



Generation of Human Epidermis-Derived Mesenchymal Stem Cell-like Pluripotent Cells (hEMSCPCs)

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We isolated human epidermis-derived mesenchymal stem cell-like pluripotent cells (hEMSCPCs) and demonstrate efficient harvesting, maintenance *in vitro* for at least 30 passages, reprogramming into multiple phenotypes *in vivo*, and integration into adult host tissues after injection into the mouse blastocyst to create chimeras. Cell phenotype was examined by karyotyping, immunostaining, immunofluorescence, and flow cytometry. A nested PCR protocol using primers specific for human SRY genes was designed to detect hEMSCPC-derived cells in female chimeric mice. FISH was used to validate the results of nested PCR. Results indicated that hEMSCPCs were derived from epidermis but were distinct from epidermal cells; they resembled mesenchymal stem cells (MSCs) morphologically and expressed the main markers of MSCs. About half of all female offspring of mice implanted with embryos injected with hEMSCPCs at the blastocyst stage harbored the human Y chromosome and tissue-specific human protein, thereby demonstrating the transdifferentiation of hEMSCPCs.

Stem cells can be derived from the embryo (embryonic stem cells, ESCs), from adult tissues (adult stem cells, ASCs), and by induction of fibroblasts (induced pluripotent stem cells, iPSCs). However, ethical problems, immunological rejection, and difficulties in obtaining human tissues limit the use of ESCs in clinical medicine^{1,2}, while iPSCs are difficult to maintain *in vitro* and carry a greater risk of tumor formation. The maintenance and propagation of these cells is especially difficult in the clinic due to the complex harvesting, isolation, and culture conditions required^{3–10}. In contrast, ASCs can be isolated from several adult tissues and present the possibility of self-transplantation for the clinical treatment of a variety of human diseases.

Recently, several ASCs have been successfully isolated and cultured *in vitro*, including hematopoietic stem cells (HSCs)¹¹, mesenchymal stem cells (MSCs)^{12,13}, epidermis stem cells¹⁴, neural stem cells (NSCs)¹⁵, adipose-derived stem cells (ADSCs)^{16–18}, islet stem cells^{19,20}, and germ line stem cells^{21–23}. Human mesenchymal stem cells originate mainly from bone marrow^{24,25}, cord blood^{26–28}, placenta^{29–31}, and endometrium³², but epidermis-derived MSCs have not yet been isolated. In the present study, we isolated small spindle-shaped cells with strong proliferative potential from human epidermis. They resembled MSCs morphologically and demonstrated pluripotency *in vivo*; thus, we defined these cells as human epidermis-derived mesenchymal stem cell-like pluripotent cells (hEMSCPCs). These hEMSCPCs expressed many typical markers of MSCs and NSCs, demonstrated good bio-safety³³, and could differentiate into neural-like cells³⁴ and immunocyte-like cells³⁵ under appropriate conditions. In the current study, we demonstrate that hEMSCPCs cells can be reprogrammed after injection into the mouse blastocyst cavity to form heterogeneous chimeras. Indeed, hEMSCPC-derived cells were present in several organs of the postnatal (1–5-month-old) mouse and expressed organ-specific functional proteins. Consequently, we have not only successfully isolated and cultured a new type of ASC with strong viability *in vitro*, but also demonstrated reprogramming and transdifferentiation after blastocyst cavity injection. These hEMSCPCs fulfill many of the requirements for clinical cell therapy, including large-scale harvesting, prolonged expansion *in vitro*, biocompatibility and safety, and pluripotency.

Results

Derivation of hEMSCPCs and morphology *in vitro*. To obtain human epidermis-derived mesenchymal stem cell-like pluripotent cells (hEMSCPCs), we first designed a selective culture medium (hEMSCPC-specific



medium). We obtained eight foreskin specimens from surgical patients confirmed negative for HIV, hepatitis, and leptospira infection. After treating the foreskin tissue with a digestive solution, the tissue was washed at least five times in PBS to prevent hypodermal cell contamination, and the epithelial layer isolated from the basilar membrane and treated with a digestive solution. Individual epithelial cells were then obtained by mechanical trituration, resuspended in hEMSCPC-specific medium, and cultured. On day two, the culture medium was replaced and non-adherent cells removed. Spindle-shaped cells with small cell bodies were observed after 7–10 days in vitro (P0 7d; Fig. 1A). While the majority of cells died, polygonal epithelial-like cells grew in some cultures. Between days 5 and 10, the culture medium was replaced (as indicated by acidification) with gentle agitation to remove dead cells. The number of spindle-shaped cells with small cell bodies gradually increased over the next days and weeks (Fig. 1A, P0 12d & P0 15d). These spindle-shaped cells were harvested at two to three weeks in vitro as they were more easily detached from the culture plates than the polygonal epithelial-like cells. Thus, we could selectively separate these two cell types by controlling the digestion time.

Isolated spindle-shaped cells with small cell bodies were then cultured alone in hEMSCPC-specific medium. Cells were split at 1:3 every 1.5–3 days (Fig. 1B, P2 3d) and they continued to proliferate rapidly up to passage 30. Most of these cells were short and spindle-shaped before passage 10 (Fig. 1B, P2 2d), but showed greater morphological heterogeneity after passage 10 (Fig. 1C–E). Most had two to three projections (Fig. 1C, round figure on the left, indicated by the arrow), while a few had several projections (Fig. 1C, round figure on the right, indicated by the arrow). In some specimens, most of the hEMSCPCs had short projections and only a few had longer slender projections (Fig. 1D, round figure on the left, indicated by the arrow),

while hEMSCPCs obtained from other specimens had mostly long slender projections. When hEMSCPCs formed a monolayer, they took on a vortex pattern resembling MSCs (Fig. 1F). Karyotyping between passages 30 to 32 revealed 46 chromosomes with X and Y (Supplementary Fig. 1).

Marker protein expression patterns revealed by immunocytochemistry. Past studies have not found mesenchymal stem cells in the epidermis, so the origin of these hEMSCPCs was initially uncertain. We surmised that epidermal stem cells might transdifferentiate into hEMSCPCs in the microenvironment supplied by the special medium. To investigate this possibility and to examine phenotypic changes during long-term culture, we examined the expression of several markers known to be expressed by skin-derived cells, including the MSC markers CD73, CD90, and CD105, the fibroblast marker vimentin, the NSC marker nestin, the neuronal marker β -III tubulin, the glial marker GFAP, the immunocyte markers CD3, CD19, CD16, and CD45, the HSC marker CD34, the epithelial cell marker CK19, the basilar membrane cell marker CD10³⁶, the vascular endothelial cell markers CD31 and VEGF-R2, and the human histocompatibility antigens HLA-DR and HLA-I. We assessed expression patterns at passages 2, 10, 20, and 30 by immunohistochemistry. Only a fraction of hEMSCPCs expressed CD90 and nestin (Fig. 2A) at passage 2, but almost all expressed CD90 (Fig. 2I) and nestin (Fig. 2C) at passages 10, 20, and 30. At all passages, most hEMSCPCs expressed CD73 (Fig. 2J) and vimentin (Fig. 2B, D), while a significant fraction expressed CD105 (Fig. 2K), and a few cells expressed β -III tubulin, GFAP, CK19 (Fig. 2E), or CD10 (Fig. 2F). Very few expressed the immunocyte markers CD3 (Fig. 2G), CD19, CD16, or CD45, or the HSC marker CD34. No cells at passage 2 expressed the vascular endothelial cells markers CD31

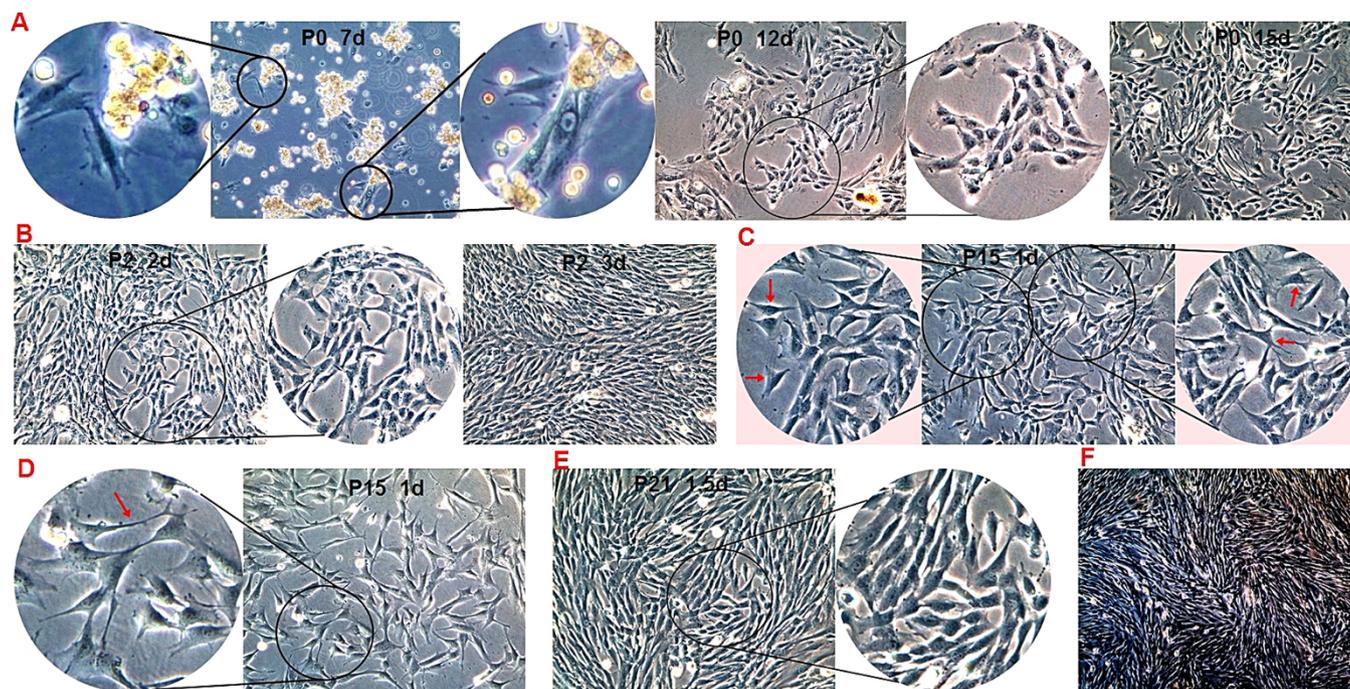


Figure 1 | Morphology of foreskin-derived cells of the epidermal layer during culture in specialized hEMSCPC media. (A): When cells from adult foreskin epidermis were cultured in vitro for 7–10 days, small fusiform cells appeared (P0 7d), while other cell types almost completely disappeared. With prolonged incubation, these small fusiform cells continued to increase in number (P0 12d and P0 15d). (B): Before passage 10, most cells were short and spindle-shaped (P2 2d). Within the first 30 passages, cells generally formed a single layer within 1.5–3 days after replating (P2 3d). (C–E): After passage 10, most cells had short spindle or regular spindle morphologies, with two to three processes projecting from the soma (C, round figure on the left, see arrow), while a minority had multiple processes (C, round figure on the right, see arrow). In some specimens, most of the hEMSCPCs had short processes, while only a minority had long and slim processes (D, round figure on the left, see arrow). In other specimens, however, most of the hEMSCPCs had long and slim processes. (F): When hEMSCPCs formed a monolayer, they took on a swirling arrangement resembling that of mesenchymal stem cells.

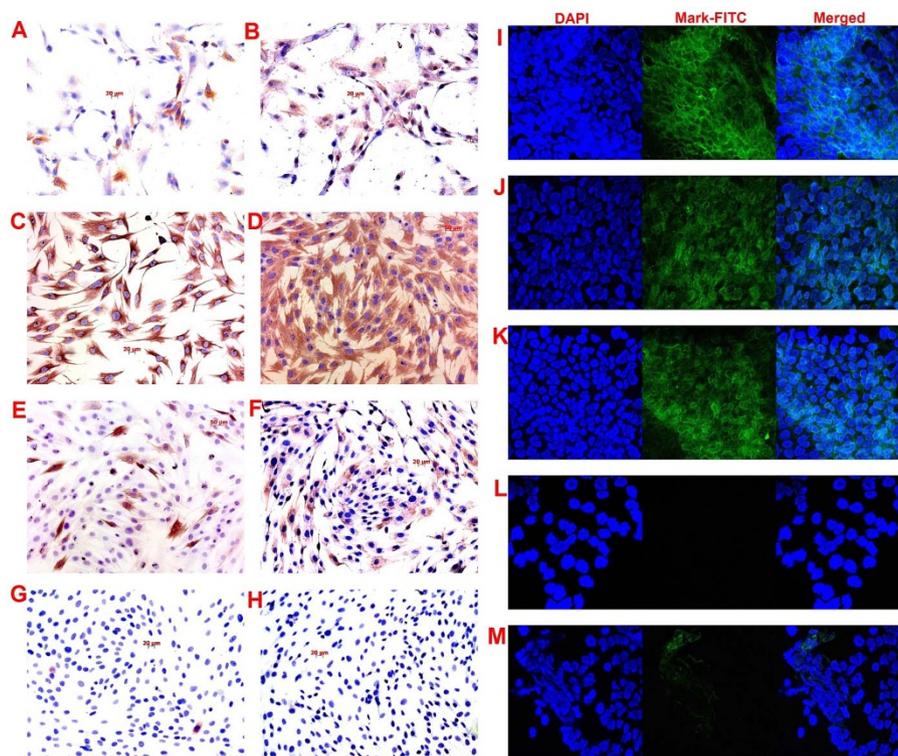


Figure 2 | Immunohistochemical expression patterns of hEMSCPCs over 30 passages. (A): Some cells at P2 showed nestin positive immunoreactivity (brown color). (B): Almost all cells at P2 were vimentin positive (brown color). (C): Almost all cells at P10, P20, and P30 showed nestin immunoreactivity (brown color). (D): Almost all cells at P10, P20, and P30 were vimentin immunoreactive (brown color). (E): At P2, P10, P20, and P30, a small number of cells were CK19 immunoreactive (brown color). (F): At P2, P10, P20, and P30, a limited number of CD10 immunoreactive cells were observed (brown color). (G): At P2, P10, P20, and P30, a few individual cells were CD3 immunoreactive (brown color). (H): At P2, no CD31-positive or VEGF R2-positive cells were seen. (I): At P10, P20, and P30, almost all cells expressed CD90 (green fluorescence). (J): At P2, P10, P20, and P30, most cells expressed CD73 (green fluorescence). (K): At P2, P10, P20, and P30, a fraction of cells expressed CD105 (green fluorescence). (L): At P10, P20, and P30, some specimens had no HLA-DR-positive cells. (M): At P10, P20, and P30, a small number of HLA-I-positive cells were observed in some specimens (green fluorescence).

and VEGF-R2 (Fig. 2H), while only a few CD31 and VEGF-R2 positive cells were found at passages 10, 20, and 30. Some hEMSCPCs were positive for human histocompatibility antigens HLA-DR and HLA-I at passage 2 (Fig. 2M), but expression decreased with passage and time in culture. Some cultures showed no HLA-DR-positive cells at passages 10, 20, or 30 (Fig. 2L).

Quantitative analysis of marker protein expression changes by flow cytometry. For quantitative analysis, we detected marker expression at passages 2, 10, 20, and 30 by flow cytometry. The results confirmed immunohistochemistry results. Cultured hEMSCPCs expressed abundant CD73 and vimentin, moderate CD105, low levels of β -III tubulin, GFAP and CD10, while only sporadic cells expressed CD3, CD19, CD16, CD45, CD34, CD31, VEGF-R2, or CK19 at passages 2, 10, 20, and 30. Cultured hEMSCPCs expressed moderate CD90 and nestin at passage 2, but highly expressed CD90 and nestin at passage 10, 20, and 30. Expression of the human histocompatibility antigens HLA-DR and HLA-I was moderate to low at passage 2, but HLA-positive cells became scarce as the passage number increased. Indeed, no HLA-DR expression was observed in some specimens at passages 10, 20, or 30 (Fig. 3 and supplementary Table 1).

A few CK19-positive hEMSCPCs were detected by immunohistochemistry, but no CK19-positive cell were detected by flow cytometry, possibly because these are epithelial-like cells that cannot be easily detached from the culture flask by trypsin, and so are lost before flow cytometry analysis.

Construction of chimeras and multipotency of hEMSCPCs. To determine if these hEMSCPCs were multipotent like other ACSs,

we injected these cells into the blastocyst cavities of mouse embryos and followed the development of chimeric mice. As hEMSCPCs expressed only very low levels of the human histocompatibility antigens HLA-DR and HLA-I, we surmised that mouse blastocyst cavity injection would be a viable strategy to study the multipotency of these cells. We injected passage 10–13 hEMSCPCs into the blastocyst cavity, and cultured the embryos in vitro for 13 to 15 hours. Well-developed embryos were transplanted into the uteruses of pseudopregnant female mice and allowed to develop to term. We obtained 135 offspring (73 male and 62 female) that grew without obvious health defects over 1–5 months. We designed two primer pairs specific for human SRY genes³⁷ of the Y chromosome and used them to detect the human Y chromosomes in blood, brain, heart, lung, spleen, liver and kidney of female mouse offspring by nested PCR. Furthermore, to validate the detection by nested PCR, we performed fluorescence in situ hybridization (FISH) using specific probes for the human Y chromosome and examined immunofluorescence staining for specific markers of human blood, brain, heart, lung, spleen, liver, and kidney cells under confocal laser scanning microscopy.

Results of the nested PCR showed that 27 of 62 female offspring were positive (43.5%) for SRY genes. The distribution of positive organs (Supplementary Table 2) revealed that 15 of 62 (24.2%) female offspring had blood cells positive for SRY genes (Fig. 4A), 4 of 62 (6.5%) had SRY-positive brain tissue (Fig. 4B left), 6 of 62 (9.7%) SRY-positive heart tissue (Fig. 4B right), 4 of 62 (6.5%) SRY-positive kidney tissue (Fig. 4C left), 4 of 62 (6.5%) SRY-positive spleen tissue (Fig. 4C right), 8 of 62 (12.9%) SRY-positive lung tissue (Fig. 4E left), and 3 of 62 (4.8%) SRY-positive liver tissue (Fig. 4E right).

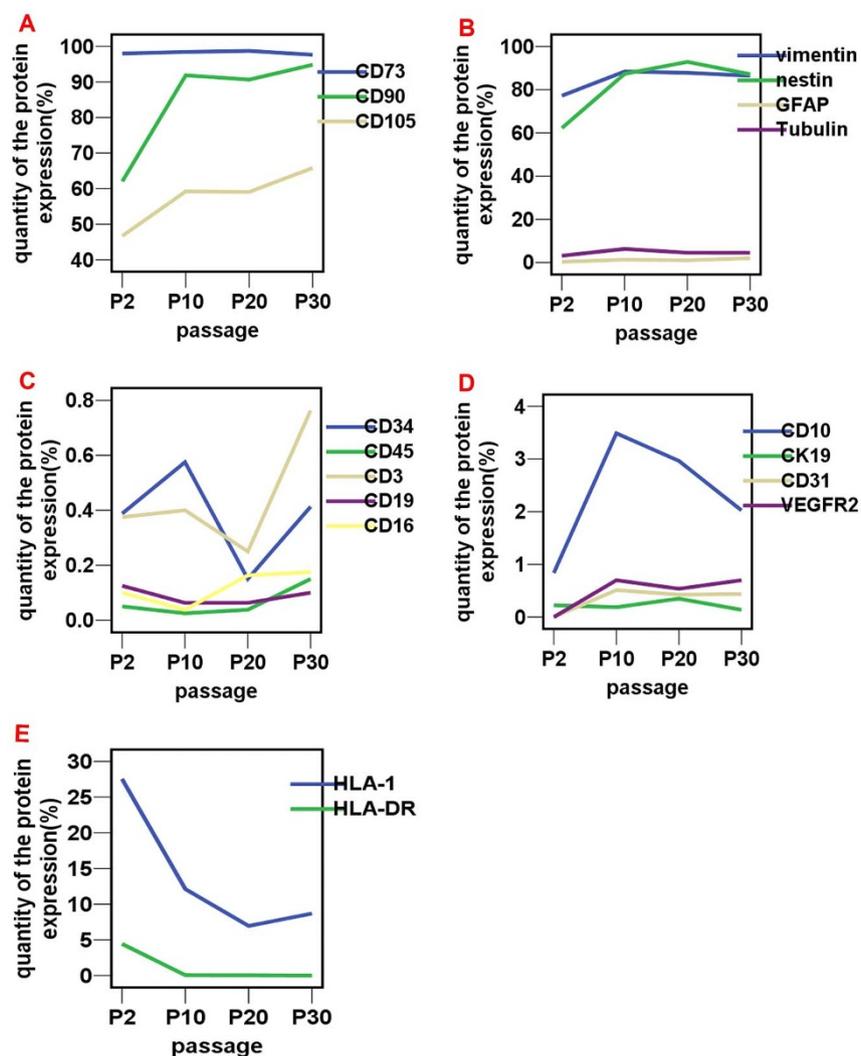


Figure 3 | Flow cytometry analysis of cell marker expression over time in culture. Flow cytometry plots for various markers at P2, P10, P20, and P30. (A): CD73 was always highly expressed at P2, P10, P20, and P30; CD90 was moderately expressed at P2, and highly expressed at P10, P20, and P30; CD105 was moderately expressed at P2, P10, P20 and P30. (B): Substantial vimentin expression was observed at P2, P10, P20, and P30. Nestin expression mirrored CD90 expression. Nestin expression was moderate at P2 but high at P10, P20, and P30, whereas GFAP and β -III tubulin showed minimal expression throughout the culture period. (C): Human EMSCPCs expressed very low levels of CD34, CD45, CD3, CD19 and CD16. (D): CD10 expression was always low, while CK19, CD31, and VEGF R2 were barely detectable. (E): The tissue compatibility antigens HLA-DR and HLA-I were moderately or minimally expressed at P2; with increasing number of passages, fewer and fewer cells expressed these markers. No HLA-DR-positive cells were detected in some specimens at P10, P20, and P30.

Cultured hEMSCPCs and cells from caudal vein blood of nested PCR-positive mice were analyzed by in situ hybridization, and results confirmed those of nested PCR. Over 90% of the hEMSCPCs were human Y-positive (Fig. 5A), and the positive karyocytes in the caudal vein blood of positive female offspring had differently shaped nuclei (Figure 5 B–E).

We also used human Y-specific probes for in situ hybridization and tissue-specific antibodies for immunofluorescence staining to test for the presence of hEMSCPC-derived cells expressing tissue-appropriate proteins. Indeed, blood, brain, cardiac muscle, lung, spleen, liver, and renal glomerulus cells that were SYR-positive by nested PCR were also positive by in situ hybridization and expressed several tissue-specific human antigens (Supplementary Fig. 2). These antigens included CD3 and CD19 in peripheral blood cells, CD16 in peripheral blood cells and spleen cells, MAP2 and β -III tubulin in brain cells, troponin I in cardiac muscle cells, SP-C and CD31 in lung cells, ALB in liver cells, and VEGF-R2 in renal glomerulus cells. The fraction of cells expressing tissue-specific antibodies was consistent with the fraction expressing the human SRY gene. In 5 randomly

chosen human SRY-positive mouse blood samples, $21.821 \pm 6.394\%$ of cells were positive for CD3, $14.995 \pm 6.178\%$ for CD16, and $17.926 \pm 4.528\%$ for CD19 (Supplementary Table 3). In contrast, these specific antigens were undetectable by immunofluorescence staining in cultured hEMSCPCs of the same passage as those injected into the blastocyst cavity (except for a few cells expressing MAP2 and β -III tubulin, results not shown). Thus, hEMSCPCs not only survived in vivo but could reprogram and transdifferentiate into tissue-specific phenotypes. These tissues include ectoderm (brain), mesoderm (blood, heart, spleen and kidney), and endoderm (lung and liver). Therefore, hEMSCPCs are multipotent like all ASCs, can be reprogrammed by the in situ microenvironment of the mouse blastocyst cavity, and have pluripotency to generate all three germ layers.

Discussion

Unlike embryonic stem cells (ESCs), neither adult stem cells (ASCs) nor iPSCs can proliferate rapidly in vitro. Furthermore, ASCs and iPSCs cannot be harvested in large quantities and the difficulty of deriving and maintaining these cells makes routine clinical use

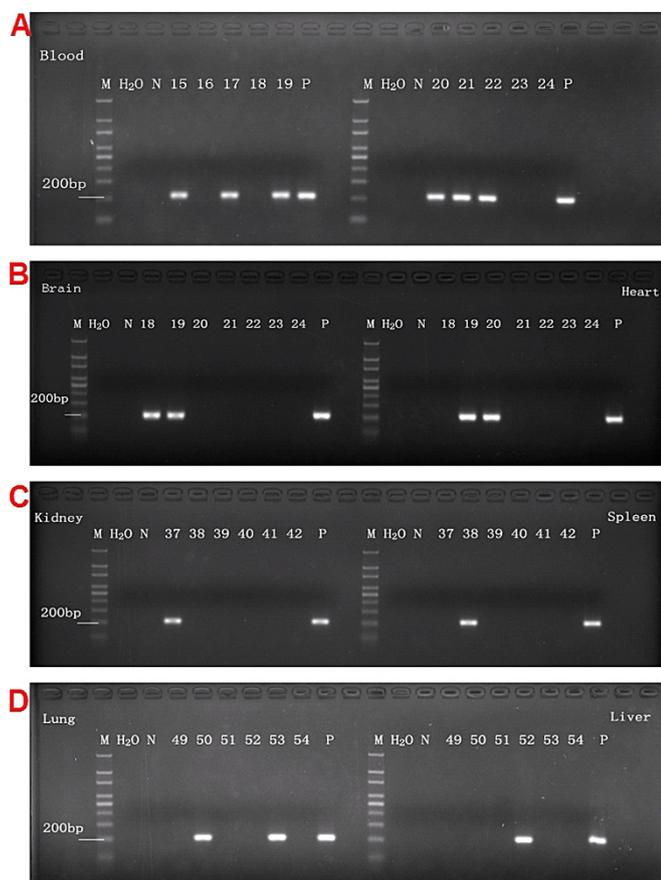


Figure 4 | Electrophoretic plots from nested PCR detection in chimeric mice. Electrophoretic plots showing SRY gene expression as revealed by nested PCR in blood (A), brain (B, on the left), heart (B, on the right), kidney (C, on the left), spleen (C, on the right), lung (D, on the left) and liver (D, on the right) of chimeric mice. The negative control (H₂O) line shows the nested PCR products from ultrapure water. The N lane is the nested PCR products from wild type mice and P the nested PCR products from the positive control (hEMSCPCs). The numbers identify the specific mouse tested.

unfeasible. While ESC can be harvested and maintained, access is restricted by ethical issues. Thus, there is currently no convenient source of non-embryonic pluripotent stem cells than can be harvested in sufficient quantities and maintained with sufficient ease to satisfy clinical demand and meet the technical requirements for routine hospital maintenance or commercial production. For the current study, we designed a special medium consisting of easily obtainable ingredients and developed isolation and culture methods to obtain human epidermis-derived mesenchymal stem cell-like pluripotent cells (hEMSCPCs) from the adult epithelium of human foreskin. These hEMSCPCs demonstrated strong vitality over multiple passages. On average, we obtained $>2 \times 10^{16}$ cells from a single circumcised foreskin sample. These cells could be stored frozen and then thawed without loss of proliferative capacity. Most importantly, these cells were able to integrate into multiple tissues and express tissue-specific markers. Thus, hEMSCPCs fulfill many of the requirements for clinical cell therapy, including large-scale harvesting, prolonged expansion in vitro, biocompatibility and safety, and pluripotency.

Previous studies have not found mesenchymal stem cell-like cells in the human epidermis, so the question arises as to the origin of these cells. In light of these morphological, immunohistochemical, and flow cytometry results, we suggest that hEMSCPCs are not blood-derived immunocytes or HSCs. These hEMSCPC did not

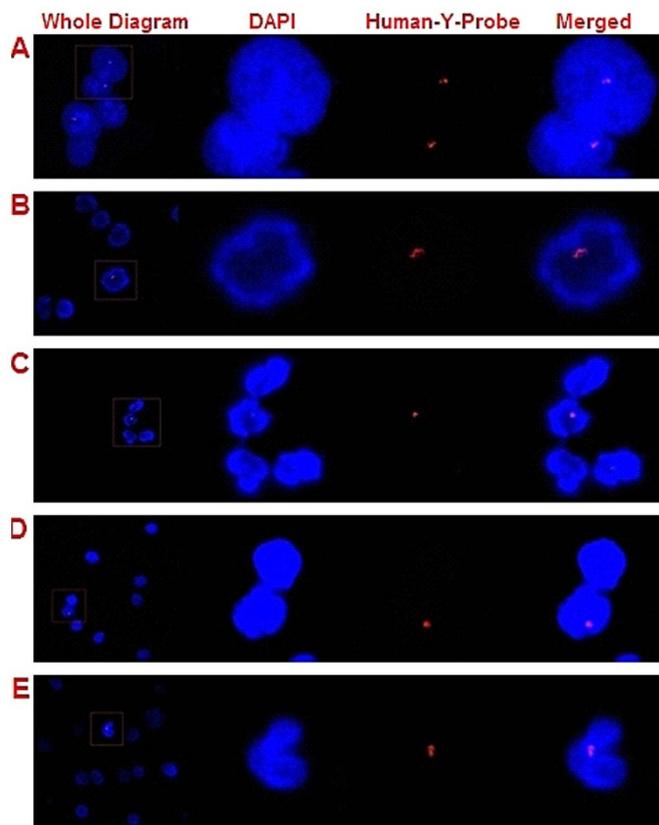


Figure 5 | Human Y FISH for hEMSCPCs and for cells from the caudal vein blood of human Y-positive female chimeras. The human Y chromosome is seen as red spots. hEMSCPCs are human Y positive (A). Cells from caudal vein blood of human Y positive female chimeras had differently shaped nuclei: sublobe nucleus (B), giant nucleus (C), spherical nucleus (D), and renal type nucleus (E).

resemble blood cells morphologically and only sporadic cells expressed hematopoietic stem cell and immunocyte markers. Furthermore, these cells were not vascular endothelial cells (VEC) because they did not express the main VEC markers at passage 2. They were not skin-derived neural cells based on morphological characteristics and the expression of MSC and fibroblast markers. They were not dermis-derived MSCs or fibroblasts as we removed the dermis before culture and strictly controlled for contamination by hypodermal cells by washing the cuticular layers many times with PBS before isolation of the epithelium. In addition, unlike dermis-derived MSCs and fibroblasts, these hEMSCPCs highly expressed the NSC marker nestin and had a tendency to take on a neural cell morphology. In contrast, hEMSCPCs at passages 2, 10, 20, and 30 expressed only low levels of the neuronal marker β -III tubulin and the glial cell marker GFAP. Cuticular layers were isolated during primary cell culture, so we presume that epidermal stem cells in the basilar membrane may transdifferentiate into hEMSCPCs in the microenvironment supplied by the special growth medium. Immunohistochemistry revealed that hEMSCPCs at passages 2, 10, 20, and 30 expressed low levels of CD10 and CK19, implying that hEMSCPCs are related to the epidermal cells of the basilar membrane. In summary, hEMSCPCs were derived from epidermis but were distinct from epidermal cells; their shapes resembled MSCs and they expressed the main markers of MSCs and NSCs. Further studies are needed to define marker expression patterns that can reveal the origin of these cells.

Current studies suggest that ASCs can transdifferentiate into other tissue-specific cells^{38–40}. Epithelial cells can also transdifferentiate into cells similar to mesenchymal stem cells in an appropriate



microenvironment^{41–43}. The special medium developed for selection and propagation of hEMSCPCs contains nutrition supplements vital for the growth of mesenchymal stem cells (bFGF and SCF), and indeed the formula is similar to those developed for the culture of mesenchymal stem cells in previous studies^{44–46}. We suggest that epidermal stem cells transdifferentiated into hEMSCPCs under the special microenvironment supplied by this defined medium, although this proposal requires much further study.

Stem cell proliferation and transdifferentiation are dependent on the local microenvironment. In fact, each developmental stage (e.g., embryonic vs. adult) provides a distinct set of microenvironments for implanted stem cell differentiation that are superior to any in vitro culture environment yet developed. When stem cells are injected into the mouse blastocyst cavity, they undergo genetic reprogramming and transdifferentiation. As embryonic development progresses, these stem cells can redistribute and undergo further transdifferentiation under the influence of site-specific microenvironments. Thus, mice blastocyst injection and chimeras production is the best tool to determine the multi-directional differentiation potential of stem cells⁴⁷. We found that hEMSCPCs injected into the mice blastocyst cavity formed heterogeneous chimeras. Implanted cells survived, were reprogrammed, and transdifferentiated into cells of all three germ layers as indicated by the expression of tissue-specific protein markers not detected in hEMSCPCs in vitro (prior to injection). Indeed, hEMSCPC-derived cells were present in several organs of the postnatal (1–5-month-old) mouse and expressed organ-specific functional proteins, suggesting that hEMSCPCs could be used for cell-based therapies. The efficiency of such treatment would depend on the number of hEMSCPCs that survive, transdifferentiate, and integrate into the appropriate target tissue. Further studies must be directed at enhancing survival and quantifying site-specific differentiation of hEMSCPCs using flow cytometry.

In conclusion, we have successfully isolated and cultured a type of mesenchymal stem cell-like pluripotent cell from epidermis with strong proliferative capacity in vitro. Past reports indicated that epidermis does not contain mesenchymal stem cell-like pluripotent cells, so we presume these cells transdifferentiated from epidermal stem cells in the special culture medium, although this conclusion requires additional experimental support.

Methods

Derivation of hEMSCPCs and maintenance in culture. Foreskin tissue was obtained from healthy circumcision patients, soaked in sterile PBS containing 1,000 U/mL gentamycin, and sent to the culture facility within one hour. The tissue was then stored in an 8–10°C refrigerator for 30 min to 4 hours for subsequent treatment. Tissue was thoroughly rinsed with PBS to remove residual blood and the loose subcutaneous tissue was resected. The tissue was again rinsed at least five times (×5) in PBS to remove blood and tissue debris, and cut into 2 mm-wide strips or 3 mm × 3 mm pieces. These tissue sections were again cleaned with PBS (×5), transferred to sterilized containers (such as 15 mL centrifugation tube), and incubated in 6–8 mL of 2 U/mL dispase II DMEM solution (Gibco, USA) at 6–8°C for 14–16 hours and then at 36.8 ± 0.2°C for 1 hour. These partially digested sections were placed onto a sterilized plate and washed with PBS (×5) to remove the remaining tissue debris. The epidermis was separated from the dermis using forceps and placed onto another sterilized plate, rinsed (PBS × 5), cut and crushed, suspended in 10 mL PBS, transferred to 15 mL centrifugation tubes, triturated with glass pipettes, and left to stand for 5–10 min to allow tissue fragments to settle to the bottom. The top fluid layer was removed, the epidermal tissue re-suspended in 10 mL PBS, and again gently pipetted. The tissue was then centrifuged at 1,200 rpm for 5 min and the supernatant discarded. The tissue pellet was suspended in 5–6 mL PBS containing 0.25% trypsin, gently pipetted, and incubated at 36.8 ± 0.2°C for 30–40 min, during which time the suspension was lightly swayed twice. To each digested sample, 5–6 mL PBS was added, followed by two additional cycles of centrifugation and resuspension in PBS. The cell pellet was then suspended in a special hEMSCPC medium containing 18–20 mL fetal bovine serum (FBS; Si Jiqing Ltd, China), 1 mL 100× non-essential amino acids (Gibco, USA), 1 mL PBS containing 0.03 g/mL L-glutamine (Gibco, USA), and 150 μL of a solution containing 40,000 U/mL gentamycin (Guangdong Succhi Pharmaceutical Group Ltd.). To this suspension was added DMEM (Gibco) containing 4.5 g/L glucose to a final volume of 100 mL. This cell suspension was supplemented with 2 ng/mL human stem cell factor (hSCF) and 10 ng/mL hbFGF (both from Perpotech, USA). After gentle pipetting, the cell

suspension from each patient sample was transferred into 3 or 4, 25 cm² culture flasks. To each culture flask was added 5–6 mL hEMSCPC medium.

Cells were incubated at 36.8 ± 0.2°C under a 5% CO₂ atmosphere with 100% humidity. The primary flasks remained unremoved for 48 hours. The medium was then replaced to remove non-adherent cells. The flasks were incubated for 5–10 days before media replacement as indicated by media acidification (yellowing). Within 7–10 days, small fusiform cells appeared and their numbers continued to increase thereafter. After 2–3 weeks, a digestion solution containing 0.25% trypsin and 0.02% EDTA (Gibco) in PBS was used for deplating and passaging. At this stage, epithelial-like cells were the minority and could be further eliminated at passage because these cells were more adherent than the small fusiform cells. Isolated small fusiform cells were collected and centrifuged at 1,200 rpm for 5 min. The fusiform cell pellet was resuspended in hEMSCPC medium, followed by gentle pipetting. Cells were counted and 5 × 10⁵ cells inoculated into new 25 cm² culture flasks for propagation. During the first 30 passages, these fusiform cells grew rapidly, and generally they could be passaged every 1.5–3 days at 1 : 3. Karyotyping was performed at P30–P32.

Immunocytochemistry. Sterilized 22 mm × 22 mm glass coverslips (thickness 0.13–0.16 mm, CITOGLAS®, China) were placed in Φ35 mm plates, rinsed twice with PBS, and then the Φ35 mm plates containing the coverslips were placed into Φ100 mm plates pre-filled with a small volume of sterilized water. Dissociated hEMSCPCs (5 × 10⁴ cells/mL × 2 mL of special medium) were then added to the Φ35 mm plates, followed by incubation for 2–3 days at 36.8 ± 0.2°C under 5% CO₂ and 100% humidity. During this incubation, the medium was replaced as required based on the degree of acidification (yellowing).

Coverslips with adherent fusiform cells were gently rinsed three times in PBS preheated to 36.8 ± 0.2°C. Coverslips used for immunohistochemical DAB staining of nestin, vimentin, β-III tubulin, GFAP, CK19, CD10, CD3, CD19, CD16, CD45, CD34, CD31, and VEGF R2 (all antibodies from Abcam, USA or Invitrogen, USA) were fixed in methanol for 20 min and then gently rinsed with distilled water. Fixed cells were permeabilized with 3% Triton X-100 for 20 min at room temperature, gently rinsed with PBS, treated with 3% H₂O₂ for 10 min at room temperature to quench endogenous peroxidase activity, rinsed with PBS, blocked with normal goat serum for 10 min, dried, and then incubated in the appropriate primary antibody for one hour at 37°C. Fixed and immunostained cells on coverslips were rinsed in PBS (×3, 3 min per rinse). Immunostaining was visualized using a commercial kit (Thermoscientific, UK). Solution A (intensifier) was added drop by drop, and cells incubated at 37°C for 15 min, followed by rinsing in PBS (×3, 3 min per rinse). Solution B containing horse radish peroxidase (HRP)-conjugated Anti Ms/Rb IgG was then added and cells incubated for 20 min at 37°C, followed by rinsing in PBS (×3, 3 min per rinse). Finally, the chromogen DAB was added for 10 min at room temperature under a microscope. Coverslips were rinsed with tap water to terminate the reaction and cells counterstained in Mayer's hematoxylin for 1 min. Coverslips were rinsed in tap water, left to dry, sealed in mounting medium (Thermoscientific, UK), and observed and photographed under a regular optical microscope (Zeiss, Germany). For CD90, CD73, CD105 (Santa Cruz, USA), HLA-DR or HLA-I (Invitrogen, USA) immunostaining, coverslips were rinsed with PBS, fixed for 1 min using 3 : 1 pure methanol plus glacial acetic acid pre-cooled to 4°C, and immediately incubated with a FITC-labeled antibody according to the protocol provided by the reagent manufacturers. Coverslips were air dried, counterstained for 30 min at room temperature with a DAPI nuclear staining solution (Abbott Molecular Inc. USA), and observed and photographed under a laser confocal microscope (Zeiss, Germany) using filter sets for DAPI (405 nm excitation, 461 nm emission) and FITC (488 nm excitation, 525 nm emission).

Flow cytometry. After passage, cells were incubated in culture flasks for 40–48 hours and then deplated/dispersed with 0.25% trypsin–0.02% EDTA into a single cell suspension. Following two rinses in PBS, cells were re-suspended in a small volume of PBS and the suspension transferred to 1.5 mL microcentrifuge tubes. The specific flow cytometry protocol depended on whether direct or indirect labeling was used. Monoclonal mouse anti-human CD73, CD34, and HLA-DR antibodies (BD, USA), monoclonal mouse anti-human CD45, CD3, CD19, CD16, and HLA-I antibodies (Invitrogen, USA), and monoclonal mouse anti-human CD10, CD31, CD90, CD105, VEGF-R2, CK19 antibodies (Santa Cruz, USA), all with appropriate isotype controls (PE-, Percp-, FITCIgG1-, and FITCIgG2-isotype controls, all provided by the corresponding antibody supplier), were used for direct immunolabeling of the corresponding proteins. Monoclonal mouse anti-human vimentin (Abcam, USA) and β-III tubulin (Millipore, USA), and polyclonal rabbit anti-human nestin and GFAP antibodies (Abcam, USA) were used for indirect immunolabeling of the corresponding proteins. The secondary antibodies were goat anti-mouse-PE and goat anti-rabbit-FITC (Southern Biotech, USA). A cell fixation and permeabilization kit was used to label intracellular antigens (Invitrogen, USA). All procedures were conducted according to the manufacturer's protocols. Cell suspensions treated only with the secondary antibody were used as isotype controls for indirectly labeling experiments. After treatment, immunolabeling was detected by flow cytometry (FACSaria, BD, USA) and the data analyzed by FCS Express V3. Values for isotype controls were within 0%–1%.

Construction of chimeras. Human EMSCPCs, frozen in liquid N₂ at P10–P13 in 10% DMSO, were thawed in a 35–37°C water bath, suspended in DMEM containing 10% FBS, and centrifuged at 1,200 rpm for 5 min. The supernatant was removed and the cell pellet re-suspended in 0.5 mL serum-free DMEM, incubated at 6–8°C for 10 min,



and then injected into mouse blastocoeles under a light microscope. Each mouse blastocoele was injected with 6–8 cells. Injected mouse embryos were incubated overnight at $36.8 \pm 0.2^\circ\text{C}$ under 5% CO_2 and 100% humidity. On the second day, well-developed embryos were transferred into the uteruses of 2.5-day pseudo-pregnant female mice. In 18 ± 1 days, pups were born. Some pseudo-pregnant mice could not be impregnated, would miscarry, or give birth to deformed pups that would die soon after birth. Among pups surviving for more than one month, only females were used for examination of hEMSCPCs survival, differentiation, and integration into host tissue.

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Author contributions

B.H. (project design, generation and characterization of hEMSCPCs, and preparation of manuscript), J.G. (project design, members organize and preparation of manuscript), K.L., W.L., Y.L. and W.W. (Make up Chimeras), J.Y., L.G. and L.L. (PCR of Human SRY, and preparation of manuscript), M.Z. and Y.Z. (flow cytometry analysis and preparation of manuscript), Y.L., W.Z. and S.L. (immunocytochemistry), X.H. (FISH of Human Y), X.S. (karyotype analysis), Z.W. (project design and preparation of manuscript). All authors reviewed the manuscript.

Additional information

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