal keratinocytes from hESC. These cells readily constructed a pluristratified epidermis displaying normal human characteristics after seeding on an artificial matrix, and either secondarily grown *in vitro* or else transplanted in immunodeficient mice.

Interpretation: Human embryonic stem cells can be coaxed to differentiate into basal keratinocytes that are fully functional, i.e. construct a pluristratified epidermis. This resource may be developed to provide temporary skin substitutes to patients awaiting autologous grafts.

Introduction (3009 words)

Cell therapy has revolutionized prognosis of large burns injuries for more than two decades thanks to epidermal replacement following *in vitro* expansion of the patient's own keratinocyte stem cells. The major drawback of this technique is the three weeks delay requested to obtain the needed amount of cells, which puts patients at risk of dehydration and infection[1]. To date, the temporary wound coverage during that period is in most cases obtained using decellularized cadaver skin. The benefit of this temporary skin substitutes is hampered by the limited availability of these allografts. The tissue may also be rapidly rejected by an immune reaction of the recipient. To overcome the problem of accessibility, inert synthetic and biosynthetic matrixes have been actively searched and developed[2]. Up to now, however, these substitutes have not replaced cadaver skin in large burns[3], because they may present an increased risk of rapid graft rejection and disease transmission due, in particular, to their content of bovine collagen and adult allogenic skin cells [2].

Human embryonic stem cells (hESC) may eventually offer yet another way to meet the challenge of temporary skin replacement as they can be expanded in unlimited amounts and differentiated into any human cell phenotype *in vitro*, provided the relevant differentiation protocol has been developped[4]. Exploration of the potential of the derivatives of hESC in cell therapy is currently very active in a number of applications as diverse as Parkinson's and Huntington neurodegenerative diseases[5,6], diabetes mellitus[7] or cardiac insufficiency following ischemia[8]. The rapid maturation of these studies has been recently exemplified by the authorization given by the Food and Drug Administration for a first clinical trial in patients with spinal trauma (January 23, 2009 - Geron Corporation). It is also interesting to mention that hESC derivatives may, at least for some time, express very low levels of HLA antigens, if any, suggesting a relative invisibility to the immune system[9].

Until now, only one study reported the formation of a skin equivalent using mouse embryonic stem cells[10]. Following this report, several studies have sought differentiating hESC along the keratinocyte lineage. Epidermal cell specification using embryoid bodies or nodules produced in SCID mice was successful, but was marred by low proliferative potential. More recently, keratinocytes derived from human embryonic stem cells were obtained using short cytokine induction[10-14] but production of a pluristratified epidermis has remained elusive *in vitro* and *in vivo*, suggesting that functional basal keratinocytes had not been obtained.

hal-00417656, version 1 - 16 Sep 2009

We have now successfully met that functional challenge, by designing a multi-step sequential protocol taking into account as closely as possible the long-term succession of biological steps that lead to epidermis formation during ontogenesis.

hal-00417656, version 1 - 16 Sep 2009

Methods

hES cell culture

hESC from two cell lines, namely SA-01 (from Cellartis, Gottenborg Sweden) and H9 (from Wicell, Madison WI) were grown on a feeder layer of STO mouse fibroblasts, inactivated with 10 mg/ml mitomycin C and seeded at 30000/cm² and were cultivated as described in (6). For differentiation, hESC clumps were seeded onto mitomycin C-treated 3T3 fibroblasts in FAD medium composed of 2/3 DMEM, 1/3 HAM:F12 and 10% of fetal calf serum (FCII, Hyclone, Logan, UT, USA) supplemented with 5µg/ml insulin, 0.5µg/ml hydrocortisone, 10^{-10}M cholera toxin, 1.37ng/ml triodothyronin, 24µg/ml adenine and 10ng/ml recombinant human EGF. Three independent experiments were performed using both H9 and SA-01 hESC. The induction of ectodermal differentiation was induced by 0.5nM of human recombinant BMP-4 (R&D Systems Europe, UK) and 0.3mM ascorbic acid (Sigma-Aldrich). Cells were grown in the same medium until clones of epithelial cells were observed and isolated, at which time BMP4 and ascorbic acid were withdrawn. As a control, primary human keratinocytes (HK) were grown on mitomycin C-treated 3T3 fibroblasts in FAD medium. When keratinocytes derived from hESC were grown in serum-free medium condition without feeder layer, KGM2 medium (Lonza, Basel, Switzerland) was used in flask coated with collagen I (BD Biosciences, BDIS, San Jose, CA, USA)

Quantitative RT-PCR.

Total RNA was isolated from hESC, HK and K-hESC using RNeasy Mini extraction kit (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol. An on-column DNase I digestion was performed to avoid genomic DNA amplification. RNA level and quality were checked using the Nanodrop technology. A total of 500 ng of RNA was used for reverse transcription using the Superscript III reverse transcription kit (Invitrogen). Real time RT-PCR analysis was performed using a LightCycler 480 system (Roche diagnostics, Basel Switzerland) and SYBR Green PCR Master Mix (Roche Diagnostics) following the manufacturer's instructions. Quantification of gene expression was based on the DeltaCt Method and normalized on 18S expression. Melting curve and electrophoresis analysis were performed to control PCR products specificities and exclude non-specific amplification. PCR Primers are listed in webappendix Table1. Quantitative RT-PCR arrays were prepared by loading of primer mixes (1 µM final in the reaction) in duplicate wells of 96 well plates. Quantitative RT-PCR was then performed as above after addition of SYBR Green PCR Master Mix and 12.5 ng (final) cDNA.

hal-00417656, version 1 - 16 Sep 2009

Statistical analysis was presented as a mean ± SD of 3 independent experiments using each of the two H9 and SA-01 hESC lines and were analysed with Student's t test (ANOVA).

Immunocytochemistry.

Cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature before permeabilization and blocking in PBS supplemented with 0.4% Triton X-100 and 5% BSA (Sigma-Aldrich). Primary antibodies were incubated overnight at 4°C in blocking buffer. Antibodies comprised mouse anti-K18 (Chemicon, Temecula, CA, USA), mouse anti-K14, rabbit anti-K14 and mouse anti-K5 (from Novocastra Ltd.,Newcastle upon Tyne, U.K.), mouse anti-ColVII, mouse anti-integrin α6 and mouse anti-laminin5 (from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and mouse anti-integrin β4 (from BD Biosciences). Cells were stained with the species specific fluorophore-conjugated secondary antibody (Invitrogen) for 1 hour at room temperature and nuclei were visualized with DAPI. Three independent experiments were performed using H9 and three using SA-01 hESC.

FACS analyses

hESC, HK and K-hESC were detached from culture plates using Trypsin-EDTA (Invitrogen) and fixed in 2% paraformaldehyde for 15 minutes at room temperature. After PBS wash, cells were either permeabilized with 0.1% Saponin (Sigma-Aldrich) or not. Primary antibodies diluted at 1:100 were incubated for one hour at room temperature in PBS containing 0.1% FCS. Control samples were done using isotype specific or no primary antibody. Species specific secondary antibodies were added for 1 hour at room temperature and stained cells were analyzed on a FACScalibur flow cytometer using CellQuest software (BD Biosciences). Detection of MHC-I and MHC-II proteins was performed as described without addition of secondary Ab, using anti- HLA-ABC and anti HLA-DR (both from BD Biosciences). The number of events analysed for each experiment was 10,000. Three independent experiments were performed using H9 and three using SA-01 hESC

Organotypic cultures and in vivo grafting.

Organotypic epidermis was generated as previously described in (19). HK and K-hESC were performed on polycarbonate culture inserts (NUNC, Roskilde, Denmark). These cells were maintained for 11 days in Epilife medium (Invitrogen) supplemented with 1.5mM CaCl2 and

hal-00417656, version 1 - 16 Oct 2009

50µg/ml ascorbic acid (Sigma-Aldrich). The cells were exposed to the air-liquid interface for 10 days. Experiments were performed using both H9 (n=3) and SA-01 (n=2) hESC.

For grafting to immunodeficient mice, bioengineered skin equivalents were generated using fibrin matrix populated with human fibroblasts. K-hESC from H9 (n=5) were seeded on the fibrin matrix, grown immersed to confluence, and then grafted onto the back of 6-week-old female nu/nu mice (Jackson Laboratory, Bar Harbor, ME) as described[15]. Implants were harvested 10-12 weeks after grafting, and the tissue specimens fixed in 10% buffered formalin for paraffin embedding.

Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

hal-00417656, version 1 - 16 Sep 2009

hal-00417656, version 1 - 16 Oct 2009

Results

Establishment of a keratinocyte lineage

Undifferentiated hESC were maintained on mitomycin-treated 3T3 feeder cells in FAD medium, supplemented with BMP4 (0.5 nM) and ascorbic acid (0.3 nM). Characterization of hESC differentiation was performed at least in triplicate using each of the two cell lines H9 and SA-01, and was carried out every ten days for forty days by quantitative RT-PCR (q-PCR) and fluorescence activated cell sorting (FACS) analyses. The pluripotency gene markers *OCT4* and *NANOG* decreased rapidly after 5 days to finally be undetectable at 20 days (figure 1A). FACS analysis confirmed a parallel loss of the undifferentiated state marker SSEA3 at 40 days (figure 1B).

The succession of epidermal markers during ontogenesis characterized by a strict temporal expression pattern of structural molecules[16-18] was fully replicated in differentiating hESC cultures. Expression of KRT8 and KRT18 genes that encode keratin 8 and keratin 18, respectively, peaked at 10 days then decreased progressively over the following weeks (figure 1A). This time course was paralleled by the transitory expression of keratin 18, which peaked between 10 and 25 days then decreased to a much reduced basal level at 40 days (figure 1B). Expression of the KRT5 and KRT14 genes increased progressively after day 10 (figure 1A). This was again paralleled by the enrichment of the culture in the protein keratin 14 over time, up to a maximum at 40 days of differentiation (figure 1B).

Characterization of a homogenous and pure population of keratinocytes derived from hESC.

The period of induction was stopped after 40 days by withdrawing BMP4 and ascorbic acid from the medium. After passage, cells exhibiting typical pavimentous epithelial morphology spontaneously formed colonies. In those culture conditions, cells proliferated actively up to nine passages and could be frozen and thawed at will, while maintaining their phenotype (webappendix p1). We named these cells K-hESC for “keratinocytes derived from human embryonic stem cells” (figure 2A). K-hESC characterization was performed using both hESC line H9 (n=3) and SA-01 (n=3). FACS analysis after four passages revealed complete absence of keratin 18 and a cell population in which more than 95% of the cells expressed keratin 5 and keratin 14 (figure 2B).

Phenotypic characteristics of K-hESC were similar to those of human adult primary keratinocytes (HK). Gene expression levels were equivalent for all tested genes that are

characteristic of basal keratinocytes, including those encoding keratin 14, keratin 5, integrins alpha-6 and beta-4, collagen VII and laminin-5 (figure 2C). There was no immunostaining for either Oct-4 or keratin 18 in K-hESC compared to the respective controls (webappendix p2). Cytoskeletal localization of keratins 5 and 14 (figure 2D) was similar in K-hESC and in adult keratinocytes. Keratin 10, a marker of differentiated keratinocytes of suprabasal layers, was absent compared to K-hESC stimulated by calcium (webappendix p2). All data confirmed the phenotypic characterization of K-hESC as basal keratinocytes. Conversely, K-hESC were well equipped to demonstrate adhesion capacity as integrins alpha-6 and beta-4 were appropriately localized at the membrane, laminin-5 and collagen VII in the extracellular matrix (figure 2D). However, unlike adult basal keratinocytes, K-hESC revealed only very low levels of HLA-ABC antigens, and no HLA-DR (figure 3).

K-hESC could also be grown in a serum-free medium without feeder cells. In those conditions, cells showed no alteration in protein expression of keratin 5, 14, and integrins alpha-6 and beta-4. K-hESC maintained in serum-free medium demonstrated similar gene expression for keratins 5 and 14 as those grown in FAD medium with feeder cells (webappendix p3).

hal-00417656, version 1 - 16 Oct 2009

Keratinocytes derived from hESC form a pluristratified epithelium.

H9 and SA-01 derived K-hESC revealed similar to HK not only in phenotypic but also in functional terms as reconstituted epidermis could be generated *in vitro* on an artificial matrix using classical techniques[19]. After 10 days of air-liquid differentiation, haematoxylin-eosin staining of cryosection of organotypic cultures of K-hESC showed a pluristratified epithelium, with a basal layer, a stratum spinosum, a stratum granulosum containing keratohyalin granules, and a stratum corneum seen as superposed layers of dead squamous enucleated cells (figure 4A). Differentiation markers were normally expressed and localized in layers (figure 4B, webappendix p4). Keratin 14 immunostaining was observed in the basal compartment and not in the suprabasal layers. Reciprocally, keratin 10 was present only in layers overlying the basal layer (figure 4B). Involucrin and filaggrin, late markers of epidermal differentiation were detected exclusively in the uppermost layers. Appropriate addressing of proteins involved in cell adhesion was confirmed by the localization of integrins alpha-6 and beta-4 at the basal membrane of basal keratinocytes. Proteins of the basement membrane, laminin-5 and collagen VII, were also observed. PCR Array using a panel of epidermal genes confirmed that HK and K-hESC organotypic epidermis displayed very similar patterns of expression (webappendix p4).

As a final demonstration, the capability of the K-hESC to generate self-renewing epithelia was evaluated through a stringent *in vivo* test. Fibrin matrix containing adult human fibroblasts were seeded with H9 derived K-hESC (n=5) to obtain confluent epidermal layer *in vitro*. These organotypic cultures were then grafted onto the dorsal region of immunodeficient nu/nu mice by orthotopical grafting[15,20]. After 12 weeks, K-hESC-derived epidermis exhibited a pluristratified architecture, consistent with that of mature native human skin (figure 5A and webappendix p5). Human involucrin was appropriately located in spinous and granular layers (figure 5B) and few Ki67 positive cells were detected in the basal layer (figure 5C).

hal-00417656, version 1 - 16 Sep 2009

Discussion

The main result of this study is the demonstration that keratinocytes can be derived from hESC that can form a pluristratifed epithelium replicating normal human epidermis *in vitro* and following grafting *in vivo*. This has been obtained using a protocol that combined co-culture with particular feeder cells and pharmacological treatment over 40 days. Growing human epidermis from hESC may bear clinical relevance as an unlimited resource for temporary skin replacement in patients with large burns awaiting autologous grafts.

Our results establishing the differentiation of K-hESC capable of forming a pluristratified epidermis extend those previously published that demonstrated acquisition *in vitro* of phenotypic keratinocyte characteristics by hESC derivatives. Specification of the keratinocyte lineage from hESC was first demonstrated using a differentiation protocol based upon embryoïd bodies, but the cells exhibited too low proliferative potential for subsequent tissue development[11]. The embryoid body protocol has since been avoided in other studies. In the absence of a direct differentiation of hESC using the pharmacological protocol that they had successfully applied on mouse ES cells[10], Aberdam and colleagues obtained some success by engineering the cells in order to force the expression of p63[21], a protein that drives keratinocyte differentiation during ontogenesis[16-18]. Expression of p63 is, however, likely not sufficient by itself to induce full functional maturation of keratinocytes as these cells did not enable full epidermis reconstitution[13]. A relatively pure population of keratinocytes was recently obtained from hESC by others under defined conditions in two dimensional culture[14], although again with no demonstration of reconstruction of a pluristratified epidermis. This latter protocol relied on BMP4 induction like ours, but differed in that retinoic acid was added instead of ascorbic acid and the time schedule of induction was only 6 days instead of 40 days. The cytokine BMP4 has been shown to inhibit neural induction and to maintain epithelial commitment[22], which makes it a good candidate for coaxing hESC along the epidermal lineages during the first steps of the differentiation protocol. How and whether it may act later on in the process is yet unknown. Ascorbic acid was specifically added in our protocol in order to help final steps of differentiation as it has been shown to stimulate full keratinocytic differentiation in the absence of retinoic acid[23,24]. Last, but quite distinctively, our protocol involved the continuous application of the pharmacological treatment up to the demonstration of a full differentiation of basal keratinocytes in the cell cultures, which required 40 days, whereas other authors used much shorter induction times. It is interesting to mention that this time schedule is in keeping with the chronobiology of the epidermis formation during embryonic development.

hal-00417656, version 1 - 16 Sep 2009

Cell therapy for large burns has successfully made use of autologous epidermis reconstructed *in vitro*. However, during the delay of 3 weeks for patient's keratinocytes culture that will provide for autografts, allografts are used as a temporary cover for full thickness burns, in most cases obtained from cadaver skin. In order to minimize disease transmission decellularisation and cryopreservation of the cadaver skin is performed[25], but this does not prevent rejection[26]. Access to cadaver skin is also a major concern, as this resource is highly limited[27]. There are other clinically useful, in general semi-synthetic products composed of bovine collagen, allogenic fibroblasts and/or keratinocytes that are powerful to establish a basement membrane facilitating the reconstruction of the dermal-epidermal junction. However, these biosynthetic substitutes are less protective than cadaver skin for large burns and may as well induce rapid immune rejection as they contain adult donor cells[28].

The epidermis reconstructed by K-hESC may meet some of the requisites of a complementary skin substitute. First, combination protocols involving dermis and epidermis replacement might be considered and analysed. Accessibility should not be a limiting problem due to the full *in vitro* preparation of the epidermis out of immortal cell lines that provide an unlimited original resource. Furthermore, the differentiated K-hESC are able to support a large number of passages, opening for further expansion. Risks of infections should also be constrained by industrialisation of the production process. K-hESC also produce and release all components needed to form an epidermal-dermal junction. Last, likely due to their early developmental stage K-hESC express little antigen if any of the major histocompatibility complex, suggesting a low immunogenicity of the skin substitute during the three week time period of its potential use in patients.

Whether this time period of treatment can eventually be extended and K-hESC be used for permanent allografts when no autologous graft is possible –e.g. for patients with genodermatoses- will have to be further studied. Maturation of K-hESC, if it does replicate normal development, should eventually be accompanied by expression of a full set of MHC antigens. It is interesting to mention, however, that skin cells obtained from foetuses following abortion –that also express very low MHC class I antigens- were successfully used for long term treatment of patients with large burns and presented no sign of rejection up to 21 months[29]. K-hESC allografts may also be eventually permitted by the establishment of banks containing cell lines with appropriately selected haplotypes[30]. Calculations indicate that less than a hundred lines would be sufficient to cover 90% of the Japanese population, and just 10 about 50% of the British people[30]. It would require, however, selecting those rare donors that are triple homozygous for HLA-A, HLA-B and HLA-DR.

hal-00417656, version 1 - 16 Sep 2009

Author Contributions

G.Waksman was principal investigator of the project. C. Baldeschi was the coordinator of the experiments. H. Guenou designed the protocol of differentiation to obtain K-hESC. X. Nissan, J. Feteira, G Lemaitre, M Saidani and C. Baldeschi isolated and characterized K-hESC populations. X. Nissan performed molecular characterization of K-hESC differentiation process and statistical analyses. J. Feteira performed FACS analyses. M. Saidani and C. Baldeschi performed immunocytochemistry. C.C. Barrault and F.X. Bernard performed organotypic cultures *in vitro*. M.D. Rio.and F. Larcher did the *in vivo* studies.
C. Baldeschi, H. Guenou, G. Lemaitre and M. Peschanski, prepared the manuscript.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The authors wish to thank Dr G. Meneguzzi and Dr L. Gagnoux for kindly providing biological resources, Dr M. Martin for constant support and Dr C. Hechard for managing partnerships of this project.
This work was supported by the Institut national de la santé et de la recherche médicale (INSERM), University Evry Val d'Essonne (UEVE), Association Française contre les Myopathies (AFM), Fondation René Tourraine and Genopole.

hal-00417656, version 1 - 16 Sep 2009

References

1. Gallico GG, 3rd, O'Connor NE, Compton CC, Kehinde O, Green H. Permanent coverage of large burn wounds with autologous cultured human epithelium. *N Engl J Med* 1984;**311**(7)**:**448-51.
2. Metcalfe AD, Ferguson MW. Bioengineering skin using mechanisms of regeneration and repair. *Biomaterials* 2007;**28**(34)**:**5100-13.
3. Alsbjorn B. In search of an ideal skin substitute. *Scand J Plast Reconstr Surg* 1984;**18**(1)**:**127-33.
4. Odorico JS, Kaufman DS, Thomson JA. Multilineage differentiation from human embryonic stem cell lines. *Stem Cells* 2001;**19**(3)**:**193-204.
5. Perrier AL, Tabar V, Barberi T, et al. Derivation of midbrain dopamine neurons from human embryonic stem cells. *Proc Natl Acad Sci U S A* 2004;**101**(34)**:**12543-8.
6. Aubry L, Bugi A, Lefort N, Rousseau F, Peschanski M, Perrier AL. Striatal progenitors derived from human ES cells mature into DARPP32 neurons in vitro and in quinolinic acid-lesioned rats. *Proc Natl Acad Sci U S A* 2008;**105**(43)**:**16707-12.
7. Baetge EE. Production of beta-cells from human embryonic stem cells. *Diabetes Obes Metab* 2008;**10 Suppl 4:**186-94.
8. Tomescot A, Leschik J, Bellamy V, et al. Differentiation in vivo of cardiac committed human embryonic stem cells in postmyocardial infarcted rats. *Stem Cells* 2007;**25**(9)**:**2200-5.
9. Drukker M, Katz G, Urbach A, et al. Characterization of the expression of MHC proteins in human embryonic stem cells. *Proc Natl Acad Sci U S A* 2002;**99**(15)**:**9864-9.
10. Coraux C, Hilmi C, Rouleau M, et al. Reconstituted skin from murine embryonic stem cells. *Curr Biol* 2003;**13**(10)**:**849-53.
11. Iuchi S, Dabelsteen S, Easley K, Rheinwald JG, Green H. Immortalized keratinocyte lines derived from human embryonic stem cells. *Proc Natl Acad Sci U S A* 2006;**103**(6)**:**1792-7.
12. Green H, Easley K, Iuchi S. Marker succession during the development of keratinocytes from cultured human embryonic stem cells. *Proc Natl Acad Sci U S A* 2003;**100**(26)**:**15625-30.
13. Aberdam D. Epidermal stem cell fate: what can we learn from embryonic stem cells? *Cell Tissue Res* 2008;**331**(1)**:**103-7.
14. Metallo CM, Ji L, de Pablo JJ, Palecek SP. Retinoic acid and bone morphogenetic protein signaling synergize to efficiently direct epithelial differentiation of human embryonic stem cells. *Stem Cells* 2008;**26**(2)**:**372-80.
15. Del Rio M, Larcher F, Serrano F, et al. A preclinical model for the analysis of genetically modified human skin in vivo. *Hum Gene Ther* 2002;**13**(8)**:**959-68.
16. Byrne C, Tainsky M, Fuchs E. Programming gene expression in developing epidermis. *Development* 1994;**120**(9)**:**2369-83.
17. Fuchs E. Epidermal differentiation and keratin gene expression. *J Cell Sci Suppl* 1993;**17:**197-208.
18. Turksen K, Troy TC. Epidermal cell lineage. *Biochem Cell Biol* 1998;**76**(6)**:**889-98.
19. Poumay Y, Dupont F, Marcoux S, Leclercq-Smekens M, Herin M, Coquette A. A simple reconstructed human epidermis: preparation of the culture model and utilization in in vitro studies. *Arch Dermatol Res* 2004;**296**(5)**:**203-11.
20. Xu RH. In vitro induction of trophoblast from human embryonic stem cells. *Methods Mol Med* 2006;**121:**189-202.
21. Medawar A, Virolle T, Rostagno P, et al. DeltaNp63 is essential for epidermal commitment of embryonic stem cells. *PLoS ONE* 2008;**3**(10)**:**e3441.

hal-00417656, version 1 - 16 Sep 2009

22. Stottmann RW, Berrong M, Matta K, Choi M, Klingensmith J. The BMP antagonist Noggin promotes cranial and spinal neurulation by distinct mechanisms. *Dev Biol* 2006;**295**(2)**:**647-63.
23. Bamberger C, Pollet D, Schmale H. Retinoic acid inhibits downregulation of DeltaNp63alpha expression during terminal differentiation of human primary keratinocytes. *J Invest Dermatol* 2002;**118**(1)**:**133-8.
24. Catani MV, Savini I, Rossi A, Melino G, Avigliano L. Biological role of vitamin C in keratinocytes. *Nutr Rev* 2005;**63**(3)**:**81-90.
25. Blome-Eberwein S, Jester A, Kuentscher M, Raff T, Germann G, Pelzer M. Clinical practice of glycerol preserved allograft skin coverage. *Burns* 2002;**28 Suppl 1:**S10-12.
26. Burd A, Chiu T. Allogenic skin in the treatment of burns. *Clin Dermatol* 2005;**23**(4)**:**376-87.
27. Metcalfe AD, Ferguson MW. Tissue engineering of replacement skin: the crossroads of biomaterials, wound healing, embryonic development, stem cells and regeneration. *J R Soc Interface* 2007;**4**(14)**:**413-37.
28. Kearney JN. Clinical evaluation of skin substitutes. *Burns* 2001;**27**(5)**:**545-51.
29. Hohlfeld J, de Buys Roessingh A, Hirt-Burri N, et al. Tissue engineered fetal skin constructs for paediatric burns. *Lancet* 2005;**366**(9488)**:**840-2.
30. Nakatsuji N, Nakajima F, Tokunaga K. HLA-haplotype banking and iPS cells. *Nat Biotechnol* 2008;**26**(7)**:**739-40.

hal-00417656, version 1 - 16 Sep 2009

hal-00417656, version 1 - 16 Sep 2009

Figure Legends.

Figure 1: Establishment of a keratinocyte lineage.

(A) Quantitative RT-PCR analysis of *OCT4/NANOG*, *KRT8/KRT18*, *KRT5/KRT14* of derived embryonic stem cells (hESC) during the 40 days of differentiation. Data are presented as a mean ± SD of 3 independent experiments using each of the two cell lines H9 and SA-01, and were analysed with Student's t test (ANOVA), * (p value ≤ 0,05), **(p value ≤ 0,01) and *** (p value ≤ 0,001).

(B) Representative FACS analysis of SSEA3, keratins 18 and 14 (K18, K14) in derived H9-hESC during the 40 days of differentiation.

Figure 2: Characterization of a homogenous population of keratinocytes derived from hESC.

(A) Microscopy analysis of human primary keratinocytes (HK), and keratinocytes derived from H9 (K-hESC) after subsequent culture.

(B) Representative FACS analysis of keratins 18, 5 and 14 (K18, K5, K14) in HK and K-hESC derived from H9. .

(C) Quantitative RT-PCR analysis of *OCT4/NANOG*, *KRT8/KRT18*, *KRT5/KRT14*, *ITGA6/ITGB4* and *LAMB3/Col7A1* in HK and K-hESC. Data are presented as a mean ± SD of 3 independent experiments using each of the two cell lines H9 and SA-01, and were analysed with Student's t test, (ANOVA) * (p value ≤ 0,05), **(p value ≤ 0,01) and *** (p value ≤ 0,001).

(D) Immunofluorescence of K5, K14, integrins alpha-6 and beta-4, laminin-5 and collagen VII in K-hESC derived from H9. Scale bar is 20 μm.

Figure 3: Expression of MHC class I (HLA-ABC) and class II (HLA-DR) proteins in hESC, K-hESC and HK.

Representative FACS analysis of MHC class I (HLA-ABC) and class II (HLA-DR) expression in hESC, HK and K-hESC derived from H9.

.

Figure 4: Reconstruction of a pluristratified epidermis using K-hESC.

(A) Haematoxylin-eosin staining of organotypic cultures of HK and K-hESC derived from SA-01. Scale bar is 50μm.

(B) Immunofluorescence analysis of the expression and localisation of keratin 14 (K14), keratin 10 (K10), involucrin, filaggrin, integrins alpha-6 and beta-4, laminin-5 and collagen VII in the K-hESC derived from SA-01 organotypic epidermis. Scale bar is 50µm.

Figure 5: Long-term *in vivo* human epidermal regeneration following xenografting to immunodeficient mice.

(A) Haematoxylin-eosin staining of artificial skin implants grafted with K-hESC derived from H9. Scale bar is 50 µm.

(B) Immunoperoxydase staining using mAb SY-5 directed against human involucrin on artificial skin implants grafted with K-hESC derived from H9 is appropriately located in spinous and granular layers. Note that dermal background could be observed, due to the anti-mouse secondary antibody. Scale bar is 50 µm.

(C) Immunoperoxydase staining for Ki67 on artifical skin implants grafted with K-hESC derived from H9. Scale bar is 50 µm.

hal-00417656, version 1 - 16 Sep 2009

Webappendix, Table 1: sequence of primers

Gene Name	Ref	Amplicon length (bp)	Forward primer	Reverse primer
18S	NM_022551.2	146	GAGGATGAGGTGGAACGTGT	TCTTCAGTCGCTCCAGGTCT
NANOG	NM_024865.2	158	CAAAGGCAAACAACCCACTT	TCTGCTGGAGGCTGAGGTAT
OCT4	NM_002701.4	169	CTTGCTGCAGAAGTGGGTGGAGGAA	CTGCAGTGTGGGTTTCGGGCA
KRT18	NM_199187.1	129	GAGTATGAGGCCCTGCTGAACATCA	GCGGGTGGTGGTCTTTTGGAT
KRT8	NM_002273	69	GATCGCCACCTACAGGAAGCT	ACTCATGTTCTGCATCCCAGACT
KRT14	NM_000526.3	79	GGCCTGCTGAGATCAAAGACTAC	CACTGTGGCTGTGAGAATCTTGTT
KRT5	NM_000424.3	74	ATCTCTGAGATGAACCGGATGATC	CAGATTGGCGCACTGTTTCTT
LAMB3	NM_000228.2	241	GACAGGACTGGAGAAGCGTGTG	CCATTGGCTCAGGCTCAGCT
COL7A1	NM_000094.3	198	GATGACCCACGGACAGAGTT	ACTTCCCGTCTGTGATCAGG
ITGA6	NM_000210.2	112	GCTGGTTATAATCCTTCAATATCAATTGT	TTGGGCTCAGAACCTTGGTTT
ITGB4	NM_000213.3	220	CTGTACCCGTATTGCGACT	AGGCCATAGCAGACCTCGTA

hal-00417656, version 1 - 16 Sep 2009

hal-00417656, version 1 - 16 Sep 2009

Legends of appendix

Appendix 1: Stable phenotype of K-hESC up to nine passages.

Representative quantitative RT-PCR analysis of K-hESC derived from SA-01 at successive passages, up to 9, showed stable expression of genes associated to the keratinocyte phenotype, including *KRT5, KRT14, ITGA6* and *ITGB4*.

Appendix 2: Characterization of a homogenous population of keratinocytes derived from hESC. Immunofluorescence analysis using:

(A) Oct4 staining in K-hESC and undifferentiated hESC.

(B) K18 staining in K-hESC and hESC differentiated for 10 days.

(C) K10 staining in K-hESC and in K-hESC stimulated by calcium.

Scale bar is 20µm

Appendix 3: Homogenous profile of K-hESC in semi-defined serum-free medium.

(A) Immunofluorescence analysis of K-hESC derived from SA-01 growing without feeders showed a homogenous expression of keratins 5, 14 and integrins alpha-6 and beta-4. Scale bar is 50µm.

(B) Quantitative RT-PCR analysis of K-hESC maintained in semi-defined serum-free medium, and in FAD medium with feeder cells demonstrated similar expression of the transcripts of keratin 5 and 14 (*KRT5* and *KRT14*). Data are presented as a mean ± SD of 3 independent experiments using each of the two cell lines H9 and SA-01, and were analysed with Student's t test (ANOVA), *** (p value ≤ 0,001).

Appendix 4: Expression profile of epidermis markers of organotypic epidermis derived from HK and K-hESC.

(A) Immunofluorescence of HK organotypic epidermis showed normal distribution of epidermis markers: keratin 14, integrins alpha-6, beta-4 in the basal layer, keratin 10 in all differentiated layers, involucrin and filaggrin exclusively in the upper most layers, and laminin-5 and collagen VII at the dermal-epidermal junction. Scale bar is 50µm.

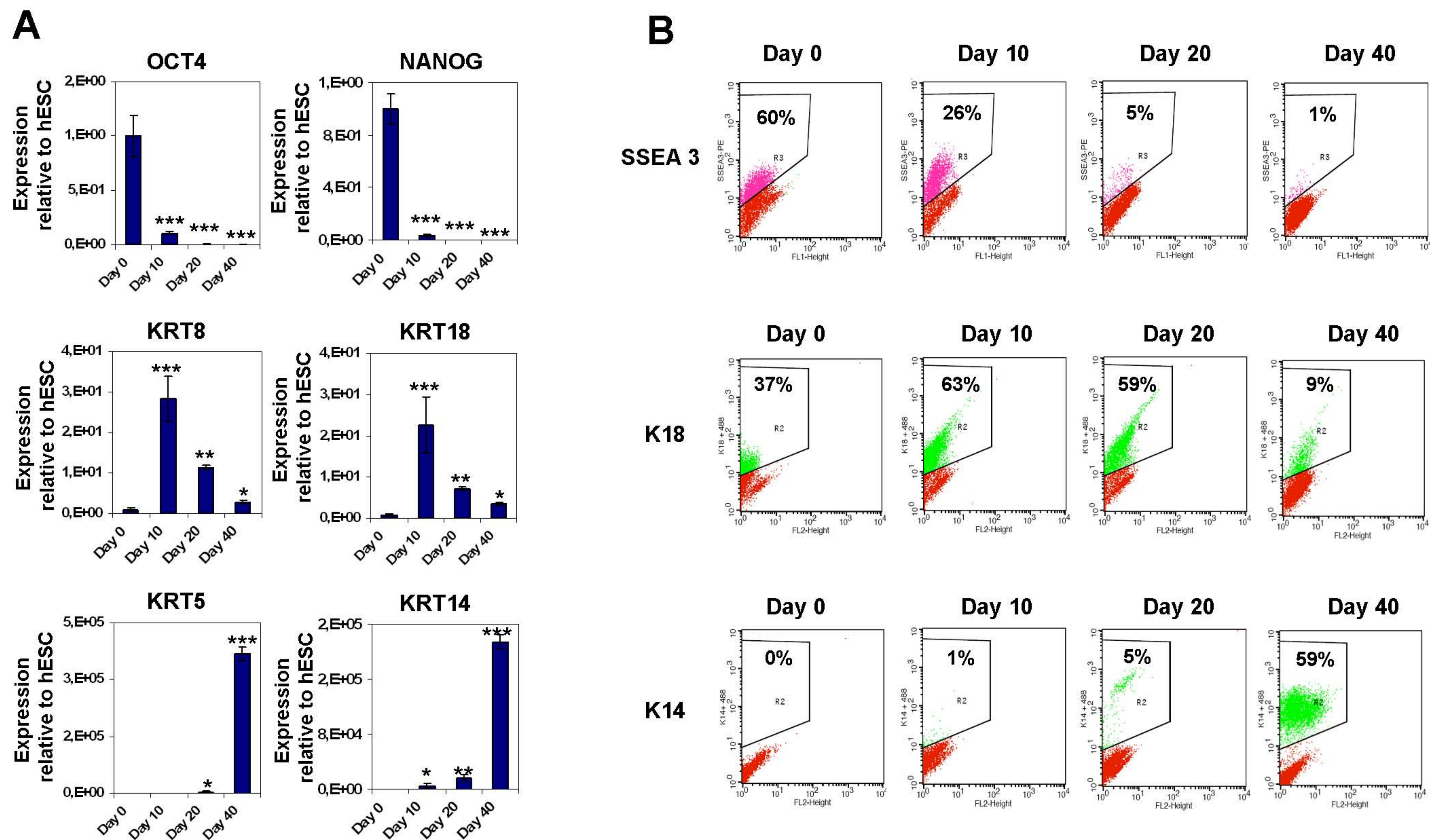
(B) PCR Array on organotypic HK and K-hESC epidermis. A large panel of epidermis genes has been tested on cDNA extracted from HK and K-hESC organotypic epidermis.

Data were collected using home made keratinocyte-focused primer quantitative PCR arrays and heat map analysis performed on Array Assist software. The two organotypic epidermis presented very similar patterns of expression.

Appendix 5: Long-term *in vivo* human epidermal regeneration following xenografting to 4 immunodeficient mice.

Haematoxylin-eosin staining of artificial skin implants grafted with K-hESC from H9. Scale bar is 100µm.

hal-00417656, version 1 - 16 Sep 2009



hal-00417656, version 1 - 16 Sep 2009

Figure 1: Establishment of a keratinocyte lineage

(A) Quantitative PCR analysis of *OCT4/NANOG*, *KRT8/KRT18*, *KRT5/KRT14* of derived embryonic stem cells (hESC) during the 40 days of differentiation. Data are presented as a mean ± SD of 3 independent experiments using each of the two cell lines H9 and SA-01, and were analysed with Student's t test (ANOVA), * (p value ≤ 0,05), **(p value ≤ 0,01) and *** (p value ≤ 0,001).

(B) Representative FACS analysis of SSEA3, keratins 18 and 14 (K18, K14) in derived H9-hESC during the 40 days of differentiation.

Figure 2 revised

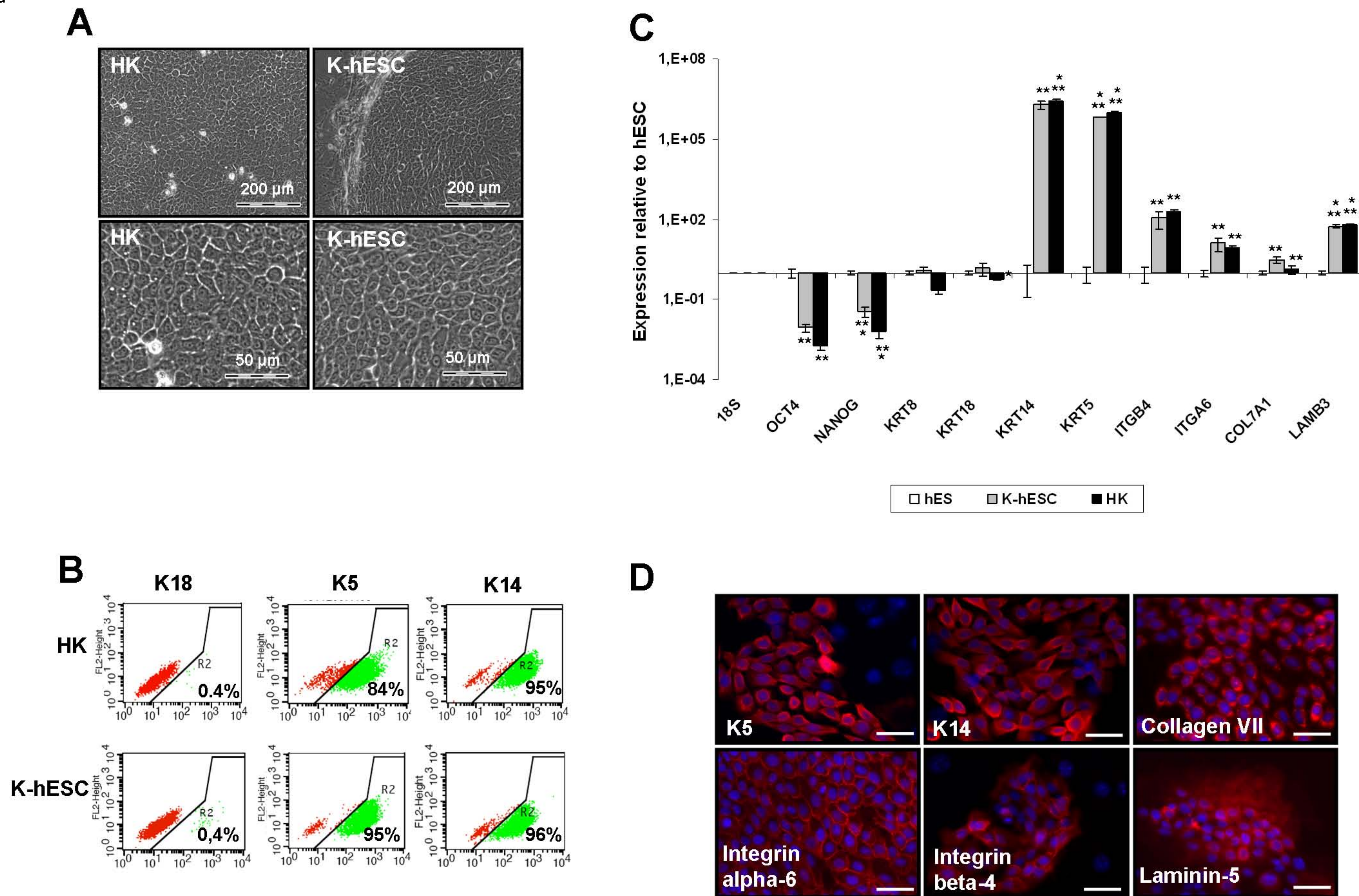


Figure 2 : Characterization of a homogenous population of keratinocytes derived from hESC.

(A) Microscopy analysis of human primary keratinocytes (HK), and keratinocytes derived from H9 (K-hESC) after subsequent culture.

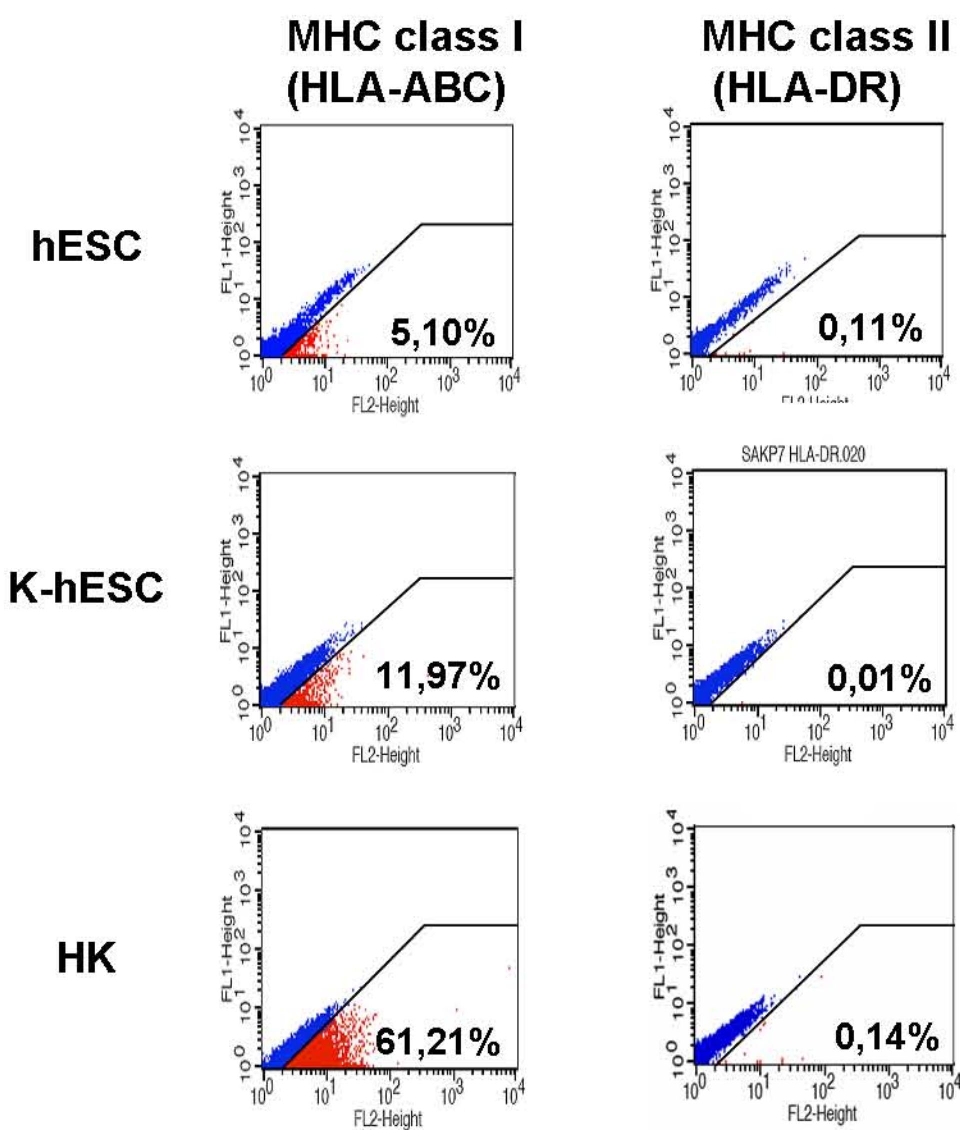
(B) Representative FACS analysis of keratins 18, 5 and 14 (K18, K5, K14) in HK and K-hESC derived from H9. .

(C) Quantitative PCR analysis of *OCT4/NANOG*, *KRT8/KRT18*, *KRT5/KRT14*, *ITGA6/ITGB4* and *LAMB3/Col7A1* in HK and K-hESC. Data are presented as a mean ± SD of 3 independent experiments using each of the two cell lines H9 and SA-01, and were analysed with Student's t test (ANOVA), * (p value ≤ 0,05), **(p value ≤ 0,01) and *** (p value ≤ 0,001).

(D) Immunofluorescence of K5, K14, integrins alpha-6 and beta-4, laminin-5 and collagen VII in K-hESC derived from H9. Scale bar is 20 µm.

hal-00417656, version 1 - 16 Oct 2009

hal-00417656, version 1 - 16 Sep 2009



<u>Figure 3: Expression of MHC class I (HLA-ABC) and class II (HLA-DR) proteins in hESC, K-hESC and HK.</u>
Representative FACS analysis of MHC class I (HLA-ABC) and class II (HLA-DR) expression in hESC, HK and K-hESC derived from H9.

.

Figure 4 revised

hal-00417656, version 1 - 16 Sep 2009

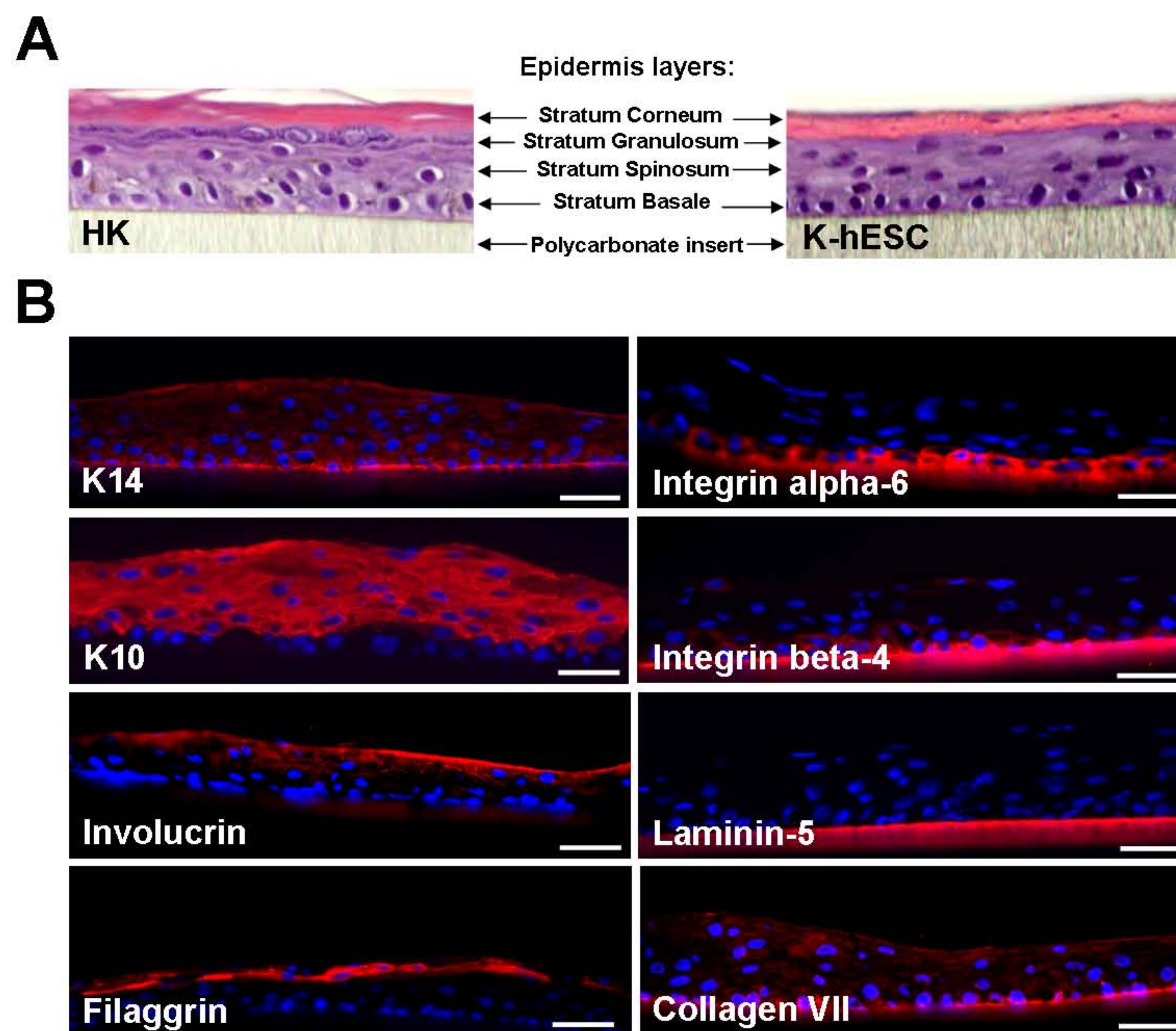


Figure 4: Reconstruction of a pluristratified epidermis using K-hESC.

(A) Haematoxylin-eosin staining of organotypic cultures of HK and K-hESC derived from SA-01.Scale bar is 50µm.

(B) Immunofluorescence analysis of the expression and localisation of keratin 14 (K14), keratin 10 (K10), involucrin, filaggrin, integrins.alpha-6 and beta-4, laminin-5 and collagen VII in the K-hESC derived from SA-01 organotypic epidermis. Scale bar is 50µm.

Figure 5 revised

hal-00417656, version 1 - 16 Sep 2009

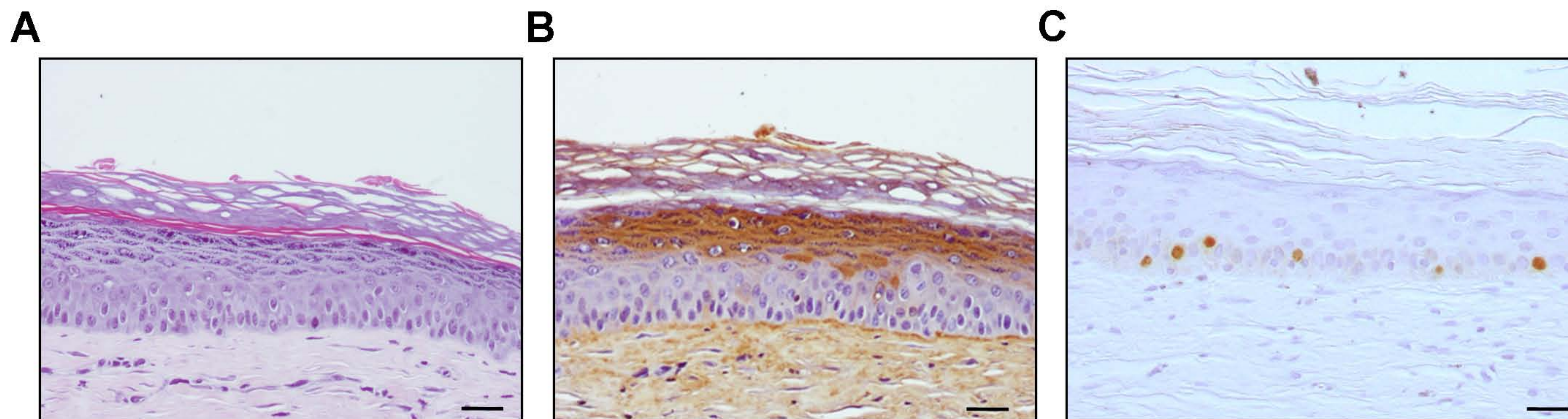


Figure 5: Long-term *in vivo* human epidermal regeneration following xenografting to immunodeficient mice.

(A) Haematoxylin-eosin staining of artificial skin implants grafted with K-hESC derived from H9. Scale bar is 50 µm.

(B) Immunoperoxydase staining using mAb SY-5 directed against human involucrin on artificial skin implants grafted with K-hESC derived from H9 is appropriately located in spinous and granular layers. Insert human involucrin staining in the human-mouse skin boundary. Note that dermal background could be observed, due to the anti-mouse secondary antibody. Scale bar is 50 µm.

(C) Immunoperoxydase staining for Ki67 on artifical skin implants grafted with K-hESC derived from H9. Scale bar is 50 µm.

webappendix table 1

Webappendix, Table 1: sequence of primers

Gene Name	Ref	Amplicon length (bp)	Forward primer	Reverse primer
18S	**NM_022551.2**	**146**	**GAGGATGAGGTGGAACGTGT**	**TCTTCAGTCGCTCCAGGTCT**
NANOG	**NM_024865.2**	**158**	**CAAAGGCAAACAACCCACTT**	**TCTGCTGGAGGCTGAGGTAT**
OCT4	**NM_002701.4**	**169**	**CTTGCTGCAGAAGTGGGTGGAGGAA**	**CTGCAGTGTGGGTTTCGGGCA**
KRT18	**NM_199187.1**	**129**	**GAGTATGAGGCCCTGCTGAACATCA**	**GCGGGTGGTGGTCTTTTGGAT**
KRT8	**NM_002273**	**69**	**GATCGCCACCTACAGGAAGCT**	**ACTCATGTTCTGCATCCCAGACT**
KRT14	**NM_000526.3**	**79**	**GGCCTGCTGAGATCAAAGACTAC**	**CACTGTGGCTGTGAGAATCTTGTT**
KRT5	**NM_000424.3**	**74**	**ATCTCTGAGATGAACCGGATGATC**	**CAGATTGGCGCACTGTTTCTT**
LAMB3	**NM_000228.2**	**241**	**GACAGGACTGGAGAAGCGTGTG**	**CCATTGGCTCAGGCTCAGCT**
COL7A1	**NM_000094.3**	**198**	**GATGACCCACGGACAGAGTT**	**ACTTCCCGTCTGTGATCAGG**
ITGA6	**NM_000210.2**	**112**	**GCTGGTTATAATCCTTCAATATCAATTGT**	**TTGGGCTCAGAACCTTGGTTT**
ITGB4	**NM_000213.3**	**220**	**CTGTACCCGTATTGCGACT**	**AGGCCATAGCAGACCTCGTA**

hal-00417656, version 1 - 16 Oct 2009