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Supplemental Information

Characterization of Fetal Keratinocytes, Showing Enhanced Stem Cell-Like Properties: A Potential Source of Cells for Skin Reconstruction

Kenneth K.B. Tan, Giorgiana Salgado, John E. Connolly, Jerry K.Y. Chan, and E. Birgitte Lane

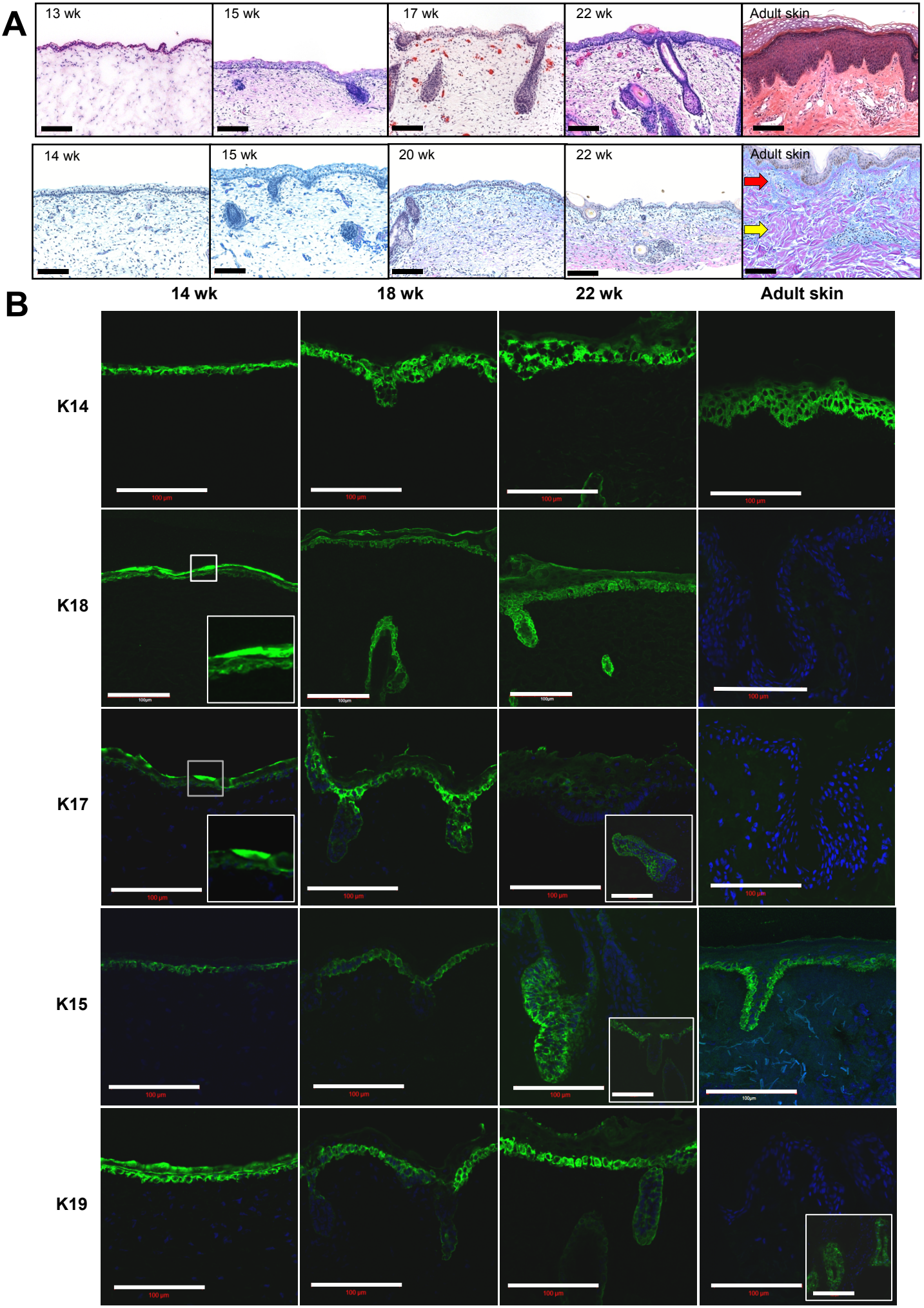

A
13 wk
15 wk
17 wk
22 wk
Adult skin
14 wk
15 wk
20 wk
22 wk
Adult skin
B
14 wk
18 wk
22 wk
Adult skin
K14
K18
K17
K15
K19

Figure S1

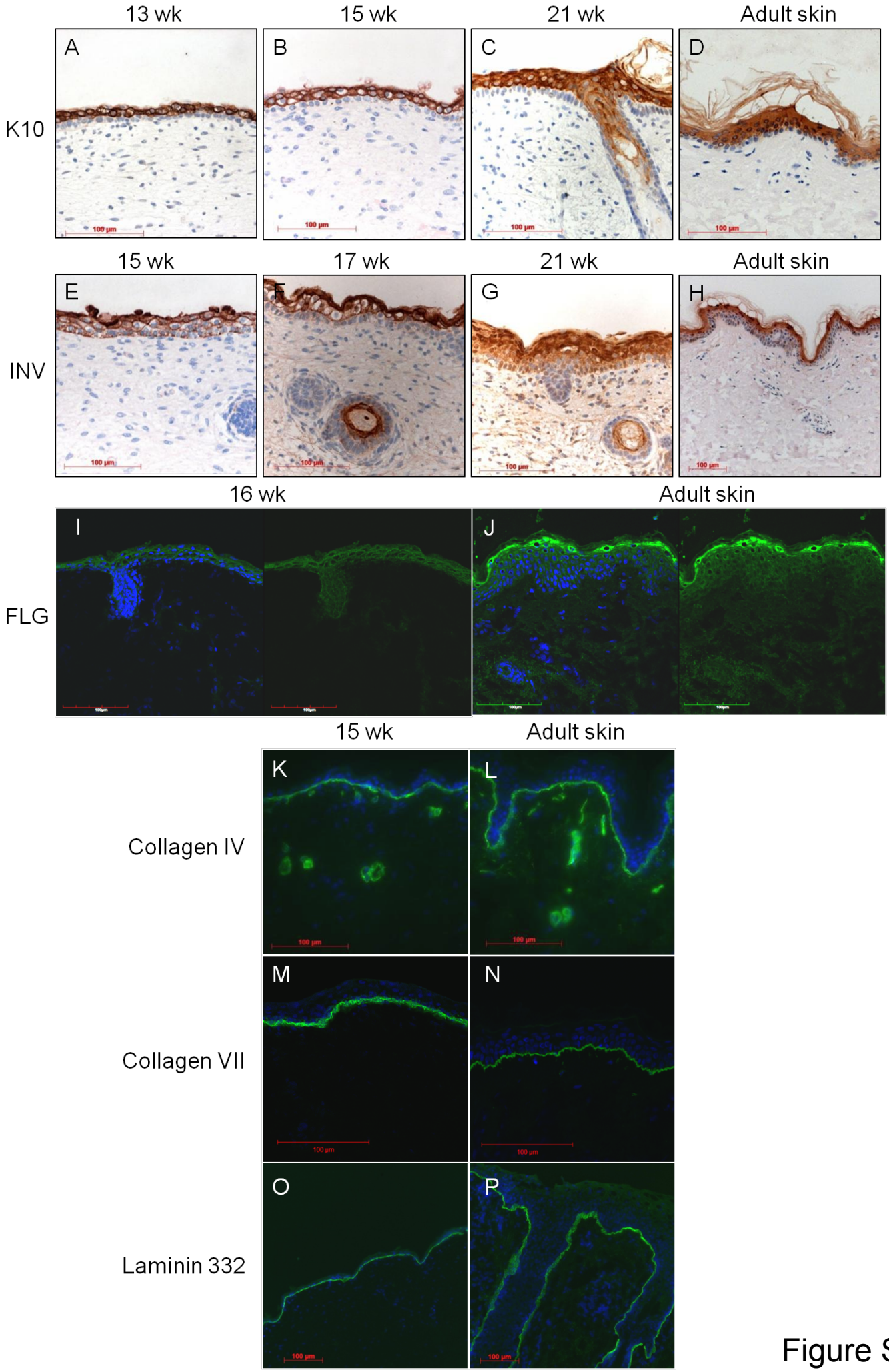

13 wk
15 wk
21 wk
Adult skin
K10
A
B
C
D
15 wk
17 wk
21 wk
Adult skin
INV
E
F
G
H
16 wk
Adult skin
FLG
I
J
15 wk
Adult skin
Collagen IV
K
L
Collagen VII
M
N
Laminin 332
O
P

Figure S2

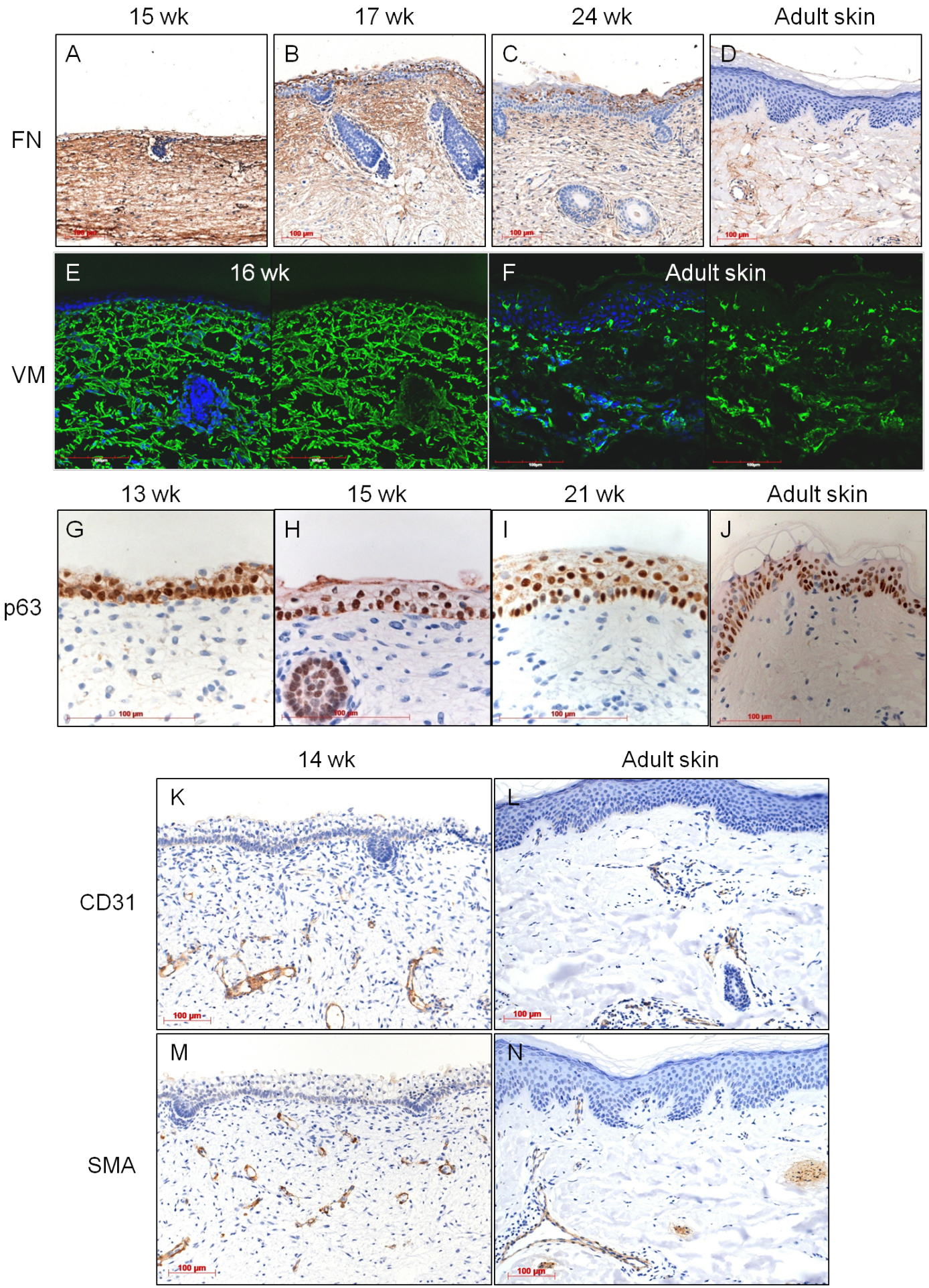

15 wk
17 wk
24 wk
Adult skin
FN
A
B
C
D
VM
E
16 wk
F
Adult skin
13 wk
15 wk
21 wk
Adult skin
p63
G
H
I
J
14 wk
Adult skin
CD31
K
L
SMA
M
N
100 µm

Figure S3

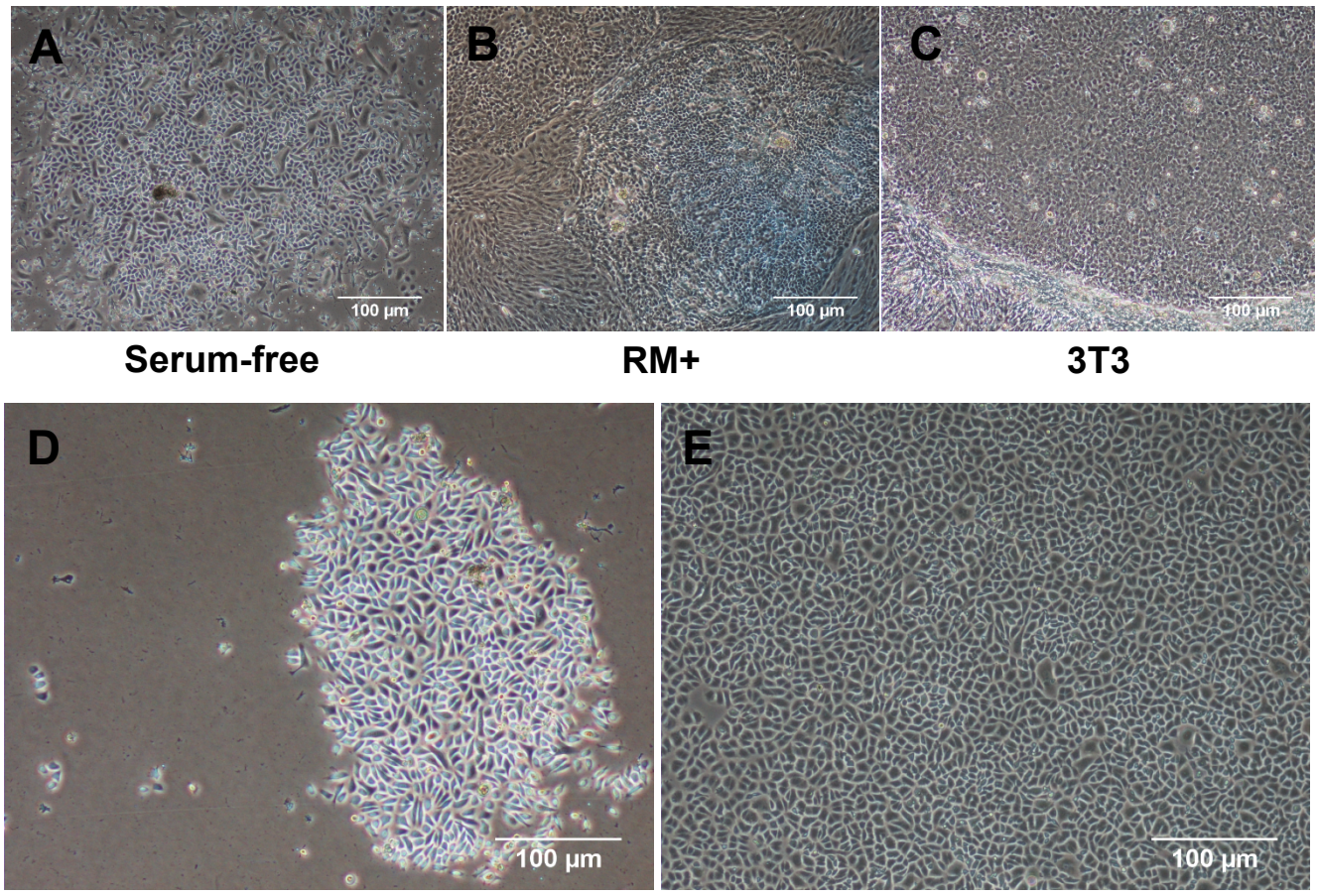
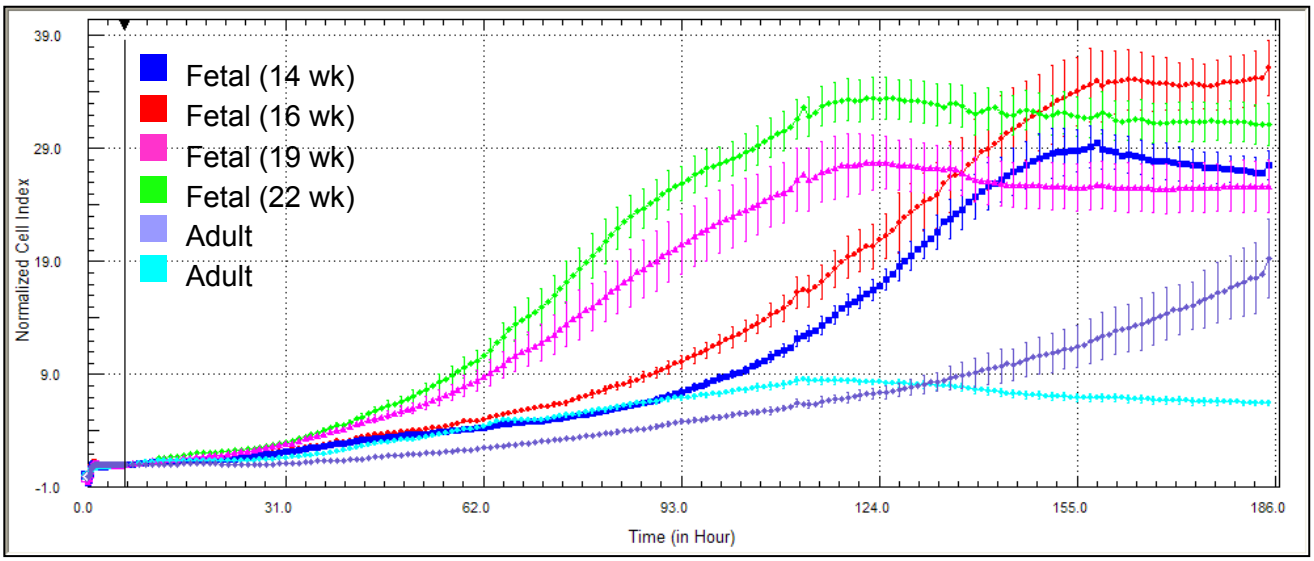

A
B
C
100 µm
100 µm
100 µm
Serum-free
RM+
3T3
D
E
100 µm
100 µm
F
Average Cell Diameter of Keratinocytes
Cell Diameter (µm)
24
22
20
18
16
14
Fetal (n=3)
Adult (n=3)
G
Average Cell Volume of Keratinocytes
Cell Volume (pL)
6
4
2
0
Fetal (n=3)
Adult (n=3)

Figure S4

A


Fetal (14 wk)
Fetal (16 wk)
Fetal (19 wk)
Fetal (22 wk)
Adult
Adult
Normalized Cell Index
39.0
29.0
19.0
9.0
-1.0
0.0
31.0
62.0
93.0
124.0
155.0
186.0
Time (in Hour)

B

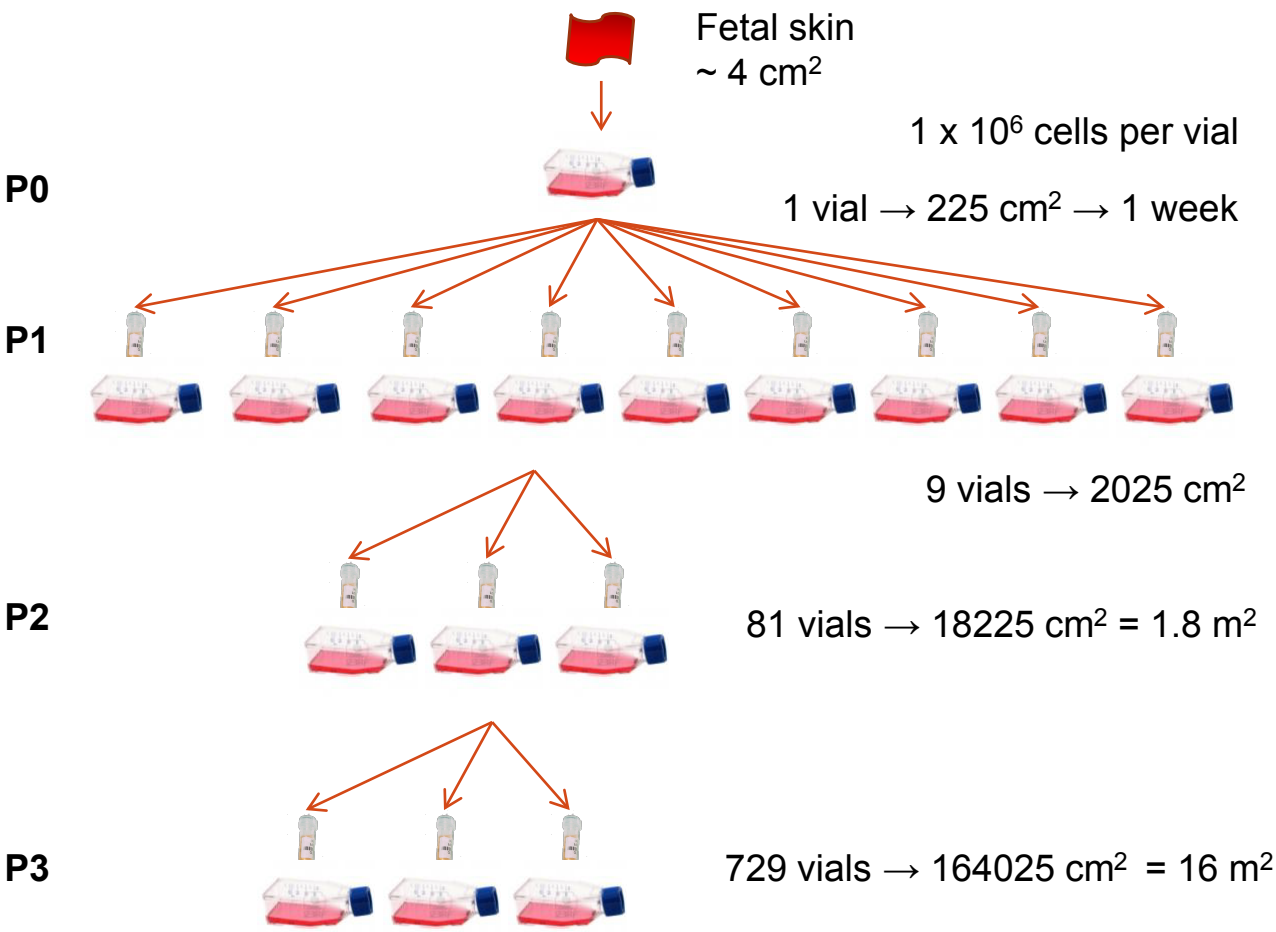

Fetal skin
~ 4 cm²
1 x 10⁶ cells per vial
P0
1 vial → 225 cm² → 1 week
P1
9 vials → 2025 cm²
P2
81 vials → 18225 cm² = 1.8 m²
P3
729 vials → 164025 cm² = 16 m²

Figure S5

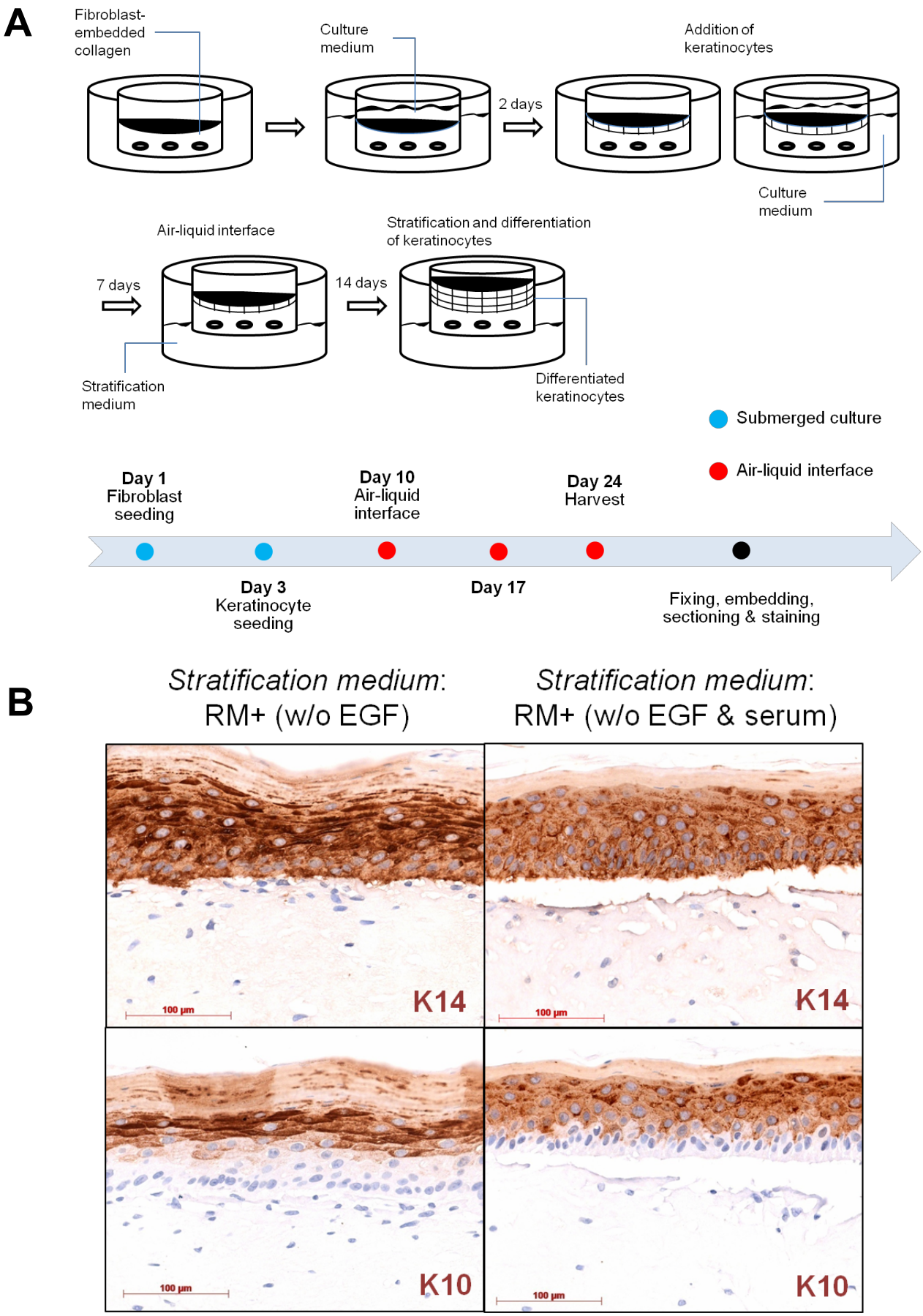

A
Fibroblast-embedded collagen
Culture medium
2 days
Addition of keratinocytes
Culture medium
Air-liquid interface
Stratification and differentiation of keratinocytes
7 days
14 days
Stratification medium
Differentiated keratinocytes
Submerged culture
Air-liquid interface
Day 1
Fibroblast seeding
Day 3
Keratinocyte seeding
Day 10
Air-liquid interface
Day 17
Day 24
Harvest
Fixing, embedding, sectioning & staining
B
Stratification medium: RM+ (w/o EGF)
Stratification medium: RM+ (w/o EGF & serum)
K14
K14
K10
K10
100 µm

Figure S6

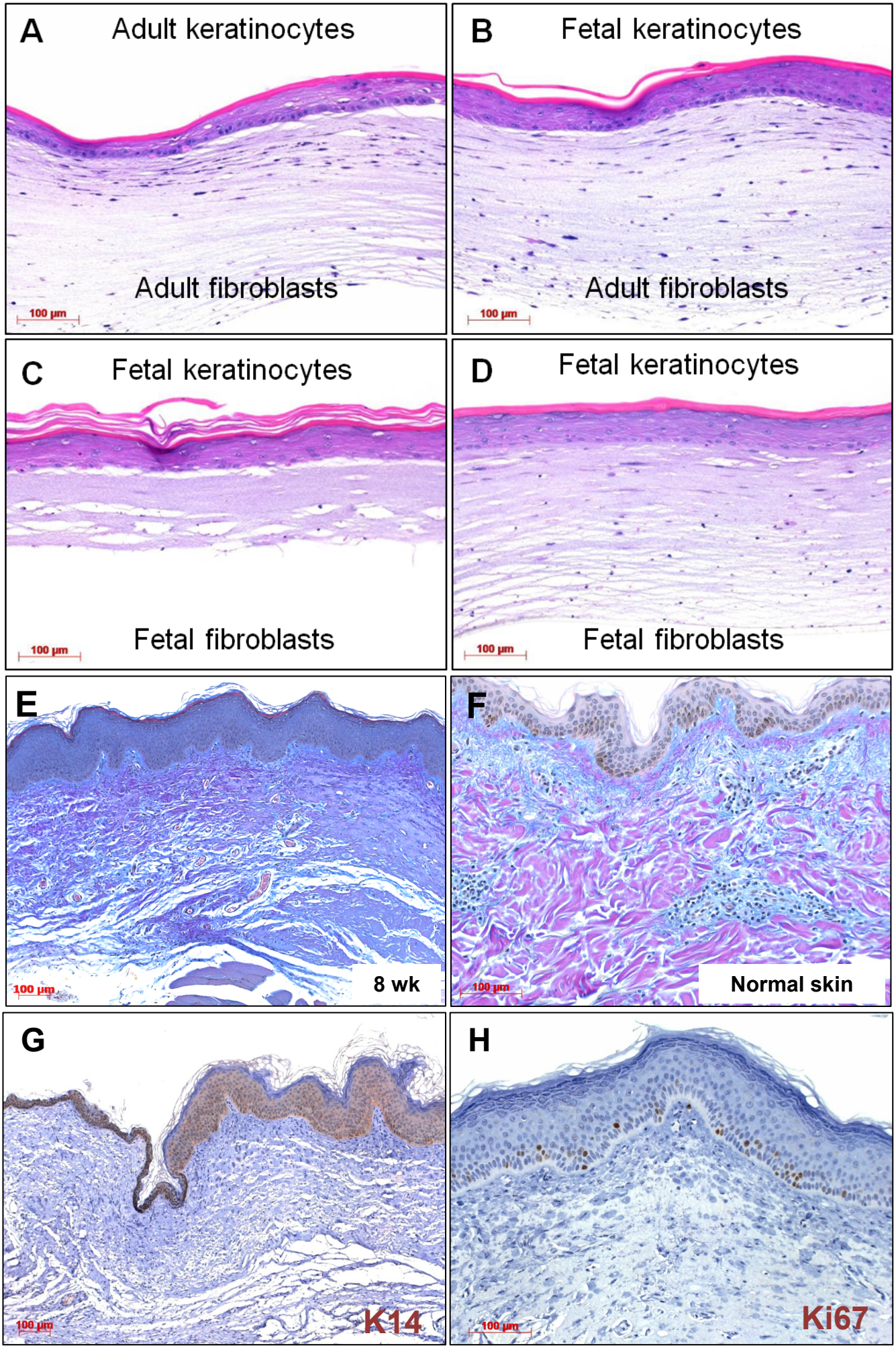

A
Adult keratinocytes
Adult fibroblasts
100 µm
B
Fetal keratinocytes
Adult fibroblasts
100 µm
C
Fetal keratinocytes
Fetal fibroblasts
100 µm
D
Fetal keratinocytes
Fetal fibroblasts
100 µm
E
8 wk
100 µm
F
Normal skin
100 µm
G
K14
100 µm
H
Ki67
100 µm

Figure S7

SUPPLEMENTAL FIGURE LEGENDS

Figure S1

Characterization of fetal skin, related to Figure 1. (A) *Top panel:* Histology of 13, 15, 17, and 22 weeks' gestation fetal skin and of adult skin. Fetal epidermis at 13 – 14 weeks gestation consists of a basal layer, a single intermediate/suprabasal layer and the overlying periderm (Figure 1A). Fetal dermis has a high density of fibroblasts and finely fibrillar matrix. From 15 weeks gestation, hair pegs can be seen projecting into the dermis, developing into recognizable hair follicles by about 17 weeks. The number of epidermal cell layers continues to increase with gestation, with the stratum corneum forming from 19 weeks gestation. By comparison, adult skin is thicker, with the epidermis consisting of 4-6 cell layers of outwardly-moving and progressively differentiating keratinocytes. *Bottom panel:* Herovici's staining of 14, 15, 20 and 22 weeks' gestation fetal (dorsal) skin and of adult skin. A higher ratio was observed of type III collagen (stained blue, red arrows) to type I collagen (stained pink, yellow arrows) in fetal skin than adult skin. With increasing gestation however, collagen I becomes more prominent especially in the reticular dermis as in the adult skin. Scale bar, 100 μm. (B) Immunofluorescence staining of keratins in fetal and adult epidermis. K18, K17, and K19 were present in fetal epidermis but not in adult epidermis. Scale bar, 100 μm.

Figure S2

Immunostaining of differentiation markers and basement membrane proteins in fetal and adult skin, related to Figure 1. K10, involucrin (INV), and filaggrin (FLG) in fetal (A-C, E-G, I) and adult (D, H, J) epidermis. The presence of differentiated keratinocytes was detected as early as 13 weeks. Presence of collagen IV, collagen VII and laminin 332 in fetal (K, M, O) and adult epidermis (L, N, P). Scale bar, 100 μm.

Figure S3

Immunostaining of fibronectin (FN), vimentin (VM), and p63, and blood vessels in fetal and adult skin, related to Figure 1. Fetal (A-C, E, G-I) and adult epidermis (D,F,J). Fetal dermis contains more fibronectin and vimentin compared to adult dermis. p63 is expressed in the nuclei of epidermal cells, more so in the basal cells in both fetal and adult epidermis. Dermal tissue did not contain any individual p63-positive cells. (K-N) Presence of blood vessels in 14 week gestation fetal skin stained with CD31 and α-smooth muscle actin (SMA). Scale bar, 100 μm.

Figure S4

Culture of fetal keratinocytes, related to Figures 1 and 2. Isolated cells can be cultured in (A) serum-free keratinocyte media, (B) complete RM+ media, or (C) co-cultured with irradiated 3T3 murine fibroblasts. (D) Removal of contaminating fibroblasts in serum-free media. The serum-free method of culture was adopted in all the experiments. (E) Confluent keratinocytes in serum-free media. RM+ medium consists of a mixture of 3 parts Dulbecco's modified Eagle's medium (DMEM) to 1 part Ham's nutrient mixture F12 medium, supplemented with 10% fetal bovine serum, 5 μg/ml insulin, 0.4 μg/ml hydrocortisone, 5 μg/ml transferrin, 1.8×10^{-4} M adenine, 10 ng/ml epidermal growth factor (EGF) and 2×10^{-11} M triiodothyronine. Scale bar, 100 μm. (F) Fetal keratinocytes are smaller in size compared to adult keratinocytes. Average cell diameter of fetal and adult keratinocytes at P4 cultured in serum-free medium. (G) Average cell volume of fetal and adult keratinocytes at P4 cultured in serum-free medium. Data are represented as mean of 3 biological replicates.

Figure S5

Proliferative potential of fetal keratinocytes, related to Figure 3. (A) Growth curves of fetal and adult keratinocytes. The population doubling times are derived from each exponential phases of the growth curves. Fetal and adult keratinocytes were plated in the 96-well plates in 5 replicates at 2500 cells per well. Cell growth was monitored over a period of 1 week. (B) A ~4 cm^2 of fetal skin will on average generate sufficient cells to be expanded to an area of 16 m^2 within 1 week after recovering live cells from a frozen cell bank.

Figure S6

Optimization of organotypic co-cultures, related to Figure 7. (A) Schematic illustration of experimental method for organotypic co-culture. (B) Immunoperoxidase staining of organotypic co-cultures with keratin 14 (K14) and keratin 10 (K10). In all conditions, K14 was expressed at the epidermal layer. Organotypic cultures at air-liquid interface that were cultured with RM+ medium without epidermal growth factor (EGF) and serum expressed K10 in the suprabasal layers immediately above the basal layer unlike those cultured with serum, in which K10 was expressed much later at the top layers, suggesting that keratinocyte differentiation conditions were suboptimal when cultured with serum. Organotypic experiments were thus cultured without serum when cultures were raised to air-liquid interface. Scale bar, 100 μm.

Figure S7

Characterization of culture-generated grafts, related to Figure 7. (A-D) Histological appearance of culture-generated human fetal skin using fibrin gel as a dermal equivalent. Organotypic cultures made with adult or fetal cells show similar stratification *in vitro* on

fibrin gels. Fibrin gel-based constructs supported the growth and differentiation of fetal and adult keratinocytes, forming epidermal-like structure and a cornified layer. (E) Herovici's staining of culture-generated human fetal skin on back of SCID mouse. Herovici's stain is capable of distinguishing between types I and III collagen, where the fine type III collagen fibres (young collagen) stain blue, and the thick and coarser type I collagen fibres (old collagen) stain pink. At 8 weeks post-grafting, a distinct layer of blue-staining fine collagen III fibres can be observed immediately underneath the epidermis, similar to the collagen architecture of normal human skin (F). Collagen I can also be seen throughout the dermis. (G-H) Immunostaining of K14 and proliferation marker Ki67 on the regenerated human skin (8 weeks post-grafting). K14 was stained throughout the human and mouse epidermis, and the proliferation marker Ki67 was mostly confined to the basal layer of the regenerated human epidermis. Scale bar, 100 μm.

Table S1

Immunohistochemical staining of various markers in fetal and adult skin

Marker	Fetal Skin	Adult Skin	Clone
Keratins			
K14	++ (basal); + (follicle)	++	LL001
K18	++ (basal); ++ (periderm); ++ (follicle)	–	LDK18
K17	++ (basal); + (periderm); + (follicle)	–	E3
K15	++ (basal); + (follicle)	+	LHK15
K19	++ (basal); ++ (periderm); + (follicle)	– (basal); + (follicle)	LP2K
Differentiation			
K10	++ (suprabasal); + (periderm)	++ (suprabasal)	LH1
Involucrin	++ (suprabasal); ++ (periderm)	++ (suprabasal)	SY5
Filaggrin	± (suprabasal)	++ (suprabasal)	15C10
Basement membrane proteins			
Collagen IV	++ (BM); ++ (bl vessels)	++ (BM); ++ (bl vessels)	PHM12
Collagen VII	++ (BM)	++ (BM)	LH7.2
Laminin 332	++ (BM)	++ (BM)	D4B5
Extracellular matrix proteins			
Fibronectin	++ (pap dermis); + (ret dermis)	± (dermis)	568
Intermediate filament proteins in connective tissue			
Vimentin	++ (dermis)	+ (dermis)	V9

Others			
p63	++ (epidermal nuclei)	++ (epidermal nuclei)	4A4
CD31	++ (bl vessels)	++ (bl vessels)	Polyclonal
α-smooth muscle actin	++ (bl vessels)	++ (bl vessels)	1A4

BM: basement membrane; bl: blood; pap: papillary; ret: reticular

Table S2

Antibodies used for Immunochemistry

Antigen	Clone	Working dilution	Source	Species
Keratin K14	LL001[1]	undiluted culture supernatant	E B Lane labs	Mouse
Keratin K18	LDK18*	undiluted culture supernatant	E B Lane labs	Mouse
Keratin K19	LP2K[2]	undiluted culture supernatant	E B Lane labs	Mouse
Keratin K15	LHK15[3]	1:50	Gift from IM Leigh	Mouse
Keratin K17	E3[4]	1:20	Novocastra	Mouse
Collagen IV	PHM12[5]	1:100	Novocastra	Mouse
Collagen VII	LH7.2[6]	1:50	Novocastra	Mouse
Laminin 332	D4B5[7]	1:50	Chemicon	Mouse
Fibronectin	568	1:100	Novocastra	Mouse
Vimentin	V9[8]	1:50	Novocastra	Mouse
Ki67	MM-1[9]	1:50	Novocastra	Mouse
Involucrin	SY5[10]	1:50	Novocastra	Mouse
Keratin K10	LH1[11]	undiluted culture supernatant	Gift from IM Leigh	Mouse
Filaggrin	15C10	1:50	Novocastra	Mouse
p63	4A4[12]	1:50	Gift from F McKeon	Mouse
Keratin K7	OV-TL 12/30	1:100	Novocastra	Mouse
HLA-ABC	W6/32[13]	1:100	DAKO	Mouse
HLA-DP, DQ,DR	CR3/43[14]	1:100	DAKO	Mouse
nucleoli	LP4N[15]	undiluted culture supernatant	E B Lane labs	Mouse
CD31	Polyclonal	1:50	Novus Biologicals	Mouse
α-smooth muscle actin	1A4	1:100	Abcam	Mouse
MTS24 / PLET-1	700738	1:50	R&D Systems	Rat
DLL1	polyclonal	1:100	Abcam	Mouse

Antibody references:

*E B Lane, unpublished.

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[5]Hancock, W.W., N. Kraft, F. Clarke, and R.C. Atkins. 1984. Production of monoclonal antibodies to fibronectin, type IV collagen and other antigens of the human glomerulus. *Pathology*. 16:197-206.

[6]Tatnall, F.M., P.C. Whitehead, M.M. Black, F. Wojnarowska, and I.M. Leigh. 1989. Identification of the epidermolysis bullosa acquisita antigen by LH 7.2 monoclonal antibody: use in diagnosis. *Br J Dermatol*. 120:533-9.

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[8]Bohn, W., Wiegers, W., Beuttenmüller, M., & Traub, P. (1992). Species-specific recognition patterns of monoclonal antibodies directed against vimentin. *Exp Cell Res., 201*(1), 1-7.

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[10]Hudson, D., Weiland, K., Dooley, T., Simon, M., & Watt, F. (1992). Characterisation of eight monoclonal antibodies to involucrin. *Hybridoma., 11*(3), 367-379.

[11]Leigh, I., Purkis, P., Whitehead, P., & Lane, E.B. (1993). Monospecific monoclonal antibodies to keratin 1 carboxy terminal (synthetic peptide) and to keratin 10 as markers of epidermal differentiation. *Br J Dermatol., 129*(2), 110-119.

[12]Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M., & Dötsch, V., et al. (1998). p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol Cell., 2*(3), 305-316.

[13]Barnstable, C., Bodmer, W., Brown, G., Galfre, G., Milstein, C., & Williams, A., et al. (1978). Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens-new tools for genetic analysis. *Cell., 14*(1), 9-20.

[14]Falini, B., Martelli, M., Tarallo, F., Moir, D., Cordell, J., & Gatter, K., et al. (1984). Immunohistological analysis of human bone marrow trephine biopsies using monoclonal antibodies. *Br J Haematol., 56*(3), 365-386.

[15]Jeppe-Jensen, D., H. Clausen, I.M. Leigh, E.B. Lane, and E. Dabelsteen. 1993. Three monoclonal antibodies differentiate human from murine epidermis. *Epithelial Cell Biol*. 2:100-6.

Table S3

Antibodies for Flow Cytometry

Antibody	Clone	Working dilution	Source	Species
HLA-ABC	W6/32	1:10	Dako	Mouse
HLA-DP, DQ,DR	CR3/43	1:10	Dako	Mouse

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Calculation of Proliferation Index in Tissues

Ki67-positive cells were counted by light microscopy (magnification 63×) at five random non-overlapping regions of each tissue section. Median number of basal cells was 30 (range 21 – 45). Values are expressed as the mean PI at each gestational age (PI = number of Ki67-positive basal cells/total basal cells ×100).

Growth Kinetics and Cell Proliferation Experiments in Culture

Fetal and adult keratinocytes were plated at 5,000 cells per cm^2 and successively subcultured at the same density when 80% confluence. Cell sizes were measured using Scepter Automated Cell Counter (Merck Millipore, Billerica, MA, USA) which uses the Coulter principle of impedance-based particle detection. After trypsinization cells were counted and cumulative cell doublings of the populations were plotted against time in culture. Population doublings were determined by counting cells at the start and end of each passage.

A real-time dynamic monitoring cell analyzer (xCELLigence, Roche Applied Science, Indianapolis, USA) was used to evaluate proliferation of fetal and adult keratinocytes. Wells of 2,500 cells were established in replicates of five. After seeding 150 µl of cell suspension into the wells of the E-plate 96 (Roche Applied Science), cells were allowed to settle for 6 hr before starting analysis. Keratinocyte growth was monitored every hour for a period of one week. Doubling times were derived from the exponential phase of the each growth curves and calculated using RTCA software 1.2.1 (Roche Applied Science).

Colony Forming Assay

To assess colony-forming ability in these feeder-free culture conditions, 200 cells or 500 cells were seeded in triplicate onto 6 cm dishes and cultured for two weeks at 37^{o}C with 5% CO_2. Cells were fixed with 10% neutral buffered formalin and stained with Rhodamine B (J.T. Baker, Pennsylvania, USA). Colony forming efficiency (CFE) was expressed as the percentage of colonies (>1mm^2) formed divided by the number of cells seeded.

Telomere Restriction Fragment Analysis

TeloTAGGG Telomere Length Assay kit (Roche Applied Science, Indianapolis, USA) was used for telomere length experiments. 2 µg of genomic DNA was isolated from keratinocytes using DNeasy® Blood & Tissue Kit (Qiagen, Germany), digested with frequent-cutter enzymes Hinf I and RsaI, then separated on a 0.9% agarose gel overnight by gel electrophoresis. Gels were immersed sequentially in depurination, denaturation and neutralization solutions, followed by overnight Southern transfer of DNA fragments to a nylon membrane by capillary action. After DNA fixation by UV-crosslinking at 120 mJ, blotted DNA fragments were hybridized with telomere-specific digoxigenin (DIG)-labeled hybridization probe (TTAGGG) at 42^{o}C overnight. Membranes were washed, blocked, and incubated with anti-DIG antibody covalently coupled to alkaline phosphatase at room temperature for 30 min. Finally, membranes were washed and incubated with CDP-Star, a highly sensitive chemiluminescent substrate. Average telomere restriction fragment (TRF) length was determined by comparing the location of TRF on the blot relative to a molecular weight standard.

Flow Cytometry Analysis

Cultured keratinocytes were trypsinized and cell suspension was fixed in 70% ethanol. Cells were immunolabeled for MHC molecules or isotype control IgG (Table S3) and sorted using BD LSR II Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ). Cells were counted (10,000 events) and data were analyzed with gating using FlowJo research software.