**Generation of Human Epidermis-Derived Mesenchymal Stem Cell-like Pluripotent Cells and their reprogramming in mouse chimeras**

Huang Bing, Li Kaijing , Yu Jie , Zhang Min , Li Yongping , Xu Xiaoping , Luo Ting , Huang Jianfa , Guan Liping , Li Weihua , Zhang wenxin , Lin Shaochun , Huang Xintao , Lin Liping , Lin Yongliang , Zhang Yichi, Wang Wencong , Wang Zhichong, Gao Qianying, Chen Xigu , Song Xinming, Ge Jian

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, iPSs). Ethical problems, immunological rejection, and difficulties in obtaining human tissues limit the use of ESCs in clinical medicine[1-2]. Induced pluripotent stem cells are difficult to maintain in vitro and carry a greater risk of tumor formation. Furthermore, the complexity of maintenance and propagation is especially difficult in the clinic[3-10]. Adult stem cells 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 cells [14], neural stem cells (NSCs) [15], adipose-derived stem cells (ADSCs)[16], islet stem cells [17], and germ line stem cells [18-20]. Human mesenchymal stem cells originate mainly from bone marrow [21-22], cord blood [23], and placenta [24-25], but epidermis-derived MSCs have not yet been isolated. We isolated small spindle-shaped cells with strong proliferative potential during the culture of human epidermis cells and designed a medium to isolate and propagate these cells. 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 present a possible new cell resource for tissue engineering and regenerative medicine.

Human EMSCPCs were passaged at 1:3 every 1.5~3 days and after about three months (around passage 30), at least $2\times10^{16}$ cells were obtained from a single adult tissue sample. These hEMSCPCs expressed the main markers of MSCs and NSCs, had good bio-safety [26], and could differentiate into neural cells [27] and immunocytes [28] under appropriate conditions. Furthermore, we found that hEMSCPCs cells could 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 mouse (1-5 months old), 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 that they can be reprogrammed after blastocyst cavity injection.

To obtain hEMSCPCs, we first designed a selective culture medium (supplementary methods). We obtained eight foreskin specimens from surgical patients who were negative for HIV, hepacivirus, and leptospira infection. After treating the foreskin tissue with a digestive solution (supplementary methods), the tissue was washed more than five times in PBS to prevent hypodermal cell contamination. The whole epithelial layer was isolated from the basilar membrane and treated with a digestive solution. Individual epithelial cells were then obtained by mechanical trituration. These epithelial cells were resuspened in hEMSCPC-specific medium and cultured in an incubator at 36.8 ± 0.2 ºC under 5% $CO_2$ and 100% humidity. On day two, the culture medium was replaced and non-adherent cells were removed. Spindle-shaped cells with small cell bodies were observed after 7-10 days in vitro (Fig. 1a, P0 7d), 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). When replacing the medium, dead cells were removed by gentle agitation with a pipetter. 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 with small cell bodies were harvested at two to three weeks in vitro as they were more easily detached from the culture plates compared to the polygonal epithelial-like cells. Thus, we could selectively separate this two cell types by controlling the digestion time. Isolated spindle-shaped cells with small cell bodies were then cultured alone in hEMSCPCs culture 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 cells 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). Chromosome detection of hEMSCPCs between passages 30 to 32 confirmed the karyotype as 46, XY (Supplementary Fig. 1).

Past reports did not find MSCs in the epidermis, so it is uncertain where these hEMSCPCs came from. 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 that are known to be expressed by skin-derived cells. These proteins included the MSC makers 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 CD10[29], the vascular endothelial cell markers CD31 and VEGF-R2, and the human histocompatibility antigens HLA-DR and HLA-Ⅰ.

We assessed expression patterns at passage 2, 10, 20, and 30 by immunohistochemisty. Only a fraction of hEMSCPCs expressed CD90 and nestin (Fig. 2a) at passage 2, but almost all expressed the MSC marker CD90 (Fig. 2i) and the NSC marker nestin (Fig. 2c) at passages 10, 20, and 30. At all passages, most hEMSCPCs cells expressed the MSC marker CD73 (Fig. 2j) and the fibroblast marker vimentin (Fig. 2b, d), a significant fraction expressed CD105 (Fig. 2k), and a few cells expressed β-III tubulin, GFAP, CK19 (Fig. 2e) and CD10 (Fig. 2f). Very few expressed the immunocyte markers CD3 (Fig. 2g), CD19, CD16, CD45, or the HSC marker CD34. No cells at passage 2 expressed the vascular endothelial cells markers CD31 and VEGF-R2 (Fig. 2h), while only a few CD31 and VEGF-R2 positive cells were found at passages 10, 20, and 30. Interestingly, 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).

For quantitative analysis, we detected marker expression at passages 2, 10, 20, and 30 by flow cytometry. The results confirmed immunohistochemisty results except for marker CK19. 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 passage 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. 3a-e and supplementary Table 1).

A few CK19 positive hEMSCPCs were detected by immunohistochemisty, but no CK19 positive cell were detected by flow cytometry, possibly because the CK19 positive hEMSCPCs belong to the epithelial-like cells, which cannot be easily detached from the culture flask by digestive solutions like trypsin, so some CK19 positive cells might be

lost before flow cytometry analysis.

In light of these morphological, immunohistochemical, and flow cytometry results, we suggest that hEMSCPCs are not blood-derived because their morphological characteristics and marker expression excluded immunocytes or HSCs. Furthermore, these cells are not vascular endothelial cells (VEC) because they do not express the main VEC markers at passages 2. They are not skin-derived neural cells based on morphological characteristics and the expression of MSC and fibroblast markers. They are 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, hEMSCPCs are distinct from the dermis-derived MSCs and fibroblasts characterized previously that 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 all 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 might transdifferentiate into hEMSCPCs in the microenvironment supplied by the special growth medium. Immunohistochemisty revealed that hEMSCPCs at passages 2, 10, 20, and 30 all expressed low levels of CD10 [29] and CK19, implying that hEMSCPCs are related to the epidermal cells of the basilar membrane.

In short, 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.

To determine if these hEMSCPCs were multipotent in vivo like other ACSs, we injected these cells into the blastocyst cavity 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 behavior of these cells in vivo. 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 uterus 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. The hEMSCPCs we obtained were all male cells, and no marker has been identified that is specific for hEMSCPCs injected into the mice blastocyst cavity. Previously, we designed two primer pairs (Supplementary methods, PCR of Human SRY) specific for human SRY genes[30] of the Y chromosomes, and used them in this study 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 of the human Y chromosomes by nested PCR, we performed in situ hybridization using specific probes for the human Y chromosome (Supplementary methods, FISH of 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. 3f), 4 of 62 (6.5%) had SRY-positive brain tissue (Fig. 3g left), 6 of 62 (9.7%) had positive heart tissue (Fig. 3g right), 8 of 62 (12.9%) positive lung tissue (Fig. 3i left), 4 of 62 (6.5%) positive spleen tissue (Fig. 3h right), 3 of 62 (about 4.8%) positive liver tissue (Fig. 3i right), and 4 of 62 (about 6.5%) SRY-positive kidney (Fig. 3h left).

The results of in situ hybridization confirmed results from nested PCR. Cultured hEMSCPCs and cells from caudal vein blood of nested PCR positive mice were analyzed by in situ hybridization. Over 90% of the hEMSCPCs were human Y-positive (Supplementary Fig. 2.a), and the karyocytes in the caudal vein blood of positive female off-spring

had differently shaped nuclei (Supplementary Figure 2.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 cells, brain cells, cardiac muscle cells, lung cells, spleen cells, liver cells, and renal glomerulus cells that were SYR-positive were also positive by in situ hybridization and expressed several specific human antigens (Fig. 4). These human-specific 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, tropolin-Ⅰ in cardiac muscle cells, SP-C and CD31 in lung cells, ALB in liver cells, and VEGF-R2 in renal glomerulus cells. In contrast, these specific antigens were not detected by immunofluorescence staining in cultured hEMSCPCs of the same passage as those injected into the blastocyst cavity, expect for a few cells expressing MAP2 and β-III Tubulin (results not shown).

Results of in situ hybridization and immunohistochemistry in chimeric mice indicated that hEMSCPCs not only survived but were reprogrammed and transdifferentiated into tissue-specific phenotypes. These tissues included ectoderm (brain), mesoderm (blood, heart, spleen and kidney), and endoderm (lung and liver). Therefore, hEMSCPCs multipotent like all ASCs, can be reprogrammed by the in situ microenvironment of the mice blastocyst cavity, and have pluripotency to generate all three germ layers like ESCs.

On the whole, we have successfully isolated and cultured a type of mesenchymal stem cells-like pluripotent cell from epidermis with strong proliferative ability in vitro. Past reports indicated that epidermis did not contain mesenchymal stem cell-like pluripotent cells, so we presume they transdifferentiated from epidermal stem cells in the special culture medium, although this conclusion requires additional experimental support. These hEMSCPCs could be injected into the mice blastocyst cavity to form heterogeneous chimeras. During mouse development, these cells survived, and were reprogrammed and transdifferentiated into all three germ layer cells. This chimeric model is a valuable tool to study the dedifferentiation of adult somatic cells in an embryonic environment and could be used to build heterogeneous organs in animals. The distribution of hEMSCPCs in heterogeneous animals requires further study.

## METHODS SUMMARY

Foreskin specimens were obtained after foreskin excision surgeries. Patients were otherwise healthy as determined by preoperative examinations. Foreskin specimens were incubated in a digestive solution to isolate the whole cuticular layer and then treated with the digestive solution again to isolate epidermal cells. Cells were cultured in a medium specially designed to maintain short spindle-shaped and spindle-shaped mesenchymal stem cells-like cells. Cells were cultured and passaged over 30 times in vitro. During the culture, morphologically defined cell types were examined under light microscopy and by karyotype, immunostaining and immunofluorescence, and by flow cytometry analysis using a battery of cell-specific markers. Once hEMSCPCs were cultured to passage 10-13, dedifferentiation and pluripotency were examined in vivo by injecting cells into the mice blastocyst cavity and allowing chimeras to develop to term. Primers specific for SRY genes of the human Y chromosome were designed to detect hEMSCPC-derived cells by nested PCR in female chimeric mice. In situ hybridization with fluorescent probes was used to validate the results of nested PCR. In addition, antibodies to a variety of tissue-specific proteins of blood cells, brain cells, cardiac muscle cells, lung cells, spleen cells, liver cells, and renal glomerulus cells were used to detect tissue-appropriate expression, thereby demonstrating the transdifferentiation of hEMSCPCs in the positive organs of female chimeric mice. Animal-related experiments were reviewed and approved by the Laboratory Animal Care and Ethic Committee of Zhongshan Ophthalmology Center affiliated to Sun Yat-sen University (2010-024).

**Figure Legends**

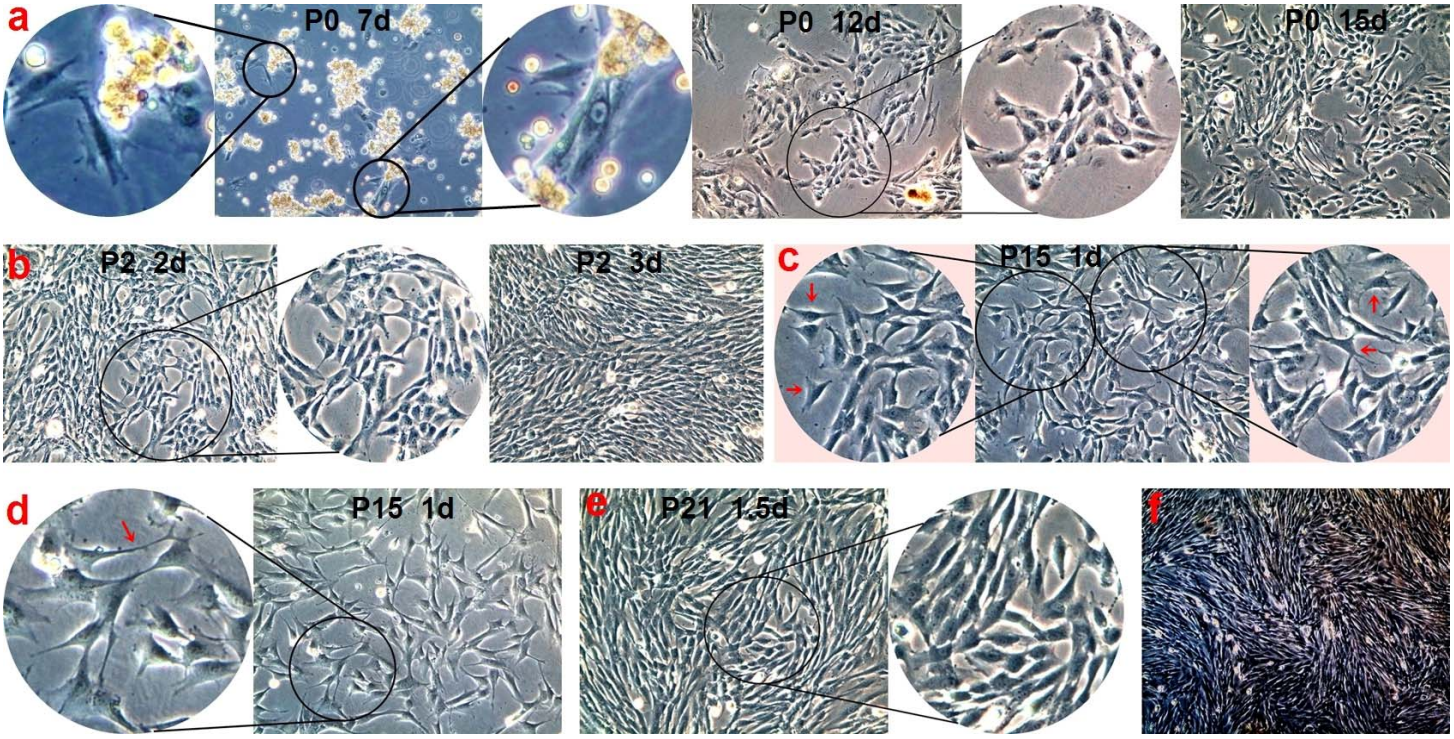


**Figure 1 | Morphology of cells cultured in vitro. a.** When primary passage cells were cultured in vitro for 7-10 days, small fusiform cells appeared (P0 7d), while other cell types almost completely disappeared. With prolonged incubation, these small fusiform cells continued to increased in number (P0 12d & 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 in most cells (**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.

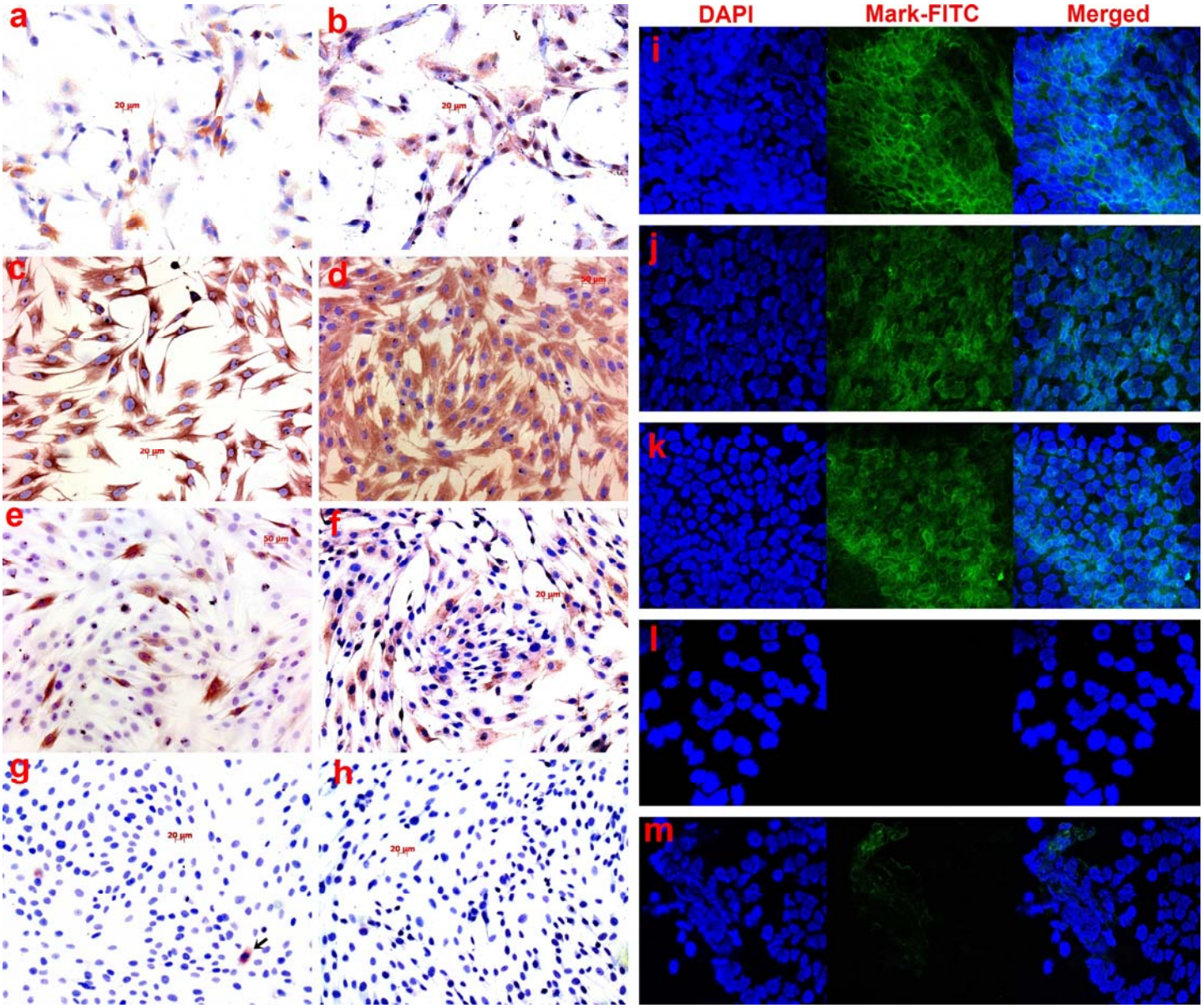


**Figure 2 | Immunohistochemical staining of cell markers and immunofluorescent images. a.** Some cells at P2 showed nestin positive immunoreactivity (brown color). **b**. Almost all cells at P2 were vimentin positive (brown color). **c**. Almost all cells at P10, P20 and P30 showed nestin immunoreactivity (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 immunoreactivity cells were observed (brown color). **g**. At P2, P10, P20, and P30, a few individual cells were CD3 immunoreactive (see brown cells arrowed). **h**. At P2, no CD31- 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). **I**. 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). Other markers with similar results are not presented.

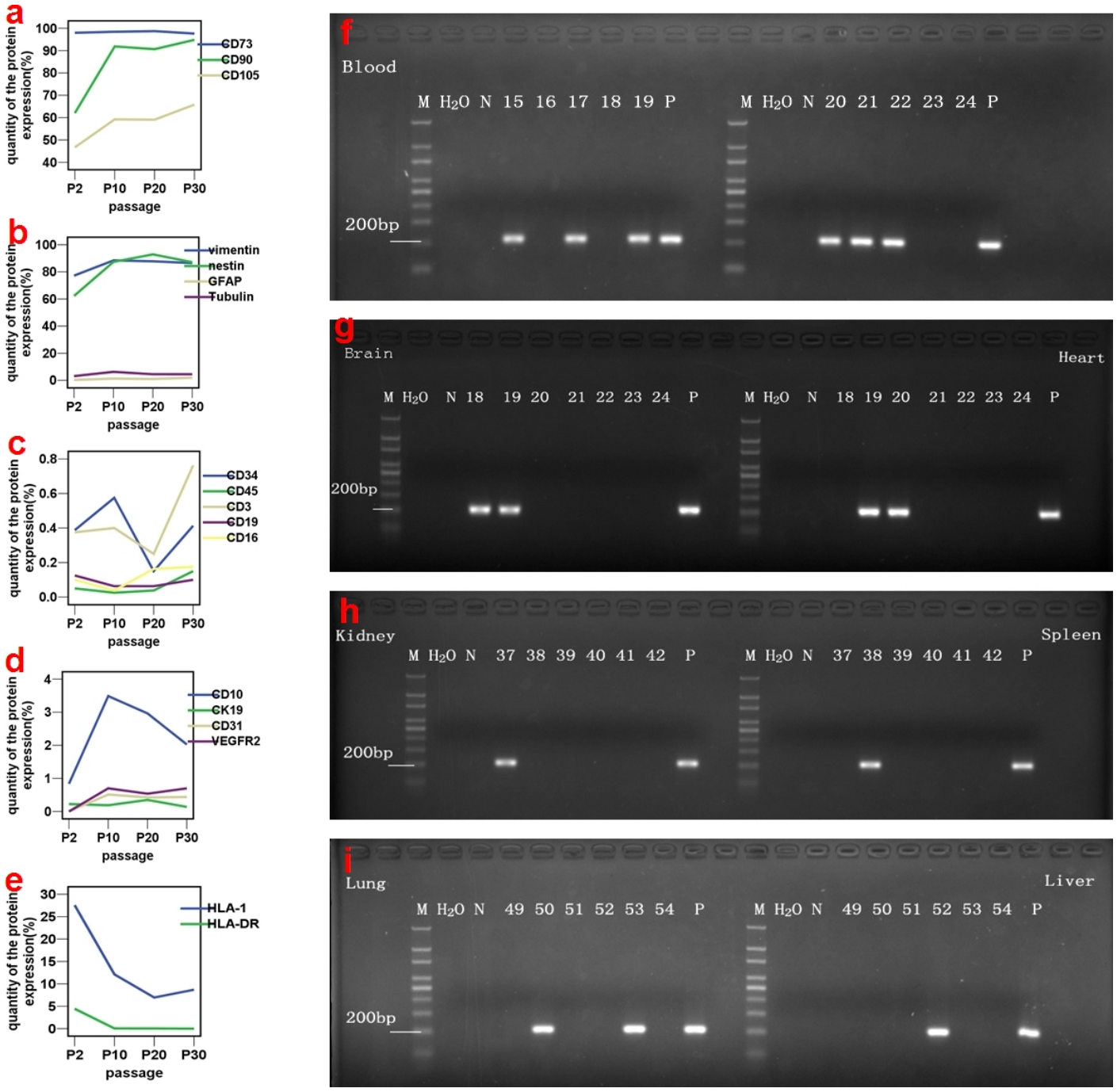


**Figure 3 | Flow cytometry analysis of cell markers over time and electrophoretic plots from nested PCR detection in chimeric mice. a-e:** Plots displaying flow cytometry data over time for various markers at P2, P10, P20, and P30. CD73 was always highly expressed at P2, P10, P20, and P30; CD90 was moderately expressed at P2, and highly expressed at P10, P20, and P30; CD105 was moderately expressed at P2, P10, P20 and P30. (**a**) Substantial vimentin expression was observed at P2, P10, P20, and P30. Nestin expression was consistent with CD90. It was moderate at P2 but high at P10, P20, and P30, whereas GFAP and β-III tubulin showed minimal expression. (**b**) Cell expressed very low levels of CD34, CD45, CD3, CD19 and CD16. (**c**) CD10 expression was always low, while CK19, CD31 and VEGF R2 were barely detectable. (**d**) The tissue compatibility antigens HLA-DR and HLA-I were moderately or lowly expressed at P2; however, with increasing number of passages, fewer and fewer cells express these markers. No HLA-DR-positive cells were detected in some specimens at P10, P20, and P30. (**e**) **f-i**.

Electrophoretic plots displaying nested PCR of SRY gene expression in human Y chromosome in blood (f), brain (g, on the left), heart (g, on the right), kidney (h, on the left), spleen (h, on the right), lung (i, on the left) and liver (i, on the right) of chimeric mice. $H_2O$ represents negative control using ultrapure water; N represents normal mother mice as negative controls; P represents positive controls with hEMSCPCs, and numbers represent the identification number of mother mouse tested.

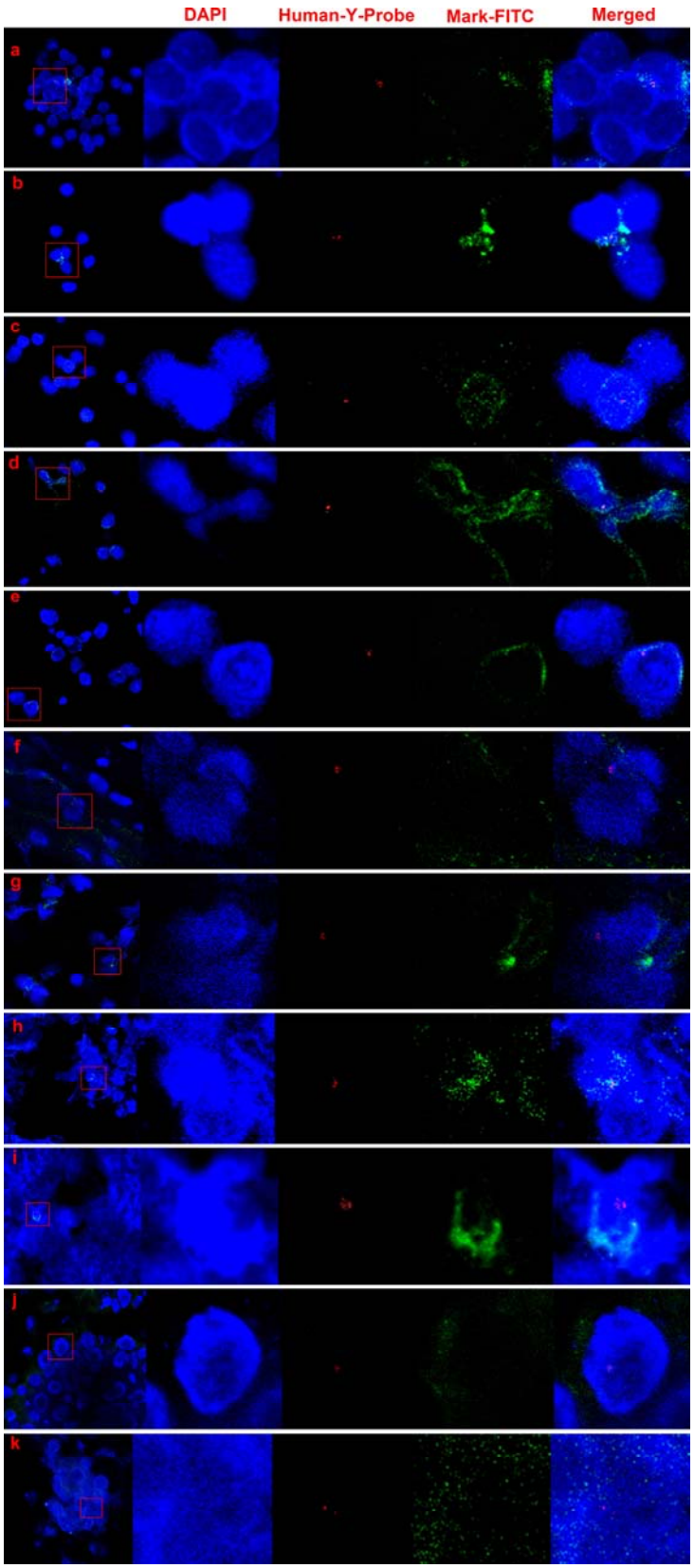


**Figure 4 | FISH on human Y chromosome in chimeric mice and immunofluorescent images using tissue-specific antibodies.** Human Y chromosome is seen as red spots, while the FITC-labeled specific antibody appears green. **a**. Peripheral blood from chimeric mice was positive for both FISH on human Y chromosome and

anti-human CD3 antibody immunofluorescence. **b**. Peripheral blood from chimeric mice was positive for both FISH on human Y chromosome and anti-human CD16 antibody immunofluorescence. **c**. Peripheral blood from chimeric mice was positive for both FISH on human Y chromosome and anti-human CD19 antibody immunofluorescence. **d**. Brain tissue from chimeric mice was positive for both FISH on human Y chromosome and anti-human MAP-2 antibody immunofluorescence. **e**. Brain tissue from chimeric mice was positive for both FISH on human Y chromosome and anti-human β-III tubulin antibody immunofluorescence. **f**. Cardiac tissue from chimeric mice was positive for both FISH on human Y chromosome and anti-human tropolin I antibody immunofluorescence. **g**. Lung tissue from chimeric mice was positive for both FISH on human Y chromosome and anti-human SP-C antibody immunofluorescence. **h**. Lung tissue from chimeric mice was positive for both FISH on human Y chromosome and anti-human CD31 antibody immunofluorescence. **I**. Splenic tissue from chimeric mice was positive for both FISH on human Y chromosome and anti-human CD16 antibody immunofluorescence. **j**. Liver tissue from chimeric mice was positive for both FISH on human Y chromosome and anti-human ALB antibody immunofluorescence. **k**. Kidney tissue from chimeric mice was positive for both FISH on human Y chromosome and anti-human VEGF R2 antibody immunofluorescence.

**References**


1. Ledford, H. Hidden toll of embryo ethics war. *Nature* **471,** 279 (2011).
2. Lo, B. & Parham, L. Ethical Issues in Stem Cell Research . *Endocr Rev.* **30(3),** 204–213(2009 ).
3. PERA, M. F. The dark side of induced pluripotency Induced pluripotent. *Nature* **471,** 46-47(2011).
4. DOLGIN, E . Flaw in induced stem-cell model. *Nature* **470,** 13 (2011).
5. Hussein, S. M. *et al.* Copy number variation and selection during reprogramming to pluripotency. *Nature* **471,** 58–62 (2011).
6.. Gore, A. *et al.* Somatic coding mutations in human induced pluripotent stem cells. *Nature* **471,** 63–67 (2011).
7. Lister, R. *et al.* Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. *Nature* **471,** 68–73 (2011).
8. Kim, K. *et al.* Epigenetic memory in induced pluripotent stem cells. *Nature* **467,** 285–290 (2010).
9. Laurent, L. C. *et al.* Dynamic Changes in the Copy Number of Pluripotency and Cell Proliferation Genes in Human ESCs and iPSCs during Reprogramming and Time in Culture. *Cell Stem Cell* **8,** 106–118 (2011).
10. Mayshar, Y. *et al.* Identification and Classification of Chromosomal Aberrations in Human Induced Pluripotent Stem Cells. *Cell Stem Cell* **7,** 521–531 (2010).
11. FINNEY ,B. *et al.* Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. *Nature Medicine* **6( 11),** 1278-1281( 2000).
12. Parekkadan, B. & Milwid, J. M. Mesenchymal stem cells as therapeutics. *Annu Rev Biomed Eng.* **12,** 87-117(2010).
13. Shi, Y. *et al.* Mesenchymal stem cells: a new strategy for immunosuppression and tissue repair. *Cell Research* **20,** 510-518 (2010).
14. Li, J. *et al.* Enrichment of putative human epidermal stem cells based on cell size and collagen type IV adhesiveness. *Cell Research* **18,**360-371 (2008).
15. Keyoung, H. M. *et al.* High-yield selection and extraction of two promoter-defined phenotypes of neural stem cells from the fetal human brain. *Nature Biotechnology* **19,**843-850(2001).
16. Estes, B.T. *et al.* Isolation of adipose-derived stem cells and their induction to a chondrogenic phenotype. *Nature Protocols* **5 (7),**1294-1311( 2010).
17. RAMIYA, V. K. *et al.* Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells. *Nature Medicine* **6( 3),** 278-282( 2000).

18. Seandel, M. *et al.* Generation of functional multipotent adult stem cells from GPR125$^{+}$ germline progenitors. *Nature* **449**, 346-350 (2007).
19. Conrad, S. *et al.* Generation of pluripotent stem cells from adult human testis. *Nature* **456,** 344-351(2008).
20. GONG, S.P. *et al.* Derivation of histocompatible stem cells from ovarian tissue. *J Reprod Dev.* **56(5),**481-94(2010).
21. Pittenger, M. F. *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* **284,** 143–147 (1999).
22. Colter, D.C. *et al.* Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. *Proc. Natl. Acad. Sci. USA* **97,**3213–3218(2000).
23. Lee, M.W. *et al.* Isolation of mesenchymal stem cells from cryopreserved human umbilical cord blood. *Int. J. Hematol.* **81,**126–130(2005).
24. Huang, Y.C. *et al.* Isolation of mesenchymal stem cells from human placental decidua basalis and resistance to hypoxia and serum deprivation. *Stem Cell Rev.* **5(3),**247-255(2009).
25. Crisan, M. *et al.* A Perivascular Origin for Mesenchymal Stem Cells in Multiple Human Organs., *Cell Stem Cell* **3,** 301–313(2008).
26. Guan,L. *et al.* Biological safety of human skin-derived stem cells after long-term *in vitro* culture. *J Tissue Eng Regen Med*. Published online in Wiley InterScience (www.interscience.wiley.com) **DOI:** 10.1002/term.290(2010).
27. Zhang, M. *et al.* Human Epidermis-Derived Mesenchymal Stem Cell-like Pluripotent Cells may Transdifferentiate into Neural-like Cells . contributing.
28. Guan,L. *et al.* In vitro differentiation of human skin-derived mesenchymal stem cells into lymphocytes: Possibility evaluation. *Journal of Clinical Rehabilitative Tissue Engineering Research* **14 (19),** 3601-3605(2010).
29. Córdoba,A. *et al.* Bcl-2 and CD10 expression in the differential diagnosis of trichoblastoma, basal cell carcinoma, and basal cell carcinoma with follicular differentiation. *International Journal of Dermatology*, **48,**713–717(2009).
30. Sinclair, A. H. *et al.* A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature* **346**, 240-244 (1990).

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**Author Information**

**Affiliations**

1. State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, GuangZhou 510060, China

Huang Bing, Li Kaijing , Yu Jie , Zhang Min , Li Yongping , Xu Xiaoping , Luo Ting , Huang Jianfa , Guan

Liping , Li Weihua , Zhang wenxin , Lin Shaochun , Huang Xintao , Lin Liping , Lin Yongliang , Zhang Yichi, Wang Wencong , Wang Zhichong, Gao Qianying, Chen Xigu ,Ge Jian

2. Genetic Laboratory of Sun Yat-sen University, GuangZhou 510080, China

Song Xinming

**Author Contributions**

Huang Bing (project design, generation and characterization of hEMSCPCs, and preparation of manuscript), Ge Jian (project design, members organize and preparation of manuscript), Li KaiJing, Li WeiHua, Lin YongLiang and Wang WenCong (Make up Chimeras), Yu Jie, Guan LiPing and Lin LiPing (PCR of Human SRY , and preparation of manuscript) , Zhang Min, Luo Ting and Zhang Yichi (Flow Cytometry Analysis, and preparation of manuscript), Li YongPing, zhang wen xin and Lin ShaoChun (immunocytochemistry), Xu XiaoPing, Huang JianFa and Huang XinTao (FISH of Human Y), Gao QianYing and Song XinMing(Karyotype analysis). Wang ZhiChong and Chen XiGu(project design, preparation of manuscript).

**Competing financial interests**

The authors declare no competing financial interests.

**Corresponding author**

Correspondence to: Huang Bing, e-mail: huangbing2000@hotmail.com ,
qiuyaxuan@126.com
Ge Jian, e-mail: gejian@mail.sysu.edu.cn

**Supplementary Information**

**Supplementary Methods**

Animal-related Experiments were reviewed and approved by the Laboratory Animal Care and Ethic Committee of Zhongshan Ophthalmology Center affiliated to Sun Yat-sen University (2010-024).

**Derivation of hEMSCPCs and Culture in Vitro**

Discarded prepuce tissue was obtained from healthy patients as indicated by clinical examination who underwent male circumcision, soaked in PBS containing 1,000 U/mL gentamycin, and was sent to the lab within one hour. The tissue was then stored in an 8-10℃ refrigerator for 30 min to 4 hours for subsequent treatment. First of all, prepuce tissue was thoroughly rinsed with PBS to remove residual blood; then loose subcutaneous tissue was resected; the tissue was again rinsed with PBS at least five times, in order to remove blood and tissue debris. Prepuce was cut into strips of 2 mm in width, or pieces of 3 mm × 3 mm in size; These strips or pieces were again rinsed with PBS for at least five times, to adequately remove blood and tissue debris. Prepared strips or pieces were transferred to sterilized

small containers (such as 15 mL centrifugation tube), and were soaked in DispaseII2U/mL DMEM solution(GIBCO, USA); each specimen was soaked in 6-8 mL solution, stored in a 6-8℃ refrigerator for 14-16 hours, and then incubated at 36.8±0.2℃ for 1 hour. Strips or pieces were placed inversely onto a sterilized plate, and washed with PBS at least five times, so as to adequately remove tissue debris; the layer of epidermis was tore off from dermis using a pair of forceps, and was placed onto another sterilized plate; the dermis is discarded; the epidermis was rinsed again with PBS at least five times to extensively remove tissue debris; the epidermis was cut and crushed, suspended with 10 mL PBS, and transferred into a 15 mL centrifugation tube; The suspension was gently pippetted and is left to stand for 5-10 min; supernatant was removed; the precipitation of epidermal tissue was re-suspended using 10 mL PBS and was gently pippetted, followed by centrifugation at 1,200 rpm for 5 min; supernatant was discarded. Precipitation of epidermal tissue was suspended with 5-6 mL PBS containing 0.25% trypsin and gently pippetted; The suspension was incubated at 36.8±0.2℃ for 30-40 min, during which the suspension was slightly swayed twice; 5-6 mL PBS was added, followed by gentle pippetting and centrifugation at 1,200 rpm for 5 min; supernatant was removed; another 10 mL PBS was used to suspend cell precipitation, followed by gentle pippetting and centrifugation at 1,200 rpm for 5 min; again, supernatant was disposed. Cell precipitation was suspended using hEMSCPCs medium [18-20 mL FBS (Si jiqing Ltd , China), 1 mL 100×non-essential amino acid (GIBCO, USA), 1 mL PBS containing L-glutamine 0.03 g/mL (GIBCO, USA), 150 μL solution containing 40,000 U/mL gentamycin (GUANGDONG SUCCHI PHARMACEUTICAL (GROUP) LTD.), added with DMEM (GIBCO, USA) with a glucose concentration of 4.5 g/L to a final volume of 100 mL; finally, hSCF is added to a final concentration of 2 ng/mL and hbFGF to a final concentration of 10 ng/mL (PERPOTECH, USA)] and gently pippetted and then transferred into a culture flask; a total of 3-4 culture flasks of 25 $cm^2$ in volume were used for epidermal cells obtained from each specimen; each culture flask was added with 5-6 mL of medium; after being gently pippetted, the cells were placed and incubated at 36.8±0.2℃、5%$CO_2$ and 100% humidity; the flask remained unmoved within 48 hours; after that, medium was replaced, and un-adherent cells disposed; incubation was continued; during the next 5-10 days, medium would be replaced based on the degree of yellowing in the medium; within 7-10 days, small fusiform cells would appears and kept increasing in number thereafter; 2-3 weeks later, digestion solution [0.25% trypsin added to 0.02% EDTA at the ratio of 1:1 (GIBCO, USA)] could be used to digest and passage the cells; Epithelial-like cells might appear in a small number of specimens; however, small fusiform cells were extremely easy to detached from the culture plates, while epithelial-like cells were not; therefore epithelial-like cells could be limited by controlling the time of digestion. Isolated small fusiform cells were collected, and centrifuged at 1,200 rpm for 5 min, and supernatant was removed; cell precipitation was suspended using medium, followed by gentle pippetting; cells were counted; a number of $5\times10^5$ cells were inoculated into a 25 $cm^2$ culture flask for further incubation. During the first 30 passages, the cells grew very fast, and generally they could be passaged every 1.5-3 days at the ratio of 1:3. Karyotyping was performed at P30-32.

**Immunocytochemistry**

Sterilized cover slips of 22 × 22 $mm^2$ were placed into Φ35mm plates; after being rinsed with PBS twice, the Φ35mm plates were placed into Φ100mm plates, which were added with a small amount of sterilized water. Digested hEMSCPCs were adjusted to a cell density of $5\times10^4$/mL using medium, and added onto Φ35mm plates pre-laid with cover slips, with 2 mL in each culture plates, followed by incubation for 2-3 days at 36.8±0.2□, 5%$CO_2$ and 100% humidity. During this incubation, medium was replaced based on the degree of yellowing in the medium.

Cover slips adhered by cultured cells were gently rinsed thrice with PBS preheated at 36.8±0.2□; cover slips used in immunohistochemical DAB staining of Nestin, Vimentin, β-□ tubulin, GFAP, CK19, CD10, CD3, CD19, CD16, CD45, CD34, CD31 and VEGF R2 (abcam, USA & Invitrogen, USA) were fixed using methanol over 20 min and then slightly rinsed with distilled water; subjected to 3%Triton x-100 over 20 min at room temperature and then slightly rinsed with PBS; treated with 3%$H_2O_2$ over 10 min at room temperature and again slightly rinsed with PBS; blocked with normal goat serum over 10 min, and were dried by shaking them; then corresponding primary antibody was added, followed by incubation at 37□ for one hour and rinsing with PBS thrice, with 3 min in each time; A solution (intensifier) was added drop by drop, followed by incubation at 37□ for 15 min; Again the cover slips were rinsed with PBS thrice, with 3 min in each time; B solution (Polymerized HRP-Anti Ms/Rb IgG) was added drop by drop, followed by incubation at 37□ for 20 min and rinsing with PBS thrice, with 3 min in each time; DAB was added to develop the color over 10 min; the cover slips were then observed under microscope, and rinsed with tap water to terminate the reaction; counterstaining was achieved using Mayer`s hematoxylin over 1 min, followed by rinsing in tap water to blue the stain; the cover slips were left to dry, sealed and observed and photographed under regular optical microscope (Zeiss, Germany). Cover slips adhered by cultured cells used in immunohistochemical staining of CD90, CD73, CD105 (Santa Cruz, USA), HLA-DR and HLA-I (Invitrogen , USA) were rinsed with PBS, and immediately incubated with FITC-labeled antibody according to the protocol provided by reagent manufacturers, and then fixed over 1 min using pure methanol and glacial acetic acid at the ratio of 3: 1 pre-cooled at 4□; They were then left to dry and counterstained with DAPI solution (Abbott Molecular Inc. USA) to achieve nuclear staining, and were blocked. They were left in room temperature for 30 min and then observed, scanned and photographed under laser confocal microscope (Zeiss, Germany)( DAPI : excitation wavelength 405nm, emission wavelength 461nm, FITC : excitation wavelength 488nm , emission wavelength 525nm ).

**Flow Cytometry Analysis**

After being passaged, the cells were further incubated for 40-48 hours, and digested with 0.25% trypsin-0.02% EDTA into single cell suspension. Following rinsing with PBS twice, it was re-suspended using a small amount of PBS, and divided into 1.5 mL EP tubes. Flow cytometry was performed for antibodies used; for directly labeled antibodies: mouse-anti-human CD73, CD34, HLA-DR monoclonal antibodies (BD, USA), mouse-anti-human CD45, CD3, CD 19, CD16, HLA-□ (Invitrogen, USA), mouse-anti-human CD10, CD31, CD90, CD105, VEGF-R2, CK19 monoclonal antibodies (Santa Cruz, USA), with isotype controls of PE-Isotype control, Percp-Isotype control, FITCIgG1-Isotype control and FITCIgG2-Isotype control (by the same suppliers as corresponding antibodies). For indirectly labeled antibodies, primary antibodies: mouse-anti-human Vimentin monoclonal antibody (abcam, USA), mouse-anti-human β-□ tubulin monoclonal antibody (Millipore, USA), rabbit-anti-human nestin polyclonal antibody and rabbit-anti-human GFAP polyclonal antibody (abcam, USA); secondary antibodies were: goat-anti-mouse-PE, goat-anti-rabbit-FITC (Southern Biotech, USA); cell fixation and permeabilization kit was used for intracellular antigen (Invitrogen, USA). All procedures were conducted according to protocols of the reagents; treatment tubes added with secondary antibody but not primary antibody were used as isotype control for indirectly labeled antibody. After the treatment, the specimens were subjected to detection in a flow cytometer (FACSaria, BD, USA), while data from flow cytometry were analyzed by FCS Express V3. Values for isotype controls were limited within 0%-1%.

**Make up Chimeras**

HEMSCPCs at P10-13 frozen in $LN_2$ were thawed in warm water at the temperature of 35-37□, and suspended with DMEM containing 10% FBS, followed by centrifugation at 1,200 rpm over 5 min; supernatant was removed. Cells were re-suspended using 0.5 mL DMEM without serum, placed in a 6-8□ refrigerator for 10 min, and were then

microscopically injected into mouse blastocoele. Each mouse blastocoeles was injected with 6-8 cells. Injected mouse embryos were incubated overnight at 36.8±0.2□, 5%$CO_2$ and 100% humidity. On the second day, well-developed embryos were transferred into the uterus of 2.5-day pseudo-pregnant mother mice. In 18±1 days, mouse pups would be born. Some of the pseudo-pregnant mice would not become pregnant or would experience miscarriage, while other might give birth to deformed pups, which would die soon. Among pups who survived for more than one month, male ones were excluded, while female ones were used for the detection.

**PCR of Human SRY**

To identify hEMSCPC-derived cells in the female mice, genomic DNA was extracted from tissue samples using the Biomiga EZgene TM Tissue gDNA Miniprep Kit. Genomic DNA from tissues was analyzed by human Y chromosome-specific DNA and the human SRY sequence was detected by a nested-PCR procedure using sequence specific primers (SRY primers: F1, 5′-CAGTGTGAAACGGGAGAAAACAGT-3′; R1, 5′- CTTCCGACGAGGTCGATACTTATA-3′; F2, 5′-TGTAATTTCTGTGCCTCCTGGAAGAATGG-3′; and R2, 5′- GAAACGGGAGAAAACAGTAAAGGCAACGT -3′). In the first PCR, a 25 μl of PCR mixture with the primers F1 and R1 was used. After the initial denaturing step at 95 °C for 10 min, 35 cycles of amplification were performed, consisting of 30 s at 95 °C, 30 s at 65 °C, and 30 s at 72 °C, followed by a final single extension step at 72 °C for 10 min. In the second PCR amplification reaction, the 25 μl reaction mixture contained the primers F2 and R2 and 0.5 μL of the first PCR product. The PCR conditions were similar to those used for the first PCR except for the cycles of amplification. In total, 32 cycles were performed. As a control, GAPDH primers were used (F, 5′- TCACTCAAGATTGTCAGCAA -3′; R, 5′-AGATCCACGACGGACACATT -3′) with the annealing temperature at 55 °C for 27 cycles. The PCR products were separated by a 1.5% agarose gel.

**PCR MATERIALS:**

We used the Biomiga EZgene TM Tissue gDNA Miniprep Kit (Biomiga,Inc., San Diego, CA,USA), 2x Taq PCR Mix (Biomiga,Inc., San Diego, CA, USA), Biowerter Regular Agarose G-10 (Gene Company LTD., Hong Kong), and a Tprofessional standard Gradient Thermocycler (Biometra GmbH,Germany). Electrophoresis was run on a LIUYI, DYY-12 (BEIJING LIUYI INSTRUMENT FACTORY, CHINA) with a VILBER LOURMAT INFINITY gel formatter (Vilber Lourmat, France). An ultraviolet Spectrophotometer was purchased from Beckman-Coulter (Model DV800; Beckman Coulter, Inc. USA).

**FISH of Human Y for hEMSCPCs**

After being incubated for 24 hours, hEMSCPCs were replaced with medium containing 0.2ug/ml colcemid when cells were 70%-80% confluent; incubation was continued on at 36.8±0.2□, 5%$CO_2$ and 100% humidity for 2-2.5 hours; medium containing colcemide was removed, and cells were rinsed with PBS twice; cells were digested, collected and counted as routine; rinsed with PBS once; cell precipitation was oscillated and blended using a vortex oscillator. An approximate of $2\times10^6$ cells were added into 3 mL 0.075 M KCl, gently blended and incubated at 37□ over 10 min; following centrifugation at 1,200 rpm for 5 min, supernatant was removed; cells were gently oscillated in a vortex oscillator to prevent cell lumping; an amount of 3 mL fixative solution freshly prepared with pure methanol and glacial acetic acid (3:1) was added while blending the suspension; after centrifugation at 1,200 rpm over 5 min, supernatant was removed; Cells were again blended gently by vortex oscillator and added with 3 mL fixative solution freshly prepared with pure methanol and glacial acetic acid (3:1) while being blended. After centrifugation at 1,200 rpm over 5 min, supernatant was removed; following a second gentle blending process, cells were added with fixative solution of pure methanol-glacial acetic acid (3:1) to a cell concentration of $1\times10^6$ cells/mL; Cell suspension was divided into EP tubes, with 100 μL in each tube. These tubes were then stored at -20℃ for subsequent use. Cells were transferred from -20℃ into room temperature, and cytospin (Shandon, Thermo Electron Corporation, UK)was performed at 2,000 rpm and moderate acceleration over 2 min; cell smears were air-dried; smears were soaked in 2×SSC/0.1%NP-40 rinsing solution (prepared based on the regimen provided by Abbott

Molecular Inc. USA) preheated at 37□ for 30 min; excessive solution on the smears was removed; gradient dehydration was performed in 70%, 85%, and 100% ethanol at room temperature, with 2 min in each gradient; the smears were air-dried, (**the following processes were to be performed light-shielded**) and added with 10 μL human-Y specific probe mixture (prepared based on the regimen provided by Abbott Molecular Inc. USA); covered with slips, the smears were subjected to co-denaturation over 6 to 7 min at 73±1□; and then placed in a sealed humid container preheated at 42□ for 16 hours; rinsed with 2×SSC/0.3%NP-40 solution (prepared based on the regimen provided by Abbott Molecular Inc. USA) preheated at 73±1□ over 2 min and then transferred to 2×SSC/0.1%NP-40 solution (prepared based on the regimen provided by Abbott Molecular Inc. USA) at room temperature for another 2 min; excessive solution was removed from the smears, which were then air-dried and counterstained with 10 μL DAPI solution (Abbott Molecular Inc. USA) and blocked by upturning the smears; these smears were observed and photographed under laser confocal microscope (Zeiss, Germany) using blue-light(excitation wavelength 405nm, emission wavelength461nm) and red-light (excitation wavelength 543nm, emission wavelength588nm) 30 min later.

**FISH of Human Y for Chimeras' blood**

An amount of 200 μL anti-coagulated blood was obtained from mice; added with 3 mL of 0.83% $NH_4Cl$ solution pre-cooled at 4□ and then gently pippetted; it was then left to stand at 37□ for 15 min; 4 mL PBS was added, followed by gentle pippetting; after centrifugation at 1,200 rpm over 5 min, supernatant was removed; 10 mL PBS was added, followed by gentle pippetting; after centrifugation at 1,500 rpm over 5 min, supernatant was removed; cell precipitation was oscillated and blended using a vortex oscillator. Processes hereafter were the same as in **FISH of Human Y for hEMSCPCs**.

**Immunofluorescence Staining & FISH of Human Y for Chimeras' blood**

Antibody would be used only when no cross-reactions were seen in tests performed in normal negative female mice.

An amount of 200 μL anticoagulated blood was obtained from mice, added with 3 mL of 0.83% $NH_4Cl$ solution pre-cooled at 4□ and gently pippetted; it was then left to stand at 37□ for 15 min; 4 mL PBS was added, followed by gentle pippetting; after centrifugation at 1,200 rpm over 5 min, supernatant was removed; 10 mL PBS was added, followed by gentle pippetting; after centrifugation at 1,500 rpm over 5 min, supernatant was removed; added with 100 μL PBS, the suspension was gently pippetted to nicely suspend the cells; (**the following processes are to be performed light-shielded**) added with FITC-labeled anti-human monoclonal antibody CD3,CD19,CD16 (Invitrogen, USA ,dosage was based on the formula provided by manufacturers) and gently pippetted; it was incubated at room temperature for 30-40 min (performed according to the procedures provided by reagent manufacturers); After 2 cycles of PBS rinsing and centrifugation at 1,800 rpm for 5 min, cell precipitation was suspended using a vortex oscillator; 1 mL fixative solution of pure methanol and glacial acetic acid (3:1) was added drop by drop while cell suspension was being gently blended; after centrifugation at 2,000 rpm over 5 min, supernatant was removed; 100 μL fixative solution of pure methanol and glacial acetic acid (3:1) was added drop by drop while cell suspension was being gently blended; cytospin (Shandon, Thermo Electron Corporation, UK) was performed at 2,000 rpm and moderate acceleration in 2 min; the smears were air-dried. Processes hereafter were the same as in **FISH of Human Y for hEMSCPCs**. Finally, smears were observed and photographed under laser confocal microscope (Zeiss, Germany) using blue-, green- and red-light (excitation wavelength 405nm/488nm/543nm, emission wavelength461nm/525nm/588nm).

**Immunofluorescence Staining & FISH of Human Y for Chimeras' Brain, Heart, Lung, Spleen, Liver & Kidney**

Antibody would be used only when no cross-reactions were seen in tests performed in normal negative female mice.

The antibodies used in this trial were as following: Primary antibodies were rabbit anti-human β-Ⅲ tubulin polyclonal

antibody and rabbit anti-human MAP2 monoclonal antibody（abcam，USA）,FITC conjugated goat anti-rabbit (SouthernBiotech, USA) served as the secondary antibody, FITC-labeled mouse or rabbit anti-human monoclonal antibody Tropolin I,SP-C,CD31, CD16,ALB, VEGF R2（Invitrogen, USA & Santa Cruz, USA & BD, USA）.

Tissues of brain, heart, lung, spleen, liver and kidney from female mice to be examined were embedded with OCT compound (Sakura Finetek, USA), and stored at -20℃ for subsequent use. Tissues of brain, heart, lung, spleen, liver and kidney with positive nested-PCR results were subjected to frozen section at the slice thickness of 4 μm; the slices were air-dried and then OCT gel was removed; to improve adhesion of tissue slices and to adequately spread tissue structures, cytospin (Shandon, Thermo Electron Corporation, UK) was performed at 2,000 rpm and moderate acceleration over 2 min; (**the following processes are to be performed light-shielded**) slices were incubated with FITC-antibody (directly labeled) or primary antibody (indirectly labeled) at room temperature for 40 min (performed according to the procedures provided by reagent manufacturers); rinsed 3-4 times with PBS pre-cooled at 6-8℃; for indirectly labeled antibody, incubation was continued on with FITC-second antibody at room temperature for 30 min (performed according to the procedures provided by reagent manufacturers); rinsed 3-4 times with PBS pre-cooled at 6-8℃. After incubation with antibodies, tissue slices were to be air-dried; soaked in 2×SSC/0.1%NP-40 solution (prepared based on the regimen provided by Abbott Molecular Inc. USA) preheated at 37□ for 30 min; excessive solution on the slices was removed; and the slices were subjected to gradient dehydration in 70%, 85% and 100% ethanol at room temperature, with 2 min in each gradient; slices were air-dried, added with 10 μL human-Y specific probe mixture (prepared based on the regimen provided by Abbott Molecular Inc. USA); covered with a cover slip, the slices underwent co-denaturation at 73±1□ in 6-7 min; and then placed in a sealed humid container pre-heated at 42□ for 16 hours; rinsed in 2×SSC/0.3%NP-40 solution (prepared based on the regimen provided by Abbott Molecular Inc. USA) preheated at 73±1□ over 2 min; transferred into 2×SSC/0.3%NP-40 solution (prepared based on the regimen provided by Abbott Molecular Inc. USA) at room temperature for 2 min; excessive solution on the slices was removed and the slices air-dried, counterstained by dripping 10-15 μL DAPI solution (Abbott Molecular Inc. USA) onto the cover slips, and blocked by upturning the slices; these slice were observed and photographed under laser confocal microscope (Zeiss, Germany) using blue-, green- and red-light(excitation wavelength 405nm/488nm/543nm, emission wavelength461nm/525nm/588nm) 30 min later.

**Supplementary Table**

Supplementary Table 1. Dynamic expression of specific markers in cultured hEMSCPCs before passage 30 as determined by flow cytometry analysis

| Marker | P2 | P10 | P20 | P30 | **P*** |
|---|---|---|---|---|---|
| CD73 | 97.975±0.689 | 98.438±0.574 | 98.713±0.296 | 97.625±0.697 | **1.182** |
| CD90** | 62.025±11.563 | 91.863±1.387[a] | 90.650±3.659[b] | 94.825±2.015[c] | **0.002** |
| CD105 | 46.700±7.356 | 59.225±8.041 | 59.075±4.730 | 65.813±3.793 | **0.171** |
| Vimentin | 77.225±5.653 | 88.425±2.884 | 87.8±4.413 | 86.475±4.463 | **0.271** |
| Nestin*** | 62.263±9.913 | 87.325±2.978[A] | 92.875±1.627[B] | 87.163±2.246[C] | **0.003** |
| GFAP | 0.325±0.269 | 1.3±0.88 | 1±0.528 | 1.975±1.062 | **0.347** |
| β-III Tubulin | 3.113±1.367 | 6.288±2.609 | 4.525±1.607 | 4.488±2.434 | **0.765** |
| CD10 | 0.838±0.404 | 3.488±1.630 | 2.963±0.968 | 2.025±0.652 | **0.227** |
| CK19 | 0.225±0.156 | 0.188±0.174 | 0.35±0.151 | 0.138±0.089 | **0.265** |
| CD34 | 0.388±0.090 | 0.575±0.185 | 0.15±0.05 | 0.413±0.146 | **0.138** |
| CD45 | 0.05±0.038 | 0.025±0.025 | 0.038±0.018 | 0.15±0.07 | **0.134** |
| CD3 | 0.375±0.096 | 0.4±0.08 | 0.25±0.06 | 0.763±0.293 | **0.093** |
| CD19 | 0.125±0.059 | 0.063±0.042 | 0.063±0.026 | 0.1±0.033 | **0.649** |
| CD16 | 0.1±0.027 | 0.038±0.018 | 0.163±0.068 | 0.175±0.077 | **0.116** |
| CD31 | 0 | 0.513±0.309 | 0.425±0.256 | 0.438±0.180 | **0.241** |
| VEGFR2 | 0 | 0.7±0.411 | 0.538±0.263 | 0.7±0.211 | **0.139** |
| HLA-DR | 4.438±3.784 | 0.05±0.032 | 0.038±0.026 | 0 | **0.284** |
| HLA-I**** | 27.563±10.104 | 12.113±7.633 | 6.95±5.576[#] | 8.688±5.151 | **0.023** |

* SPSS 13.0 was used for statistical analysis. Data from FACS are presented as mean ± standard error of the mean (SEM). Results were analyzed by two-way ANOVA with Bonferroni multiple comparison post-test for more than two groups. Differences were considered statistically significant when $P < 0.05$. n=8.

** hEMSCPCs at passage 2 had lower CD90 expression compared to passages 10, 20, and 30. [a] The comparison between passage 2 and passage 10, P= 0.009, [b] The comparison between passage 2 and passage 20, P= 0.013, [c] The comparison between passage 2

and passage 30, P=0.004.

*** hEMSCPCs at passage 2 had lower nestin expression compared to passages 10, 20, and 30. [A] P= 0.021, [B] P= 0.004, [C] P= 0.022.

**** hEMSCPCs at passage 2 had higher HLA-ǀ expression compared to passage 20, #P= 0.035.

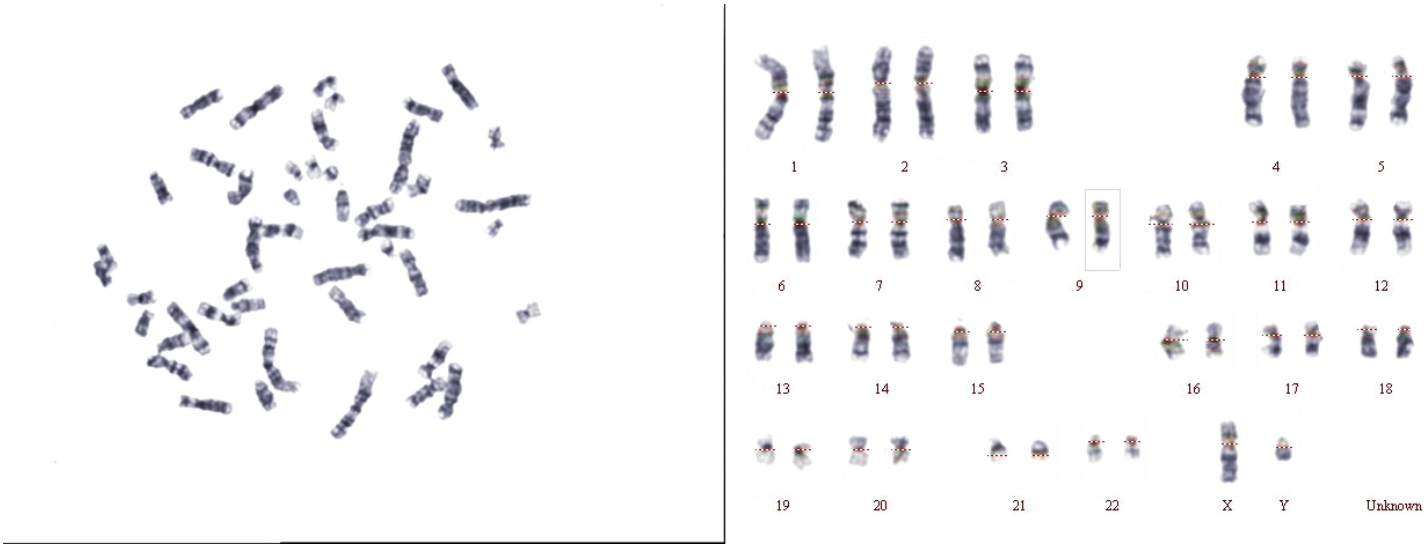
Supplementary Table 2.. Positive organs based on nested PCR of human SRY genes

| Number | Blood | Heart | Brain | Lung | Kidney | Spleen | Liver |
|---|---|---|---|---|---|---|---|
| 6 | | | + | + | + | | |
| 8 | + | | | | | | |
| 10 | + | | | | | | |
| 11 | + | | | | | | |
| 13 | + | | | | | | |
| 15 | + | | | | | | |
| 17 | + | | | | | | |
| 18 | | | + | | | + | |
| 19 | + | + | + | | | | |
| 20 | + | + | | + | | | |
| 21 | + | | | | | | |
| 22 | + | | | | | | |
| 24 | | | | | | + | |
| 32 | + | + | | | | | |
| 34 | + | | | | | | |
| 36 | | + | | | + | | + |
| 37 | | | + | + | + | | + |
| 38 | | | | | | + | |
| 39 | + | | | | | | |
| 41 | | | | + | | | |
| 42 | + | | | + | | | |
| 45 | + | | | | | | |

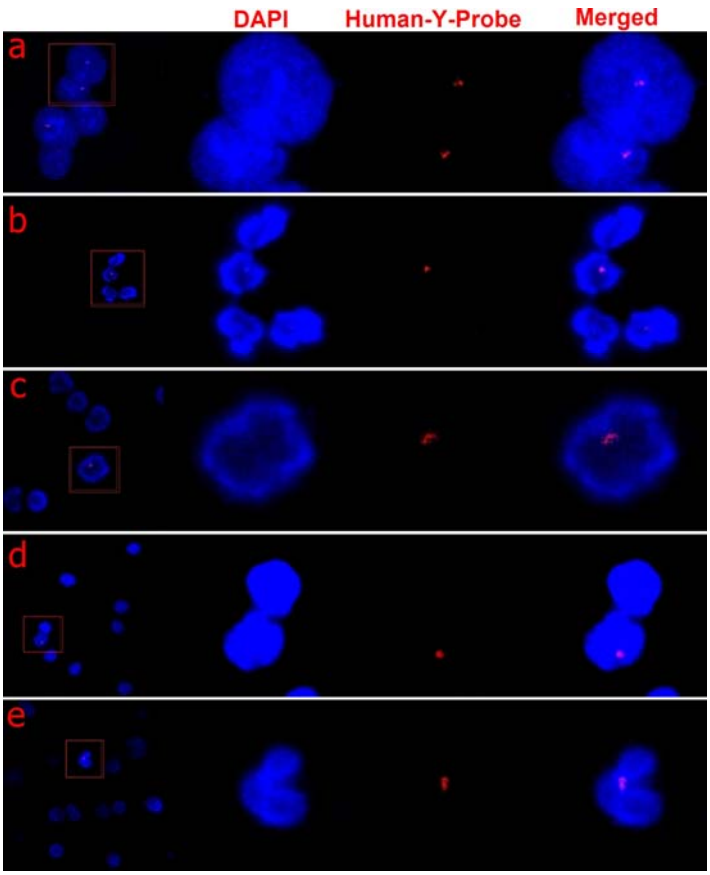
| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 50 | | | | + | | | |
| 52 | | + | | | + | | + |
| 53 | | | | + | | | |
| 63 | | + | | | | | |
| 64 | | | | | | + | |
| 66 | | | | + | | | |

Neither normal negative mice (number 1 to number 5) nor mice with 7 negative organs are shown. hEMSCPCs were most widely distributed in blood, then lung, followed by heart, brain, spleen, kidney, and finally liver (the lowest).

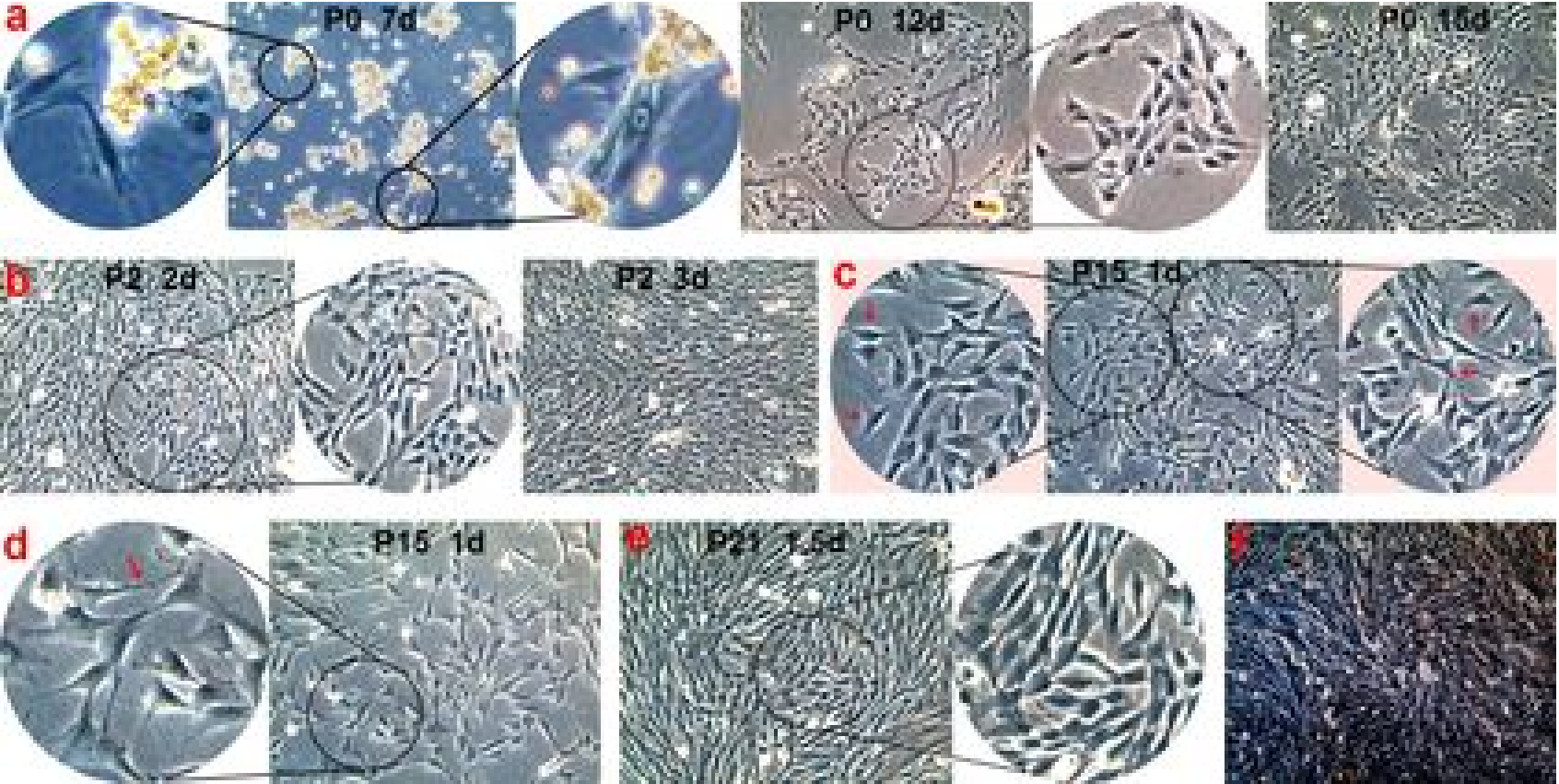
**Supplementary Figure Legends**

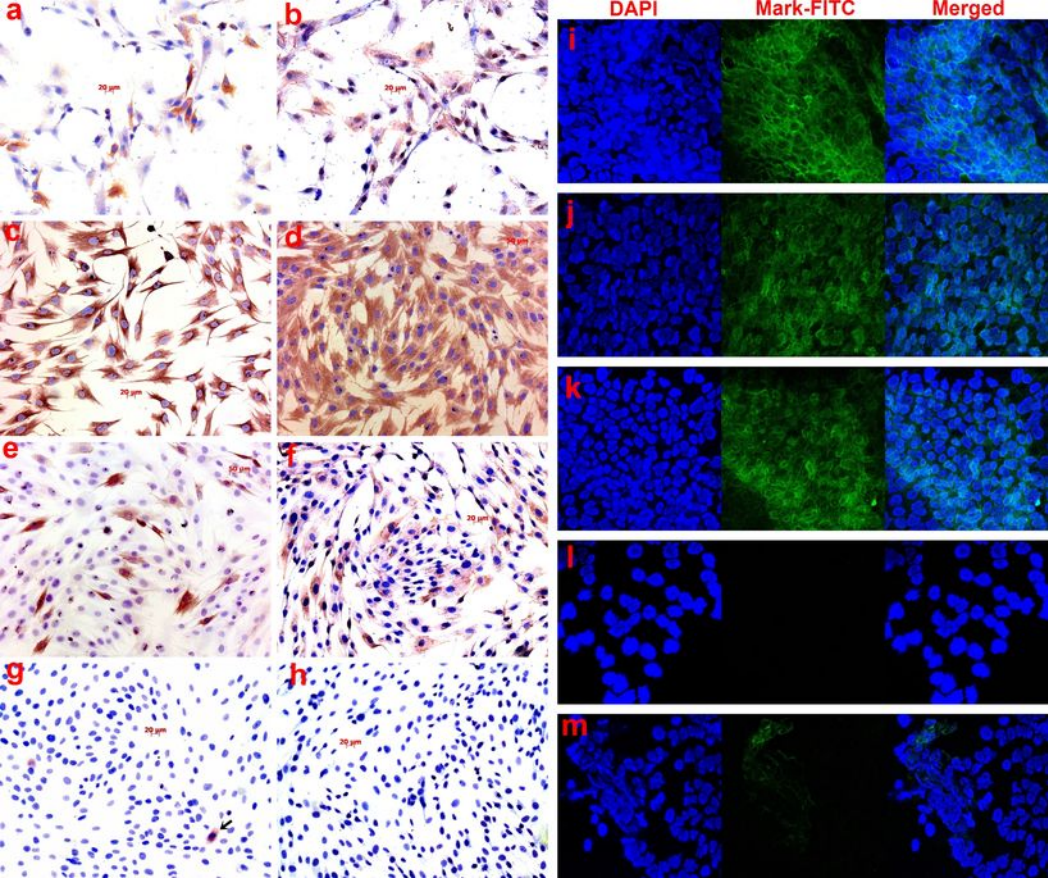


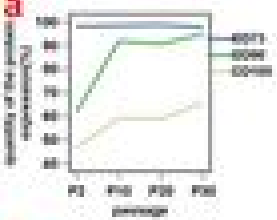

**Supplementary Figure 1. Karyotype of hEMSCPCs.** Chromosome detection of hEMSCPCs between passages 30 to 32 confirmed the karyotype as 46, XY.

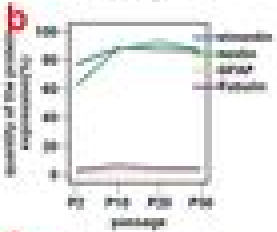


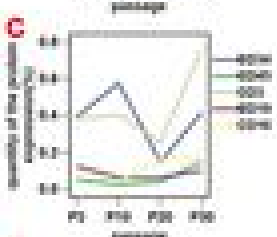

**Supplementary Figure 2. Human Y FISH for hEMSCPCs and the cells from positive caudal vein blood of female chimeras** Human Y chromosome is seen as red spots. hEMSCPCs have positive human Y (a); the cells from caudal vein blood of positive female chimeras had differently shaped nuclei: sublobe nucleus (b), giant nucleus (c), spherical nucleus (d), and renal type nucleus (e).

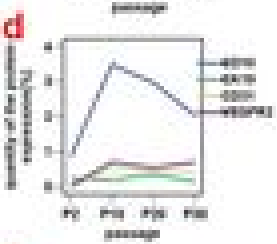

a
P0 7d
P0 12d
P0 15d
b
P2 2d
P2 3d
c
P15 1d
d
P15 1d
e
P21 1.5d
f

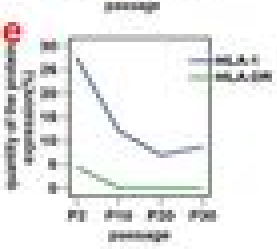

a
b
c
d
e
f
g
h
i
j
k
l
m
DAPI
Mark-FITC
Merged
20 μm


a
quantity of the protein expression(%)
100
80
60
40
20
0
P2
P10
P20
P30
passage


b
quantity of the protein expression(%)
100
80
60
40
20
0
P2
P10
P20
P30
passage


c
quantity of the protein expression(%)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
P2
P10
P20
P30
passage


d
quantity of the protein expression(%)
4
3
2
1
0
P2
P10
P20
P30
passage

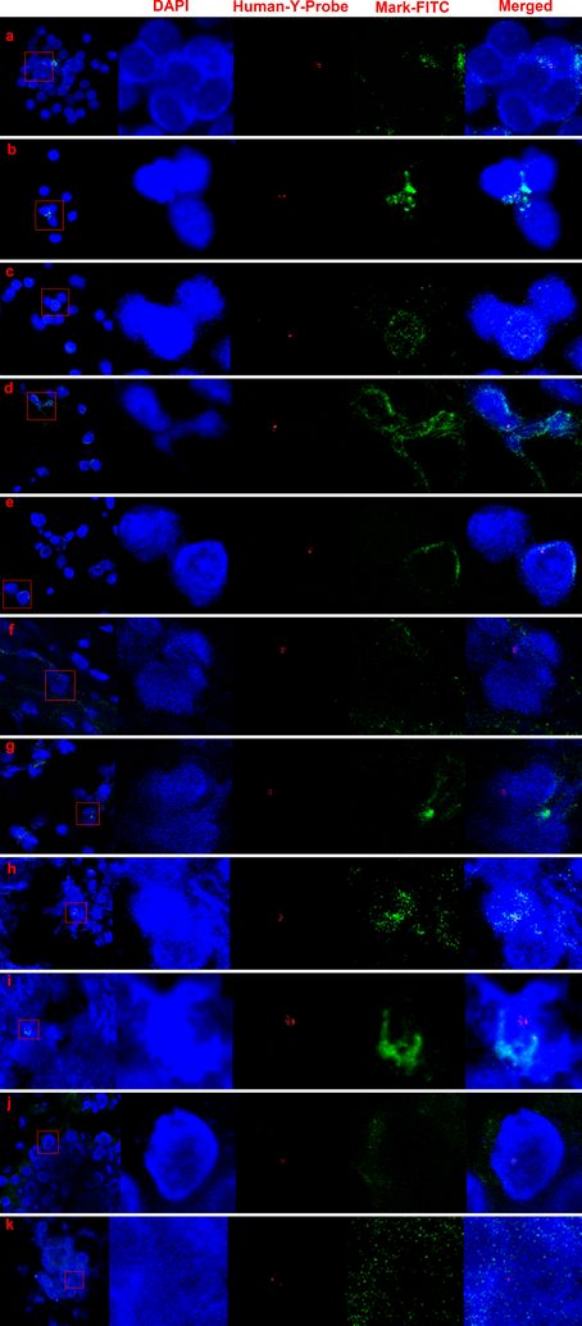

e
quantity of the protein expression(%)
80
70
60
50
40
30
20
10
0
P2
P10
P20
P30
passage

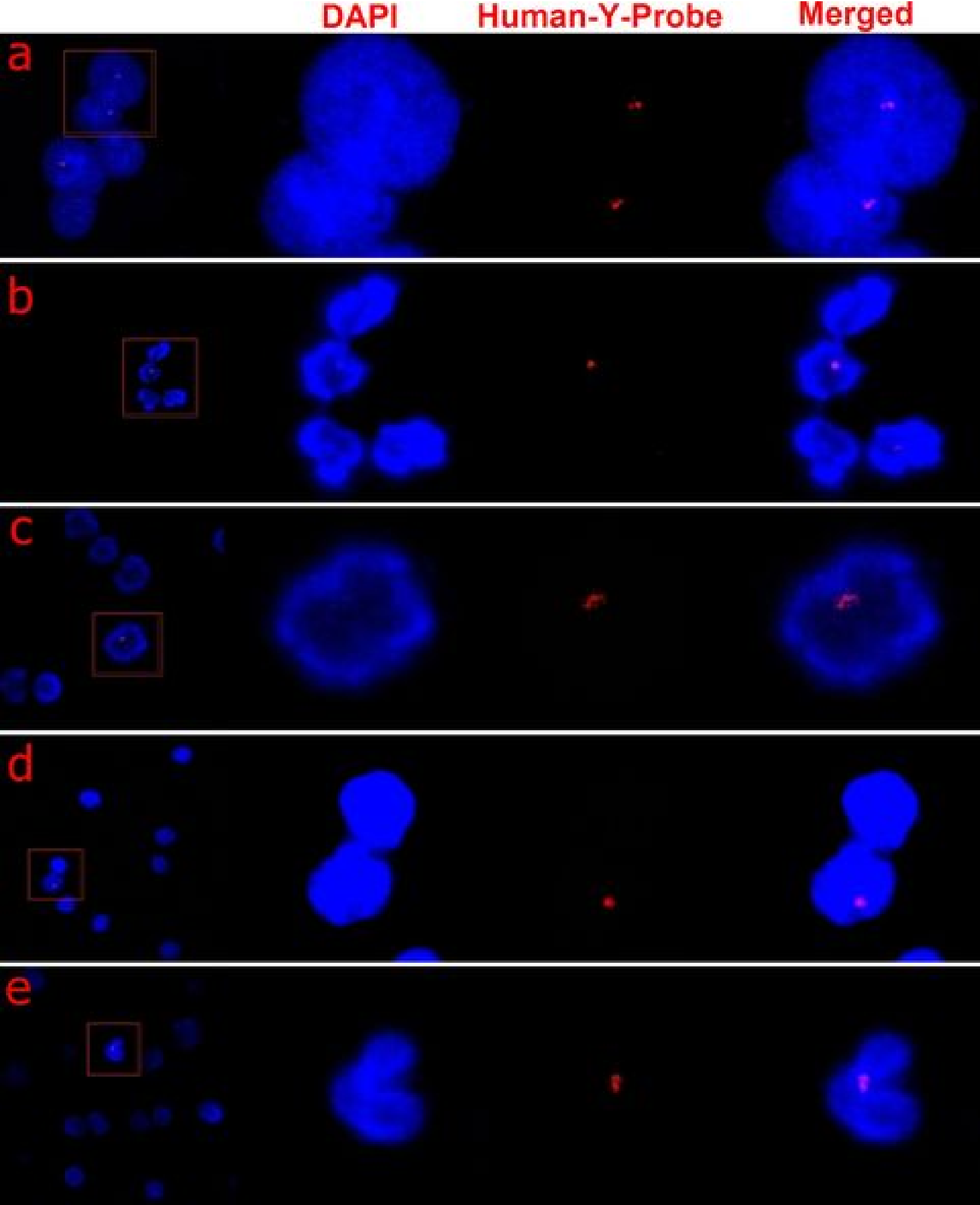

f
Blood
M H2O N 15 16 17 18 19 P
M H2O N 20 21 22 23 24 P
300bp


g
Brain
Heart
M H2O N 18 19 20 21 22 23 24 P
M H2O N 18 19 20 21 22 23 24 P
300bp


h
Kidney
Spleen
M H2O N 37 38 39 40 41 42 P
M H2O N 37 38 39 40 41 42 P
300bp


i
Lung
Liver
M H2O N 49 50 51 52 53 54 P
M H2O N 49 50 51 52 53 54 P
300bp


DAPI
Human-Y-Probe
Mark-FITC
Merged
a
b
c
d
e
f
g
h
i
j
k


DAPI
Human-Y-Probe
Merged
a
b
c
d
e