to 21 hydroxylase deficiency: from birth to adulthood. *Semin Reprod Med* 2012; **30**: 400-409 [PMID: 23044877 DOI: 10.1055/s-0032-1324724]
3 **Haring R**. Perspectives for metabolomics in testosterone replacement therapy. *J Endocrinol* 2012; **215**: 3-16 [PMID: 22547567 DOI: 10.1530/JOE-12-0119]
4 **Morris PD**, Channer KS. Testosterone and cardiovascular disease in men. *Asian J Androl* 2012; **14**: 428-435 [PMID: 22522504 DOI: 10.1038/aja.2012.21]
5 **Shelton JB**, Rajfer J. Androgen deficiency in aging and metabolically challenged men. *Urol Clin North Am* 2012; **39**: 63-75 [PMID: 22118346 DOI: 10.1016/j.ucl.2011.09.007]
6 **Staerman F**, Léon P. Andropause (androgen deficiency of the aging male): diagnosis and management. *Minerva Med* 2012; **103**: 333-342 [PMID: 23042368]
7 **Marjoribanks J**, Farquhar C, Roberts H, Lethaby A. Long term hormone therapy for perimenopausal and postmenopausal women. *Cochrane Database Syst Rev* 2012; **7**: CD004143 [PMID: 22786488]
8 **Rozenberg S**, Vandromme J, Antoine C. Postmenopausal hormone therapy: risks and benefits. *Nat Rev Endocrinol* 2013; **9**: 216-227 [PMID: 23419265 DOI: 10.1038/nrendo.2013.17]
9 **Miller WL**. Molecular biology of steroid hormone synthesis. *Endocr Rev* 1988; **9**: 295-318 [PMID: 3061784 DOI: 10.1210/edrv-9-3-295]
10 **Miller WL**, Auchus RJ. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr Rev* 2011; **32**: 81-151 [PMID: 21051590 DOI: 10.1210/er.2010-0013]
11 **Morohashi K**. The ontogenesis of the steroidogenic tissues. *Genes Cells* 1997; **2**: 95-106 [PMID: 9167967]
12 **Val P**, Martinez-Barbera JP, Swain A. Adrenal development is initiated by Cited2 and Wt1 through modulation of Sf-1 dosage. *Development* 2007; **134**: 2349-2358 [PMID: 17537799 DOI: 10.1242/dev.004390]
13 **Bandiera R**, Vidal VP, Motamedi FJ, Clarkson M, Sahut-Barnola I, von Gise A, Pu WT, Hohenstein P, Martinez A, Schedl A. WT1 maintains adrenal-gonadal primordium identity and marks a population of AGP-like progenitors within the adrenal gland. *Dev Cell* 2013; **27**: 5-18 [PMID: 24135228 DOI: 10.1016/j.devcel.2013.09.003]
14 **Hatano O**, Takakusu A, Nomura M, Morohashi K. Identical origin of adrenal cortex and gonad revealed by expression profiles of Ad4BP/SF-1. *Genes Cells* 1996; **1**: 663-671 [PMID: 9078392]
15 **Luo X**, Ikeda Y, Parker KL. A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. *Cell* 1994; **77**: 481-490 [PMID: 8187173 DOI: 10.1016/0092-8674(94)90211-9]
16 **Sadovsky Y**, Crawford PA, Woodson KG, Polish JA, Clements MA, Tourtellotte LM, Simburger K, Milbrandt J. Mice deficient in the orphan receptor steroidogenic factor 1 lack adrenal glands and gonads but express P450 side-chain-cleavage enzyme in the placenta and have normal embryonic serum levels of corticosteroids. *Proc Natl Acad Sci USA* 1995; **92**: 10939-10943 [PMID: 7479914 DOI: 10.1073/pnas.92.24.10939]
17 **Parker KL**, Schimmer BP. Steroidogenic factor 1: a key determinant of endocrine development and function. *Endocr Rev* 1997; **18**: 361-377 [PMID: 9183568 DOI: 10.1210/er.18.3.361]
18 **Lala DS**, Rice DA, Parker KL. Steroidogenic factor I, a key regulator of steroidogenic enzyme expression, is the mouse homolog of fushi tarazu-factor I. *Mol Endocrinol* 1992; **6**: 1249-1258 [PMID: 1406703 DOI: 10.1210/me.6.8.1249]
19 **Morohashi K**, Honda S, Inomata Y, Handa H, Omura T. A common trans-acting factor, Ad4-binding protein, to the promoters of steroidogenic P-450s. *J Biol Chem* 1992; **267**: 17913-17919 [PMID: 1517227]
20 **Parker KL**, Rice DA, Lala DS, Ikeda Y, Luo X, Wong M, Bakke M, Zhao L, Frigeri C, Hanley NA, Stallings N, Schimmer BP. Steroidogenic factor 1: an essential mediator of endocrine development. *Recent Prog Horm Res* 2002; **57**: 19-36 [PMID: 12017543 DOI: 10.1210/rp.57.1.19]
21 **Schimmer BP**, White PC. Minireview: steroidogenic factor 1: its roles in differentiation, development, and disease. *Mol Endocrinol* 2010; **24**: 1322-1337 [PMID: 20203099 DOI: 10.1210/me.2009-0519]
22 **Mangelsdorf DJ**, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM. The nuclear receptor superfamily: the second decade. *Cell* 1995; **83**: 835-839 [PMID: 8521507 DOI: 10.1016/0092-8674(95)90199-X]
23 **Robinson-Rechavi M**, Escriva Garcia H, Laudet V. The nuclear receptor superfamily. *J Cell Sci* 2003; **116**: 585-586 [PMID: 12538758 DOI: 10.1242/jcs.00247]
24 **Krylova IN**, Sablin EP, Moore J, Xu RX, Waitt GM, MacKay JA, Juzumiene D, Bynum JM, Madauss K, Montana V, Lebedeva L, Suzawa M, Williams JD, Williams SP, Guy RK, Thornton JW, Fletterick RJ, Willson TM, Ingraham HA. Structural analyses reveal phosphatidyl inositols as ligands for the NR5 orphan receptors SF-1 and LRH-1. *Cell* 2005; **120**: 343-355 [PMID: 15707893 DOI: 10.1016/j.cell.2005.01.024]
25 **Galarneau L**, Paré JF, Allard D, Hamel D, Levesque L, Tugwood JD, Green S, Bélanger L. The alpha1-fetoprotein locus is activated by a nuclear receptor of the Drosophila FTZ-F1 family. *Mol Cell Biol* 1996; **16**: 3853-3865 [PMID: 8668203]
26 **Fayard E**, Auwerx J, Schoonjans K. LRH-1: an orphan nuclear receptor involved in development, metabolism and steroidogenesis. *Trends Cell Biol* 2004; **14**: 250-260 [PMID: 15130581 DOI: 10.1016/j.tcb.2004.03.008]
27 **Lee YK**, Moore DD. Liver receptor homolog-1, an emerging metabolic modulator. *Front Biosci* 2008; **13**: 5950-5958 [PMID: 18508634]
28 **Lazarus KA**, Wijayakumara D, Chand AL, Simpson ER, Clyne CD. Therapeutic potential of Liver Receptor Homolog-1 modulators. *J Steroid Biochem Mol Biol* 2012; **130**: 138-146 [PMID: 22266285 DOI: 10.1016/j.jsbmb.2011.12.017]
29 **Wilson C**. Metabolism: LRH-1 is a transcriptional regulator of glucokinase in the liver. *Nat Rev Endocrinol* 2012; **8**: 566 [PMID: 22847241 DOI: 10.1038/nrendo.2012.137]
30 **Bookout AL**, Jeong Y, Downes M, Yu RT, Evans RM, Mangelsdorf DJ. Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. *Cell* 2006; **126**: 789-799 [PMID: 16923397 DOI: 10.1016/j.cell.2006.06.049]
31 **Wang ZN**, Bassett M, Rainey WE. Liver receptor homologue-1 is expressed in the adrenal and can regulate transcription of 11 beta-hydroxylase. *J Mol Endocrinol* 2001; **27**: 255-258 [PMID: 11564608 DOI: 10.1677/jme.0.0270255]
32 **Suzuki T**, Kasahara M, Yoshioka H, Morohashi K, Umesono K. LXXLL-related motifs in Dax-1 have target specificity for the orphan nuclear receptors Ad4BP/SF-1 and LRH-1. *Mol Cell Biol* 2003; **23**: 238-249 [PMID: 12482977 DOI: 10.1128/MCB.23.1.238-249.2003]
33 **Wang W**, Zhang C, Marimuthu A, Krupka HI, Tabrizizad M, Shelloe R, Mehra U, Eng K, Nguyen H, Settachatgul C, Powell B, Milburn MV, West BL. The crystal structures of human steroidogenic factor-1 and liver receptor homologue-1. *Proc Natl Acad Sci USA* 2005; **102**: 7505-7510 [PMID: 15897460 DOI: 10.1073/pnas.0409482102]
34 **Saxena D**, Escamilla-Hernandez R, Little-Ihrig L, Zeleznik AJ. Liver receptor homolog-1 and steroidogenic factor-1 have similar actions on rat granulosa cell steroidogenesis. *Endocrinology* 2007; **148**: 726-734 [PMID: 17095585 DOI: 10.1210/en.2006-0108]
35 **Heng JC**, Feng B, Han J, Jiang J, Kraus P, Ng JH, Orlov YL, Huss M, Yang L, Lufkin T, Lim B, Ng HH. The nuclear receptor Nr5a2 can replace Oct4 in the reprogramming of murine somatic cells to pluripotent cells. *Cell Stem Cell* 2010; **6**: 167-174 [PMID: 20096661 DOI: 10.1016/j.stem.2009.12.009]
36 **Yazawa T**, Inaoka Y, Okada R, Mizutani T, Yamazaki Y, Usa-

mi Y, Kuribayashi M, Orisaka M, Umezawa A, Miyamoto K. PPAR-gamma coactivator-1alpha regulates progesterone production in ovarian granulosa cells with SF-1 and LRH-1. *Mol Endocrinol* 2010; **24**: 485-496 [PMID: 20133449 DOI: 10.1210/me.2009-0352]

37 **Yazawa T**, Kawabe S, Inaoka Y, Okada R, Mizutani T, Imamichi Y, Ju Y, Yamazaki Y, Usami Y, Kuribayashi M, Umezawa A, Miyamoto K. Differentiation of mesenchymal stem cells and embryonic stem cells into steroidogenic cells using steroidogenic factor-1 and liver receptor homolog-1. *Mol Cell Endocrinol* 2011; **336**: 127-132 [PMID: 21129436 DOI: 10.1016/j.mce.2010.11.025]

38 **Yazawa T**, Mizutani T, Yamada K, Kawata H, Sekiguchi T, Yoshino M, Kajitani T, Shou Z, Miyamoto K. Involvement of cyclic adenosine 5'-monophosphate response element-binding protein, steroidogenic factor 1, and Dax-1 in the regulation of gonadotropin-inducible ovarian transcription factor 1 gene expression by follicle-stimulating hormone in ovarian granulosa cells. *Endocrinology* 2003; **144**: 1920-1930 [PMID: 12697699 DOI: 10.1210/en.2002-221070]

39 **Ikeda Y**, Lala DS, Luo X, Kim E, Moisan MP, Parker KL. Characterization of the mouse FTZ-F1 gene, which encodes a key regulator of steroid hydroxylase gene expression. *Mol Endocrinol* 1993; **7**: 852-860 [PMID: 8413309 DOI: 10.1210/me.7.7.852]

40 **Kawabe K**, Shikayama T, Tsuboi H, Oka S, Oba K, Yanase T, Nawata H, Morohashi K. Dax-1 as one of the target genes of Ad4BP/SF-1. *Mol Endocrinol* 1999; **13**: 1267-1284 [PMID: 10446902 DOI: 10.1210/me.13.8.1267]

41 **Duggavathi R**, Volle DH, Mataki C, Antal MC, Messaddeq N, Auwerx J, Murphy BD, Schoonjans K. Liver receptor homolog 1 is essential for ovulation. *Genes Dev* 2008; **22**: 1871-1876 [PMID: 18628394 DOI: 10.1101/gad.472008]

42 **Peng N**, Kim JW, Rainey WE, Carr BR, Attia GR. The role of the orphan nuclear receptor, liver receptor homologue-1, in the regulation of human corpus luteum 3beta-hydroxysteroid dehydrogenase type II. *J Clin Endocrinol Metab* 2003; **88**: 6020-6028 [PMID: 14671206 DOI: 10.1210/jc.2003-030880]

43 **Pezzi V**, Sirianni R, Chimento A, Maggiolini M, Bourguiba S, Delalande C, Carreau S, Andò S, Simpson ER, Clyne CD. Differential expression of steroidogenic factor-1/adrenal 4 binding protein and liver receptor homolog-1 (LRH-1)/fetoprotein transcription factor in the rat testis: LRH-1 as a potential regulator of testicular aromatase expression. *Endocrinology* 2004; **145**: 2186-2196 [PMID: 14736734 DOI: 10.1210/en.2003-1366]

44 **Yazawa T**, Inanoka Y, Mizutani T, Kuribayashi M, Umezawa A, Miyamoto K. Liver receptor homolog-1 regulates the transcription of steroidogenic enzymes and induces the differentiation of mesenchymal stem cells into steroidogenic cells. *Endocrinology* 2009; **150**: 3885-3893 [PMID: 19359379 DOI: 10.1210/en.2008-1310]

45 **Bland ML**, Jamieson CA, Akana SF, Bornstein SR, Eisenhofer G, Dallman MF, Ingraham HA. Haploinsufficiency of steroidogenic factor-1 in mice disrupts adrenal development leading to an impaired stress response. *Proc Natl Acad Sci USA* 2000; **97**: 14488-14493 [PMID: 11121051 DOI: 10.1073/pnas.97.26.14488]

46 **Bland ML**, Fowkes RC, Ingraham HA. Differential requirement for steroidogenic factor-1 gene dosage in adrenal development versus endocrine function. *Mol Endocrinol* 2004; **18**: 941-952 [PMID: 14726490 DOI: 10.1210/me.2003-0333]

47 **Fatchiyah M**, Shima Y, Oka S, Ishihara S, Fukui-Katoh Y, Morohashi K. Differential gene dosage effects of Ad4BP/SF-1 on target tissue development. *Biochem Biophys Res Commun* 2006; **341**: 1036-1045 [PMID: 16458255 DOI: 10.1016/j.bbrc.2006.01.058]

48 **Zubair M**, Oka S, Parker KL, Morohashi K. Transgenic expression of Ad4BP/SF-1 in fetal adrenal progenitor cells leads to ectopic adrenal formation. *Mol Endocrinol* 2009; **23**: 1657-1667 [PMID: 19628584 DOI: 10.1210/me.2009-0055]

49 **Morohashi K**, Zubair M. The fetal and adult adrenal cortex. *Mol Cell Endocrinol* 2011; **336**: 193-197 [PMID: 21130838 DOI: 10.1016/j.mce.2010.11.026]

50 **Jeyasuria P**, Ikeda Y, Jamin SP, Zhao L, De Rooij DG, Themmen AP, Behringer RR, Parker KL. Cell-specific knockout of steroidogenic factor 1 reveals its essential roles in gonadal function. *Mol Endocrinol* 2004; **18**: 1610-1619 [PMID: 15118069 DOI: 10.1210/me.2003-0404]

51 **Pelusi C**, Ikeda Y, Zubair M, Parker KL. Impaired follicle development and infertility in female mice lacking steroidogenic factor 1 in ovarian granulosa cells. *Biol Reprod* 2008; **79**: 1074-1083 [PMID: 18703422 DOI: 10.1095/biolreprod.108.069435]

52 **Paré JF**, Malenfant D, Courtemanche C, Jacob-Wagner M, Roy S, Allard D, Bélanger L. The fetoprotein transcription factor (FTF) gene is essential to embryogenesis and cholesterol homeostasis and is regulated by a DR4 element. *J Biol Chem* 2004; **279**: 21206-21216 [PMID: 15014077 DOI: 10.1074/jbc.M401523200]

53 **Gu P**, Goodwin B, Chung AC, Xu X, Wheeler DA, Price RR, Galardi C, Peng L, Latour AM, Koller BH, Gossen J, Kliewer SA, Cooney AJ. Orphan nuclear receptor LRH-1 is required to maintain Oct4 expression at the epiblast stage of embryonic development. *Mol Cell Biol* 2005; **25**: 3492-3505 [PMID: 15831456 DOI: 10.1128/MCB.25.9.3492-3505.2005]

54 **Volle DH**, Duggavathi R, Magnier BC, Houten SM, Cummins CL, Lobaccaro JM, Verhoeven G, Schoonjans K, Auwerx J. The small heterodimer partner is a gonadal gatekeeper of sexual maturation in male mice. *Genes Dev* 2007; **21**: 303-315 [PMID: 17289919 DOI: 10.1101/gad.409307]

55 **Labelle-Dumais C**, Paré JF, Bélanger L, Farookhi R, Dufort D. Impaired progesterone production in Nr5a2+/- mice leads to a reduction in female reproductive function. *Biol Reprod* 2007; **77**: 217-225 [PMID: 17409375 DOI: 10.1095/biolreprod.106.059121]

56 **Crawford PA**, Sadovsky Y, Milbrandt J. Nuclear receptor steroidogenic factor 1 directs embryonic stem cells toward the steroidogenic lineage. *Mol Cell Biol* 1997; **17**: 3997-4006 [PMID: 9199334]

57 **Yazawa T**, Mizutani T, Yamada K, Kawata H, Sekiguchi T, Yoshino M, Kajitani T, Shou Z, Umezawa A, Miyamoto K. Differentiation of adult stem cells derived from bone marrow stroma into Leydig or adrenocortical cells. *Endocrinology* 2006; **147**: 4104-4111 [PMID: 16728492 DOI: 10.1210/en.2006-0162]

58 **Jadhav U**, Jameson JL. Steroidogenic factor-1 (SF-1)-driven differentiation of murine embryonic stem (ES) cells into a gonadal lineage. *Endocrinology* 2011; **152**: 2870-2882 [PMID: 21610156 DOI: 10.1210/en.2011-0219]

59 **Sonoyama T**, Sone M, Honda K, Taura D, Kojima K, Inuzuka M, Kanamoto N, Tamura N, Nakao K. Differentiation of human embryonic stem cells and human induced pluripotent stem cells into steroid-producing cells. *Endocrinology* 2012; **153**: 4336-4345 [PMID: 22778223 DOI: 10.1210/en.2012-1060]

60 **Prockop DJ**. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 1997; **276**: 71-74 [PMID: 9082988 DOI: 10.1126/science.276.5309.71]

61 **Gojo S**, Umezawa A. Plasticity of mesenchymal stem cells--regenerative medicine for diseased hearts. *Hum Cell* 2003; **16**: 23-30 [PMID: 12971622 DOI: 10.1111/j.1749-0774.2003.tb00125.x]

62 **Le Blanc K**, Ringdén O. Mesenchymal stem cells: properties and role in clinical bone marrow transplantation. *Curr Opin Immunol* 2006; **18**: 586-591 [PMID: 16879957 DOI: 10.1016/j.coi.2006.07.004]

63 **Bernardo ME**, Locatelli F, Fibbe WE. Mesenchymal stromal cells. *Ann N Y Acad Sci* 2009; **1176**: 101-117 [PMID: 19796238 DOI: 10.1111/j.1749-6632.2009.04607.x]

64 **Friedenstein AJ**, Gorskaja JF, Kulagina NN. Fibroblast

precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol* 1976; **4**: 267-274 [PMID: 976387]
65 **Caplan AI**. Mesenchymal stem cells. *J Orthop Res* 1991; **9**: 641-650
66 **Umezawa A**, Maruyama T, Segawa K, Shadduck RK, Waheed A, Hata J. Multipotent marrow stromal cell line is able to induce hematopoiesis in vivo. *J Cell Physiol* 1992; **151**: 197-205 [PMID: 1373147 DOI: 10.1002/jcp.1041510125]
67 **Terai M**, Uyama T, Sugiki T, Li XK, Umezawa A, Kiyono T. Immortalization of human fetal cells: the life span of umbilical cord blood-derived cells can be prolonged without manipulating p16INK4a/RB braking pathway. *Mol Biol Cell* 2005; **16**: 1491-1499 [PMID: 15647378 DOI: 10.1091/mbc.E04-07-0652]
68 **Okamoto K**, Miyoshi S, Toyoda M, Hida N, Ikegami Y, Makino H, Nishiyama N, Tsuji H, Cui CH, Segawa K, Uyama T, Kami D, Miyado K, Asada H, Matsumoto K, Saito H, Yoshimura Y, Ogawa S, Aeba R, Yozu R, Umezawa A. 'Working' cardiomyocytes exhibiting plateau action potentials from human placenta-derived extraembryonic mesodermal cells. *Exp Cell Res* 2007; **313**: 2550-2562 [PMID: 17544394 DOI: 10.1016/j.yexcr.2007.04.028]
69 **Bassi G**, Pacelli L, Carusone R, Zanoncello J, Krampera M. Adipose-derived stromal cells (ASCs). *Transfus Apher Sci* 2012; **47**: 193-198 [PMID: 22818214 DOI: 10.1016/j.transci.2012.06.004]
70 **Pittenger MF**, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; **284**: 143-147 [PMID: 10102814 DOI: 10.1126/science.284.5411.143]
71 **Ding Y**, Xu D, Feng G, Bushell A, Muschel RJ, Wood KJ. Mesenchymal stem cells prevent the rejection of fully allogenic islet grafts by the immunosuppressive activity of matrix metalloproteinase-2 and -9. *Diabetes* 2009; **58**: 1797-1806 [PMID: 19509016 DOI: 10.2337/db09-0317]
72 **Amini AR**, Laurencin CT, Nukavarapu SP. Bone tissue engineering: recent advances and challenges. *Crit Rev Biomed Eng* 2012; **40**: 363-408 [PMID: 23339648 DOI: 10.1615/CritRevBiomedEng.v40.i5.10]
73 **Wang X**, Wang Y, Gou W, Lu Q, Peng J, Lu S. Role of mesenchymal stem cells in bone regeneration and fracture repair: a review. *Int Orthop* 2013; **37**: 2491-2498 [PMID: 23948983 DOI: 10.1007/s00264-013-2059-2]
74 **Haider SG**. Cell biology of Leydig cells in the testis. *Int Rev Cytol* 2004; **233**: 181-241 [PMID: 15037365 DOI: 10.1016/S0074-7696(04)33005-6]
75 **Dong L**, Jelinsky SA, Finger JN, Johnston DS, Kopf GS, Sottas CM, Hardy MP, Ge RS. Gene expression during development of fetal and adult Leydig cells. *Ann N Y Acad Sci* 2007; **1120**: 16-35 [PMID: 18184909 DOI: 10.1196/annals.1411.016]
76 **Svechnikov K**, Landreh L, Weisser J, Izzo G, Colón E, Svechnikova I, Söder O. Origin, development and regulation of human Leydig cells. *Horm Res Paediatr* 2010; **73**: 93-101 [PMID: 20190545]
77 **Hu MC**, Chou SJ, Huang YY, Hsu NC, Li H, Chung BC. Tissue-specific, hormonal, and developmental regulation of SCC-LacZ expression in transgenic mice leads to adrenocortical zone characterization. *Endocrinology* 1999; **140**: 5609-5618 [PMID: 10579324 DOI: 10.1210/en.140.12.5609]
78 **Yazawa T**, Uesaka M, Inaoka Y, Mizutani T, Sekiguchi T, Kajitani T, Kitano T, Umezawa A, Miyamoto K. Cyp11b1 is induced in the murine gonad by luteinizing hormone/human chorionic gonadotropin and involved in the production of 11-ketotestosterone, a major fish androgen: conservation and evolution of the androgen metabolic pathway. *Endocrinology* 2008; **149**: 1786-1792 [PMID: 18162527 DOI: 10.1210/en.2007-1015]
79 **Miyamoto K**, Yazawa T, Mizutani T, Imamichi Y, Kawabe SY, Kanno M, Matsumura T, Ju Y, Umezawa A. Stem cell differentiation into steroidogenic cell lineages by NR5A family. *Mol Cell Endocrinol* 2011; **336**: 123-126 [PMID: 21134412 DOI: 10.1016/j.mce.2010.11.031]
80 **Gondo S**, Yanase T, Okabe T, Tanaka T, Morinaga H, Nomura M, Goto K, Nawata H. SF-1/Ad4BP transforms primary long-term cultured bone marrow cells into ACTH-responsive steroidogenic cells. *Genes Cells* 2004; **9**: 1239-1247 [PMID: 15569155]
81 **Yanase T**, Gondo S, Okabe T, Tanaka T, Shirohzu H, Fan W, Oba K, Morinaga H, Nomura M, Ohe K, Nawata H. Differentiation and regeneration of adrenal tissues: An initial step toward regeneration therapy for steroid insufficiency. *Endocr J* 2006; **53**: 449-459 [PMID: 16807499 DOI: 10.1507/endocrj.KR-74]
82 **Tanaka T**, Gondo S, Okabe T, Ohe K, Shirohzu H, Morinaga H, Nomura M, Tani K, Takayanagi R, Nawata H, Yanase T. Steroidogenic factor 1/adrenal 4 binding protein transforms human bone marrow mesenchymal cells into steroidogenic cells. *J Mol Endocrinol* 2007; **39**: 343-350 [PMID: 17975261 DOI: 10.1677/JME-07-0076]
83 **Gondo S**, Okabe T, Tanaka T, Morinaga H, Nomura M, Takayanagi R, Nawata H, Yanase T. Adipose tissue-derived and bone marrow-derived mesenchymal cells develop into different lineage of steroidogenic cells by forced expression of steroidogenic factor 1. *Endocrinology* 2008; **149**: 4717-4725 [PMID: 18566117 DOI: 10.1210/en.2007-1808]
84 **Wei X**, Peng G, Zheng S, Wu X. Differentiation of umbilical cord mesenchymal stem cells into steroidogenic cells in comparison to bone marrow mesenchymal stem cells. *Cell Prolif* 2012; **45**: 101-110 [PMID: 22324479 DOI: 10.1111/j.1365-2184.2012.00809.x]
85 **Kawabe S**, Yazawa T, Kanno M, Usami Y, Mizutani T, Imamichi Y, Ju Y, Matsumura T, Orisaka M, Miyamoto K. A novel isoform of liver receptor homolog-1 is regulated by steroidogenic factor-1 and the specificity protein family in ovarian granulosa cells. *Endocrinology* 2013; **154**: 1648-1660 [PMID: 23471216 DOI: 10.1210/en.2012-2008]
86 **Collas P**. Programming differentiation potential in mesenchymal stem cells. *Epigenetics* 2010; **5**: 476-482 [PMID: 20574163 DOI: 10.4161/epi.5.6.12517]
87 **Tollervey JR**, Lunyak VV. Epigenetics: judge, jury and executioner of stem cell fate. *Epigenetics* 2012; **7**: 823-840 [PMID: 22805743]
88 **Mizutani T**, Yazawa T, Ju Y, Imamichi Y, Uesaka M, Inaoka Y, Matsuura K, Kamiki Y, Oki M, Umezawa A, Miyamoto K. Identification of a novel distal control region upstream of the human steroidogenic acute regulatory protein (StAR) gene that participates in SF-1-dependent chromatin architecture. *J Biol Chem* 2010; **285**: 28240-28251 [PMID: 20601698 DOI: 10.1074/jbc.M110.129510]
89 **Matsumura T**, Imamichi Y, Mizutani T, Ju Y, Yazawa T, Kawabe S, Kanno M, Ayabe T, Katsumata N, Fukami M, Inatani M, Akagi Y, Umezawa A, Ogata T, Miyamoto K. Human glutathione S-transferase A (GSTA) family genes are regulated by steroidogenic factor 1 (SF-1) and are involved in steroidogenesis. *FASEB J* 2013; **27**: 3198-3208 [PMID: 23650189]
90 **Imamichi Y**, Mizutani T, Ju Y, Matsumura T, Kawabe S, Kanno M, Yazawa T, Miyamoto K. Transcriptional regulation of human ferredoxin reductase through an intronic enhancer in steroidogenic cells. *Biochim Biophys Acta* 2014; **1839**: 33-42 [PMID: 24321386 DOI: 10.1016/j.bbagrm.2013.11.005]
91 **Strahl BD**, Allis CD. The language of covalent histone modifications. *Nature* 2000; **403**: 41-45 [PMID: 10638745 DOI: 10.1038/47412]
92 **Fischle W**, Wang Y, Allis CD. Histone and chromatin cross-talk. *Curr Opin Cell Biol* 2003; **15**: 172-183 [PMID: 12648673 DOI: 10.1016/S0955-0674(03)00013-9]
93 **Pokholok DK**, Harbison CT, Levine S, Cole M, Hannett NM, Lee TI, Bell GW, Walker K, Rolfe PA, Herbolsheimer

E, Zeitlinger J, Lewitter F, Gifford DK, Young RA. Genome-wide map of nucleosome acetylation and methylation in yeast. *Cell* 2005; **122**: 517-527 [PMID: 16122420 DOI: 10.1016/j.cell.2005.06.026]

94 **Ehrlich M**. Expression of various genes is controlled by DNA methylation during mammalian development. *J Cell Biochem* 2003; **88**: 899-910 [PMID: 12616529 DOI: 10.1002/jcb.10464]

95 **Ng RK**, Gurdon JB. Epigenetic inheritance of cell differentiation status. *Cell Cycle* 2008; **7**: 1173-1177 [PMID: 18418041 DOI: 10.4161/cc.7.9.5791]

96 **Yazawa T**, Kawabe S, Kanno M, Mizutani T, Imamichi Y, Ju Y, Matsumura T, Yamazaki Y, Usami Y, Kuribayashi M, Shimada M, Kitano T, Umezawa A, Miyamoto K. Androgen/androgen receptor pathway regulates expression of the genes for cyclooxygenase-2 and amphiregulin in periovulatory granulosa cells. *Mol Cell Endocrinol* 2013; **369**: 42-51 [PMID: 23415714 DOI: 10.1016/j.mce.2013.02.004]

97 **Ju Y**, Mizutani T, Imamichi Y, Yazawa T, Matsumura T, Kawabe S, Kanno M, Umezawa A, Kangawa K, Miyamoto K. Nuclear receptor 5A (NR5A) family regulates 5-aminolevulinic acid synthase 1 (ALAS1) gene expression in steroidogenic cells. *Endocrinology* 2012; **153**: 5522-5534 [PMID: 23024262 DOI: 10.1210/en.2012-1334]

98 **Imamichi Y**, Mizutani T, Ju Y, Matsumura T, Kawabe S, Kanno M, Yazawa T, Miyamoto K. Transcriptional regulation of human ferredoxin 1 in ovarian granulosa cells. *Mol Cell Endocrinol* 2013; **370**: 1-10 [PMID: 23435367 DOI: 10.1016/j.mce.2013.02.012]

99 **Inaoka Y**, Yazawa T, Mizutani T, Kokame K, Kangawa K, Uesaka M, Umezawa A, Miyamoto K. Regulation of P450 oxidoreductase by gonadotropins in rat ovary and its effect on estrogen production. *Reprod Biol Endocrinol* 2008; **6**: 62 [PMID: 19077323 DOI: 10.1186/1477-7827-6-62]

100 **Fukami M**, Nishimura G, Homma K, Nagai T, Hanaki K, Uematsu A, Ishii T, Numakura C, Sawada H, Nakacho M, Kowase T, Motomura K, Haruna H, Nakamura M, Ohishi A, Adachi M, Tajima T, Hasegawa Y, Hasegawa T, Horikawa R, Fujieda K, Ogata T. Cytochrome P450 oxidoreductase deficiency: identification and characterization of biallelic mutations and genotype-phenotype correlations in 35 Japanese patients. *J Clin Endocrinol Metab* 2009; **94**: 1723-1731 [PMID: 19258400 DOI: 10.1210/jc.2008-2816]

101 **Soneda S**, Yazawa T, Fukami M, Adachi M, Mizota M, Fujieda K, Miyamoto K, Ogata T. Proximal promoter of the cytochrome P450 oxidoreductase gene: identification of microdeletions involving the untranslated exon 1 and critical function of the SP1 binding sites. *J Clin Endocrinol Metab* 2011; **96**: E1881-E1887 [PMID: 21900384 DOI: 10.1210/jc.2011-1337]

**P- Reviewers**: Holan V, Lalli E, Pixley JS **S- Editor**: Qi Y
**L- Editor**: Roemmele A **E- Editor**: Zhang DN

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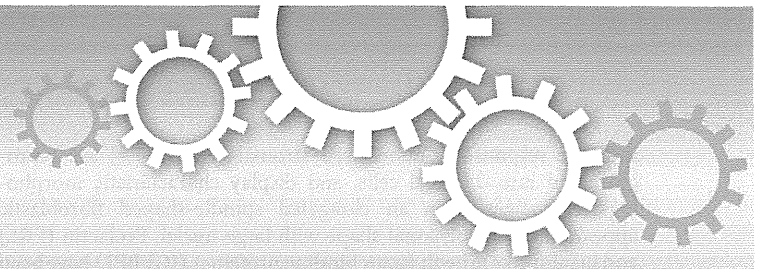

OPEN

SUBJECT AREAS:
STEM-CELL DIFFERENTIATION
MESENCHYMAL STEM CELLS

Received
6 January 2014

Accepted
10 March 2014

Published
8 April 2014

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# Derivation of human decidua-like cells from amnion and menstrual blood

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**We induced differentiation of human amnion-derived mesenchymal stem cells (AMCs) and menstrual blood-derived mesenchymal stem cells (MMCs) into endometrial stroma-like cells, which could be useful for cell therapy to support embryo implantation. Interestingly, the expression patterns of surface markers were similar among AMCs, MMCs, and endometrial stromal cells. In addition, whereas treatment with estrogen and progesterone was not very effective for decidualizing AMCs and MMCs, treatment with 8-Br-cAMP prompted remarkable morphological changes in these cells as well as increased expression of decidualization markers (prolactin and insulin-like growth factor binding protein-1) and attenuated expression of surface markers unique to mesenchymal stem cells. These results demonstrated that bone marrow-derived stem cells, which are considered a potential source of endometrial progenitor cells, as well as AMCs and MMCs show *in vitro* decidualization potential, which is characteristic of endometrial stromal cells.**

Many infertile couples have achieved pregnancy by assisted reproductive technology (ART). However, despite having good-quality embryos for transfer, there are still women who experience repeated implantation failure even after several ART attempts[1,2]. Uterine receptivity is considered as another important factor for successful implantation and many studies have attempted to identify clinically useful markers of a receptive uterine state. However, such molecular markers related to repeated implantation failure have proven difficult to find, largely because uterine receptivity is regulated via the expression of various mediators, including cell adhesion molecules (e.g. cadherins and integrins), chemical mediators (e.g. prostaglandins), and cytokines (e.g. leukemia inhibitory factor and epithelial growth factor).

Thinning of endometrium has also been correlated with implantation failure, with causes including aging[3], and repeated invasive procedures such as dilation and curettage for miscarriage or early-stage endometrial cancer. Some studies have reported clinical benefits from ascorbic acid (vitamin C), tocopherol (vitamin E), pentoxifylline (PTX), or sildenafil for repeated implantation failure in patients with thin endometrium, although the effectiveness and molecular mechanisms by which such agent could improve the implantation process is not well established[4–6].

In studies to improve implantation rates, Landgren et al.[7] developed a coculture model of embryos with endometrial cells harvested from an endometrial biopsy taken at 4, 5, and 6 days after the lutenizing hormone (LH) peak in healthy women with normal menstrual cycles. The rate of pregnancy increased for embryos transferred after the coculture of embryo with endometrium than for embryos transferred after repeat ART[8]. However it is difficult to retrieve and culture endometrial cells from women whose endometrium has already become thin. Therefore we focused on mesenchymal stem cells (MSCs) as a source for this type of cell therapy using endometrial stroma-like cells. MSCs might also support embryo implantation by excreting cytokines and chemical mediators for adhesion, migration, or immunomodulation[9–11]. Indeed, fertility was restored in a patient with severe Asherman's syndrome treated using autologous bone marrow-derived MSC populations[12].

MSCs are multipotent, adherent stem cells capable of differentiating into osteoblasts, adipocytes, and chondroblasts[13], as well as endodermal lineages such as pancreatic islands[14,15] or hepatocytes[16,17], and ectodermal lineage such as neurons[14,18]. An earlier study verified that human bone marrow-derived MSC (BMCs) are potential progenitors of endometrial stromal cells by demonstrating their ability to decidualize[19]. Decidualization is a remodeling process designed to prepare endometrium for pregnancy that is induced by progesterone secreted from the ovaries in the luteal phase. This remodeling is necessary in a successful pregnancy to regulate trophoblast invasion, resist oxidative stress, and protect the placental semi-allograft against maternal

immune responses. Endometrial stromal cells (ESCs) are reprogrammed into decidual cells, and display characteristic morphological changes from an elongated spindle-shaped fibroblastic appearance to a polygonal shape with large nuclei. Prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP1) have been widely used as phenotypic markers of decidual cells. In the current study, we hypothesized that if MSCs could differentiate into ESC-like cells, their treatment with agents known to promote decidualization could induce the expression of PRL and IGFBP1 in the MSC-derived cells. Indeed, AMCs and MMCs successfully differentiate into endometrial stroma-like cells presenting decidualization potential to the same extent as bone marrow-derived cells[19]. In this context, human amnion in particular could be a powerful therapeutic cell source because not only is it an easily accessible tissue for cell harvesting, it does not require co-administration of immunosuppressive agent or matching of MHC typing for immunological tolerance.

## Results

**Characterization of human endometrial cells.** Immunofluorescence staining and flow cytometric analysis of cultured human endometrial cells and MRC5 fibroblasts revealed both to be positive for vimentin and negative for cytokeratin, whereas a human carcinoma cell line, HeLa cells, was positive for both vimentin and cytokeratin. (Fig. 1A–E). By flow cytometry, the endometrial cells and fibroblasts were negative for CD9 and positive for CD10, whereas HeLa cells were positive for CD9 and negative for CD10. Together, these results confirmed that the endometrial cells were stromal in origin, compared to the epithelial-derived HeLa cells.

We then investigated cell surface marker expressions of ESCs, AMCs, MMCs, BMCs, and MRC5 by flow cytometric analysis on the initial day of treatment (day 0) (Fig. 1F). Although there is no marker specific to MSCs, they are known to express CD73, CD90, and CD105[20], and the AMCs and MMCs tested herein indeed expressed these cell surface markers as well as CD54, CD166 and HLA-ABC, but not HLA-DR (Supplementary Fig. S1). This result confirmed the earlier studies of our group demonstrating that both AMCs and MMCs express MSC markers such as CD29, CD44, CD59, CD73, CD105, and CD166, but not hematopoietic markers such as CD14, CD34, and CD45[21–23]. None of the cells analyzed in the present study expressed hematopoietic lineage markers such as CD34 or monocyte-macrophage antigens such as CD14 (a marker for macrophage and dendritic cells) and CD45 (leukocyte common antigen), suggesting no contaminating hematopoietic cells. Lastly, and interestingly, the surface marker expression patterns of AMCs and MMCs were very similar to those of ESCs.

**Decidual differentiation by treatment with $E_2 + P_4$.** Human ESCs showed distinct morphological changes following treatment with $E_2 + P_4$, from spindle-shaped to large round cells with large round nuclei, whereas AMCs, MMCs, and BMCs showed no remarkable morphological changes (Fig. 2A). Flow cytometric analysis of ESCs, AMCs, MMCs, BMCs, and MRC5 fibroblasts on day 14 of treatment with $E_2 + P_4$ (Fig. 2B and Supplementary Fig. S2) also showed no remarkable changes in surface marker expressions compared to those on day 0.

Immunofluorescence staining was also performed to assess the expression of decidualization markers (PRL and IGFBP1) at day 0, 7, and 14 after treatment with $E_2 + P_4$ (Fig. 3). None of these markers were expressed at day 0 in ESCs, AMCs, MMCs, and BMCs, but PRL and IGFBP1 were expressed in ESCs on days 7 and 14 following treatment with $E_2 + P_4$ (Figs. 3A and 3B). AMCs also showed clear expression of PRL on days 7 and 14 following treatment, but only faint IGFBP1 expression (Figs. 3C and 3D), while the reverse pattern was observed in MMCs (Figs. 3E and 3F). Finally, both PRL and IGFBP1 were faintly expressed in BMCs (Figs. 3G and 3H).

The qRT-PCR analysis of expression changes in *PRL* and *IGFBP1* mRNAs with $E_2 + P_4$ treatment (Figs. 2C and 2D) revealed upregulation of *PRL* in each cell type on days 7 and 14 following treatment compared to day 0 levels, whereas *IGFBP1* was upregulated only in ESCs on days 7 and 14 compared to day 0, and not in AMCs, MMCs, and BMCs.

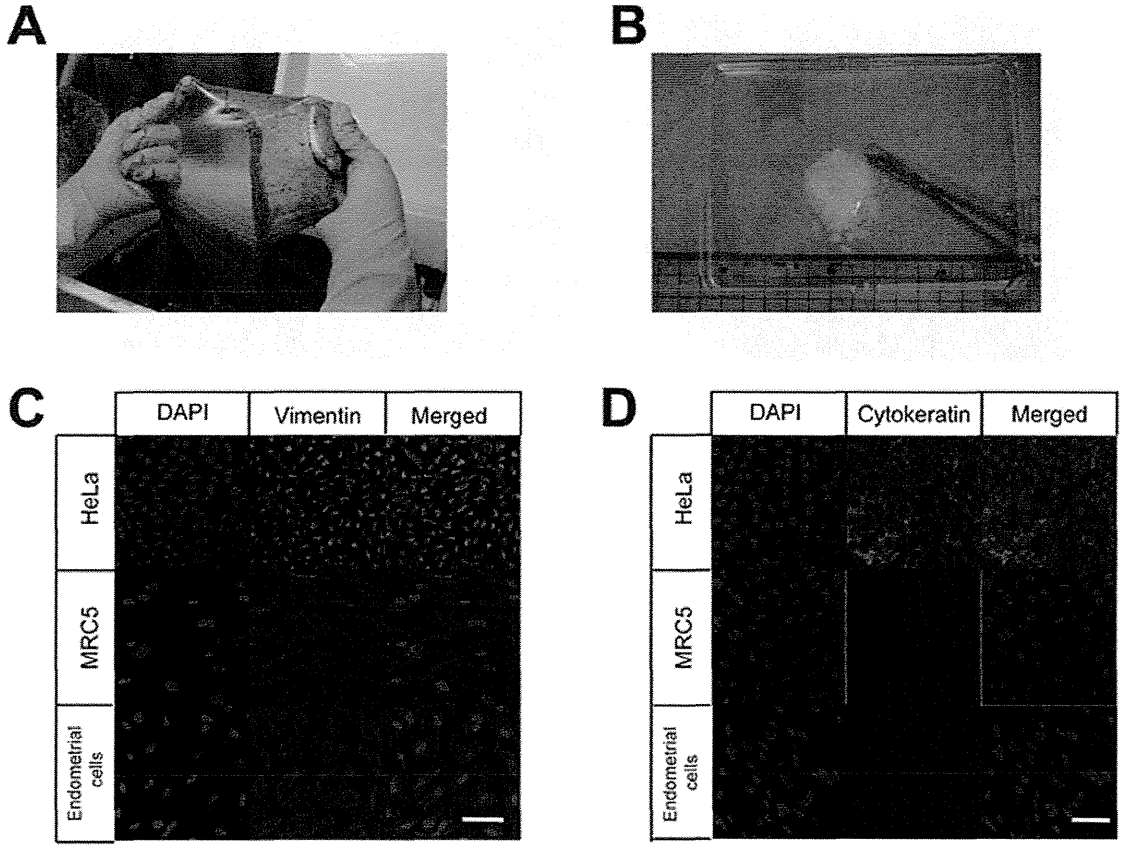
**Decidual differentiation by treatment with cAMP.** The human ESCs, AMCs, MMCs, and BMCs all showed morphological changes from a spindle-shaped appearance to large rounded cells with large round nuclei following treatment with cAMP (Fig. 4A). In addition, flow cytometry revealed that CD90, CD105, and CD166 were attenuated in the AMCs and ESCs on day 14 of the cAMP treatment (Fig. 4B and Supplementary Fig. S3) compared to day 0 levels. Together, these changes suggested that cAMP upregulates decidual differentiation in AMCs. On the other hand, CD105 and CD166 in MMCs as well as CD90 and CD166 in BMCs were unchanged, suggesting that AMCs could have more potential for differentiation to ESCs than MMCs and BMCs.

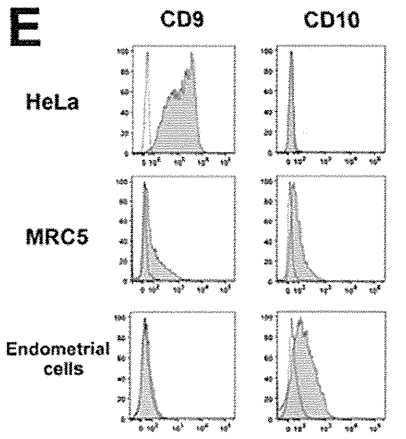
Immunofluorescence staining showed no expression of decidualization markers (PRL and IGFBP1) at day 0 in the four cell types, but positive staining for PRL on days 7 and 14 of the cAMP treatment (Fig. 5A, 5C, 5E and 5G). Although IGFBP1 was also expressed in all the types of cells on day 7 and day 14, AMCs and MMCs showed more remarkable IGFBP1 expression on day 7 compared to day 14.

Finally, the qRT-PCR analysis showed that *PRL* transcripts were more abundant in all the cell types on days 7 and 14 following cAMP treatment than on day 0 (Figs. 4C and 4D), whereas *IGFBP1* was upregulated in AMCs and ESCs, but not in MMCs and BMCs. These results suggested that AMCs treated with cAMP have the closest decidualization potential to ESCs.

## Discussion

**Insufficient response of MSCs to $E_2 + P_4$.** In this study, we investigated whether human MSCs such as AMCs and MMCs could be differentiated *in vitro* into endometrial stroma-like cells using decidualization stimuli and analysis of cellular morphology and expression of decidualization markers (PRL and IGFBP1). The MSCs and ESCs were treated with $E_2 + P_4$ to mimic the normal endocrinological condition in the secretory phase of a human menstrual cycle. ESCs and BMCs were considered as positive controls. Unexpectedly, not only the AMCs and MMCs, but also the BMCs, failed to show remarkable decidual changes with $E_2 + P_4$ treatment. Sustained expression of cell surface markers, no marked morphological changes, and little expression of IGFBP1 in MSCs treated with $E_2 + P_4$ indicated that these cells are not sufficiently responsive to such stimulation to induce differentiation, supporting previous results by Aghajanova. et al.[19] in BMCs treated with $E_2 + P_4$. The qRT-PCR in this study showed significant expression of progesterone receptors (PR) and estrogen receptors (ESR1 and ESR2) in AMCs, MMCs, and ESCs (Supplementary Fig. S5). Aghajanova et al. also demonstrated *PR*, *ESR1*, and *ESR2* mRNA expression in BMCs and ESCs, but this expression was not significantly influenced by cAMP treatment of the cells[19]. Furthermore, with respect to secretory-phase endometrium *in vivo*, decidualization of the superficial endometrial layers is only apparent ~10 days after the postovulatory increase in progesterone levels. All these observations indicate that additional signals other than the progesterone signaling are required to initiate decidualization. Interestingly, trichostatin A (TSA), a specific histone deacetylase (HDACs) inhibitor, enhanced the upregulation of PRL and IGFBP1 in a dose-dependent manner in cultured endometrial stromal cells following treatment with $E_2 + P_4$[24]. In addition, IGFBP1 expression induced by cAMP was associated with the histone acetylation status of the promoter region in human endometrial stromal cells[25]. Based on these results, we speculated that TSA might be effective in enhancing IGFBP1 expression in AMCs and MMCs treated with $E_2 + P_4$; however, AMCs and MMCs treated with $E_2 + P_4$ and several concentrations



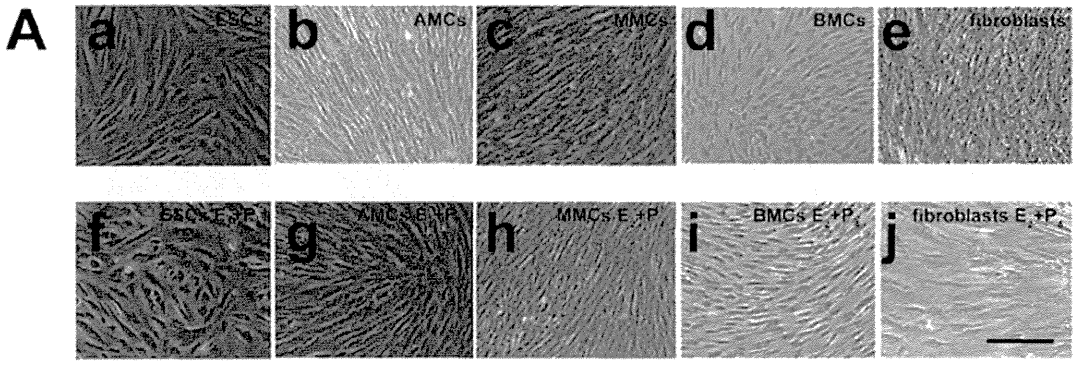

F

| Surface marker | CD 13 | CD 14 | CD 16 | CD 19 | CD 29 | CD 34 | CD 45 | CD 54 | CD 73 | CD 90 | CD 105 | CD 166 | HLA -DR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ESCs | ± | - | - | - | ± | - | - | + | ++ | + | + | + | - |
| AMCs | ± | - | - | - | - | - | - | + | ++ | + | + | + | - |
| MMCs | ± | - | - | - | - | - | - | + | ++ | + | + | + | - |
| BMCs | ± | - | - | - | + | - | ± | - | ++ | ++ | + | + | - |
| MRC5 | - | - | - | - | - | - | - | - | + | - | - | - | - |

**Figure 1 | Characterization of endometrial cells, amnion-derived cells, and menstrual blood-derived cells.** (A), Macroscopic view of human amniotic membrane. (B), The amniotic membrane was cut into pieces of approximately 2 $mm^3$ in size. (C and D), Laser confocal microscopic view of immunofluorescence staining of endometrial cells, HeLa cells, and MRC5 fibroblasts in culture with anti-vimentin (red) (C) and anti-cytokeratin antibodies (green) (D), DAPI staining (blue). Scale bars, 100 μm. (E), Surface marker (CD9 or CD10) expression of endometrial cells, HeLa cells, and MRC5 fibroblasts. White and grey areas indicate reactivity of antibodies for isotype controls and that for CD9 or CD10, respectively. (F), Summary of flow cytometric analysis of endometrial stromal cells (ESCs), amnion-derived mesenchymal stem cells (AMCs), menstrual blood-derived mesenchymal stem cells (MMCs), bone marrow-derived mesenchymal stem cells (BMCs), and MRC5 fibroblasts before treatment. The shift in peak histogram value of flow cytometric data was measured and classified into four groups: −, from negative to 2 log shift; ±, 2 log shift from negative control; +, 3 log shift from negative control; ++, 4 log shift from negative control.

of TSA showed no enhancement of IGFBP1 expression (Supplemental Fig. S4). We therefore further investigated the decidualization potential of MSCs in the current study using cAMP, a well-known and common facilitator of decidualization[19,25].

**Attenuation of cell surface markers for MSCs during successful decidualization by cAMP.** Estrogen and progesterone bind to nuclear receptor proteins for nuclear importing, where they bind DNA directly to regulate transcription, whereas cAMP activates the protein




B

| Surface marker | CD 13 | CD 14 | CD 16 | CD 19 | CD 29 | CD 34 | CD 45 | CD 54 | CD 73 | CD 90 | CD 105 | CD 166 | HLA -DR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ESCs | ± | - | - | - | + | - | - | + | + | + | + | + | - |
| AMCs | ± | - | - | - | + | - | - | + | ++ | + | + | + | - |
| MMCs | + | - | - | - | + | - | - | + | ++ | + | + | + | - |
| BMCs | ± | - | - | - | ± | - | ± | + | ++ | ++ | + | + | - |
| MRC5 | - | - | - | - | - | - | - | - | - | - | + | - | - |

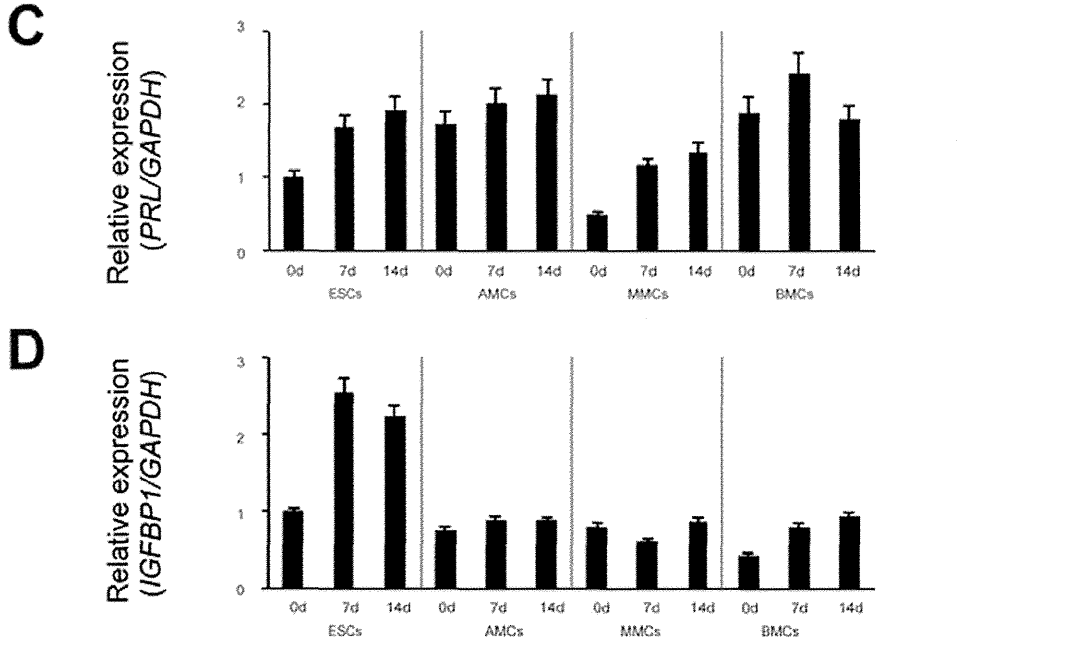



**Figure 2 | Morphological changes and alteration of flow cytometric profiles in mesenchymal stem cells treated with $E_2$ + $P_4$.** (A), Morphological changes in endometrial stromal cells (ESCs) (a, f), amnion-derived mesenchymal stem cells (AMCs) (b, g), menstrual blood-derived mesenchymal stem cells (MMCs) (c, h), bone marrow-derived mesenchymal stem cells (BMCs) (d, i), and MRC5 fibroblasts (e, j) before (a–c) and after (f–j) treatment with 10 nM $E_2$ and 1 μM $P_4$ ($E_2$ + $P_4$) for 14 days. Scale bars, 200 μm. (B), Summary of flow cytometric analysis of ESCs, AMCs, MMCs, BMCs, and MRC5 fibroblasts treated with $E_2$ + $P_4$ for 14 days. We measured the shift of peak histogram value for the flow cytometric data and defined each result by the aforementioned criteria. (C), PRL mRNA expression in ESCs, AMCs, MMCs, and BMCs treated with $E_2$ + $P_4$ for 0, 7, and 14 days. (D), IGFBP1 mRNA expression in ESCs, AMCs, MMCs, and BMCs treated with $E_2$ + $P_4$ for 14 days.

kinase A (PKA) pathway and a catalytic subunit of PKA translocates into the cell nucleus to phosphorylate cAMP response element-binding protein (CREB). Activated CREB then enhances gene transcription via cAMP response element (CRE)[26]. In this study, cAMP treatment of AMCs and MMCs produced morphological changes characteristic of decidual cells, and PRL and IGFBP1 expression increases consistent with those seen in ESCs. AMCs and MMCs were thus demonstrated to have decidualization potential.

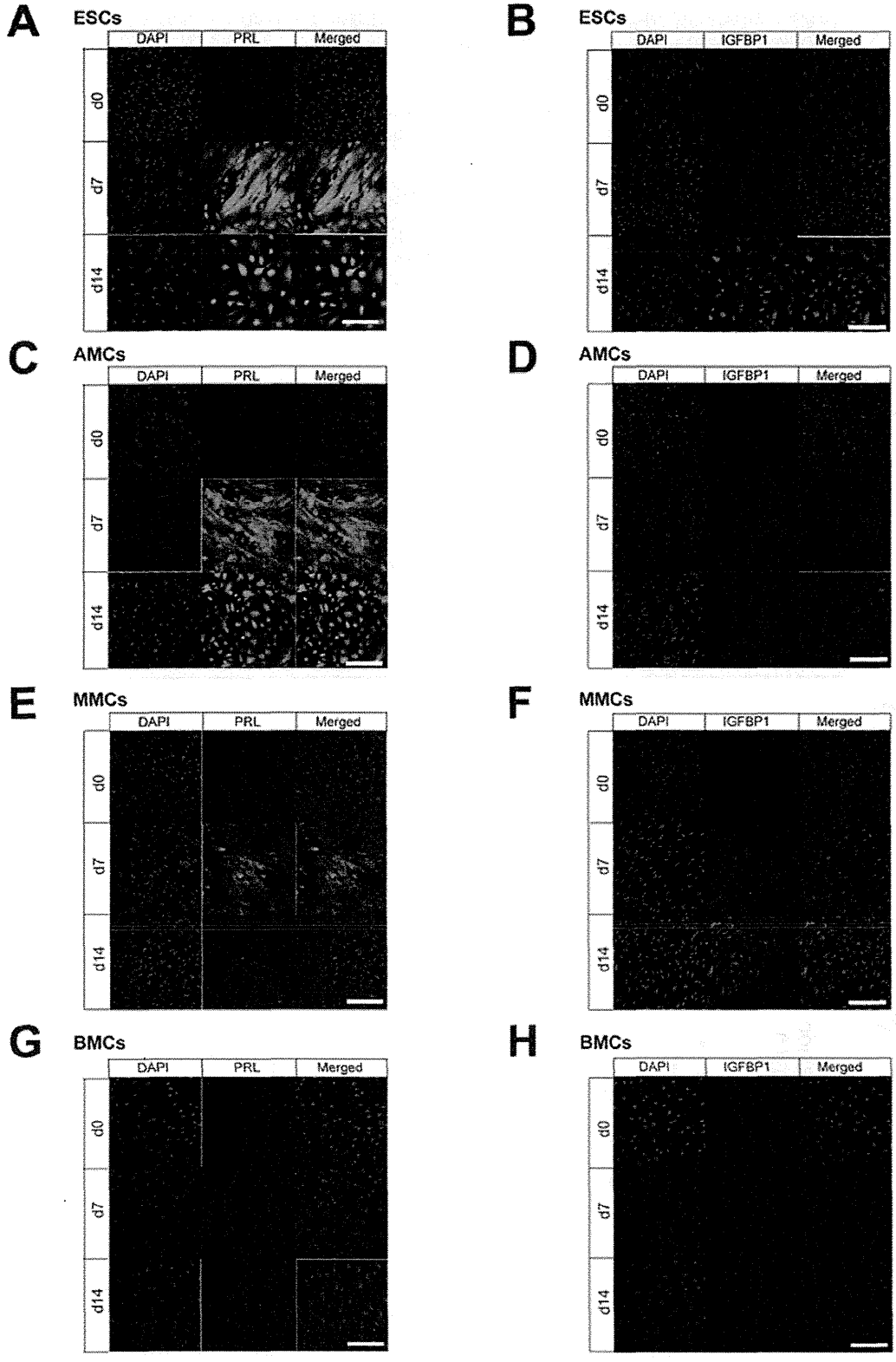
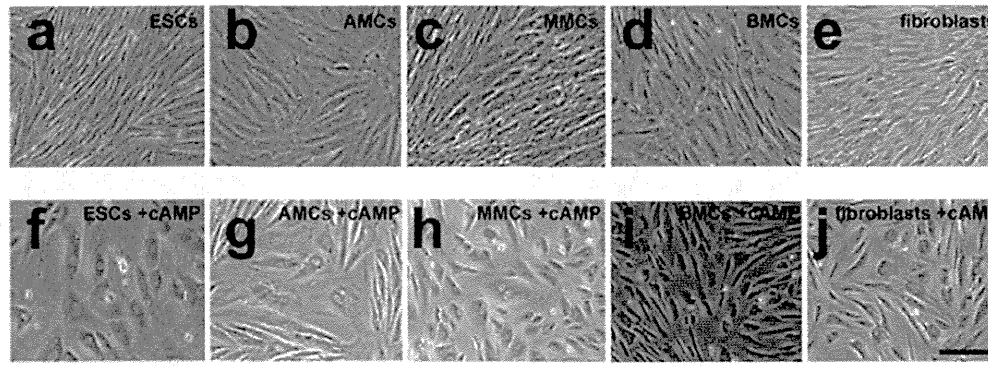



**Figure 3 | Immunofluorescence staining of decidualization markers (PRL and IGFBP1) in mesenchymal stem cells treated with $E_2 + P_4$.** Laser confocal microscopy of PRL immunofluorescence staining (green signals in A, C, E, and G) and IGFBP1 (red signals in B, D, F, H) in endometrial stromal cells (ESCs) (A and B), amnion-derived mesenchymal stem cells (AMCs) (C and D), menstrual blood-derived mesenchymal stem cells (MMCs) (E and F), and bone marrow-derived mesenchymal stem cells BMCs (G and H) treated with $E_2 + P_4$ for 0, 7, or 14 days. Scale bars, 200 μm.

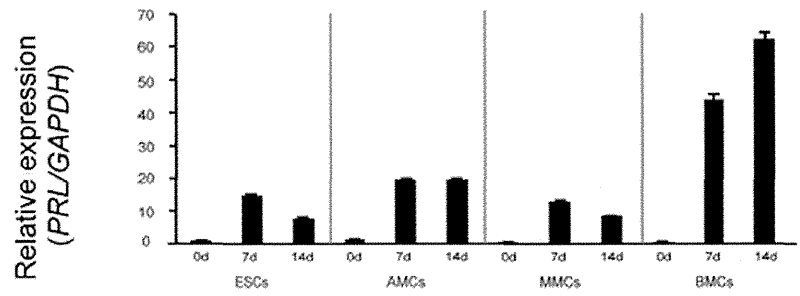
A




B

| Surface marker | CD 13 | CD 14 | CD 16 | CD 19 | CD 29 | CD 34 | CD 45 | CD 54 | CD 73 | CD 90 | CD 105 | CD 166 | HLA -DR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ESCs | - | - | - | - | - | - | - | + | ++ | - | ± | ± | - |
| AMCs | ± | - | - | - | - | - | - | ± | ++ | ± | ± | ± | - |
| MMCs | + | ± | ± | ± | ± | ± | ± | ++ | ++ | ± | + | + | ± |
| BMCs | ± | ± | - | - | ± | - | - | + | + | ++ | ± | + | - |
| MRC5 | - | - | - | - | - | - | - | - | - | - | - | - | - |

C

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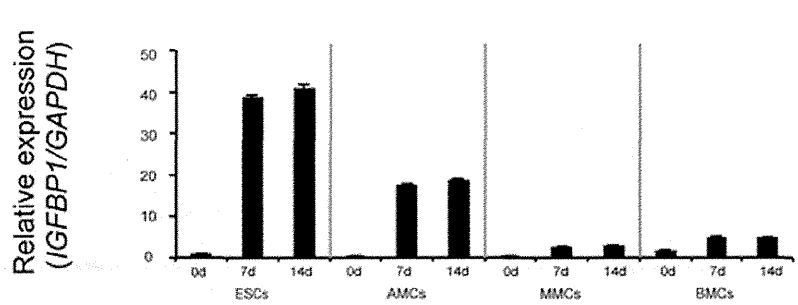


**Figure 4 | Morphological changes and alteration of flow cytometric profiles in mesenchymal stem cells treated with cAMP.** (A), Morphological changes of endometrial stromal cells (ESCs) (a, f), amnion-derived mesenchymal stem cells (AMCs) (b, g), menstrual blood-derived mesenchymal stem cells (MMCs) (c, h), bone marrow-derived mesenchymal stem cells (BMCs) (d, i), and MRC5 fibroblasts (e, j) before (a–e) and (f–j) after treatment with 1 mM 8-Br-cAMP for 14 days. Scale bars, 200 μm. (B), Summary of flow cytometric analysis of ESCs, AMCs, MMCs, BMCs, and MRC5 fibroblasts treated with 1 mM 8-Br-cAMP for 14 days. We measured the shift of peak histogram value for the flow cytometric data and defined each result by the aforementioned criteria. (C), PRL mRNA expression in ESCs, AMCs, MMCs, and BMCs treated with 1 mM 8-Br-cAMP for 0, 7, and 14 days. (D), IGFBP1 mRNA expression in ESCs, AMCs, MMCs, and BMCs treated with 1 mM 8-Br-cAMP for 0, 7, and 14 days.

In the cell surface marker analysis, the attenuation of CD90, CD105, and CD166 during decidualization was notable. Such a response by these well-defined human MSCs markers could indicate the direction of differentiation in MSCs treated with cAMP. Furthermore, CD90 is known to regulate adhesion to T cells in MSCs, and a decrease in CD90-positive cells might be a novel predictive marker of immunosuppressive activity[27]. Based on these observations, the decreased rate of CD90-positive cells with cAMP

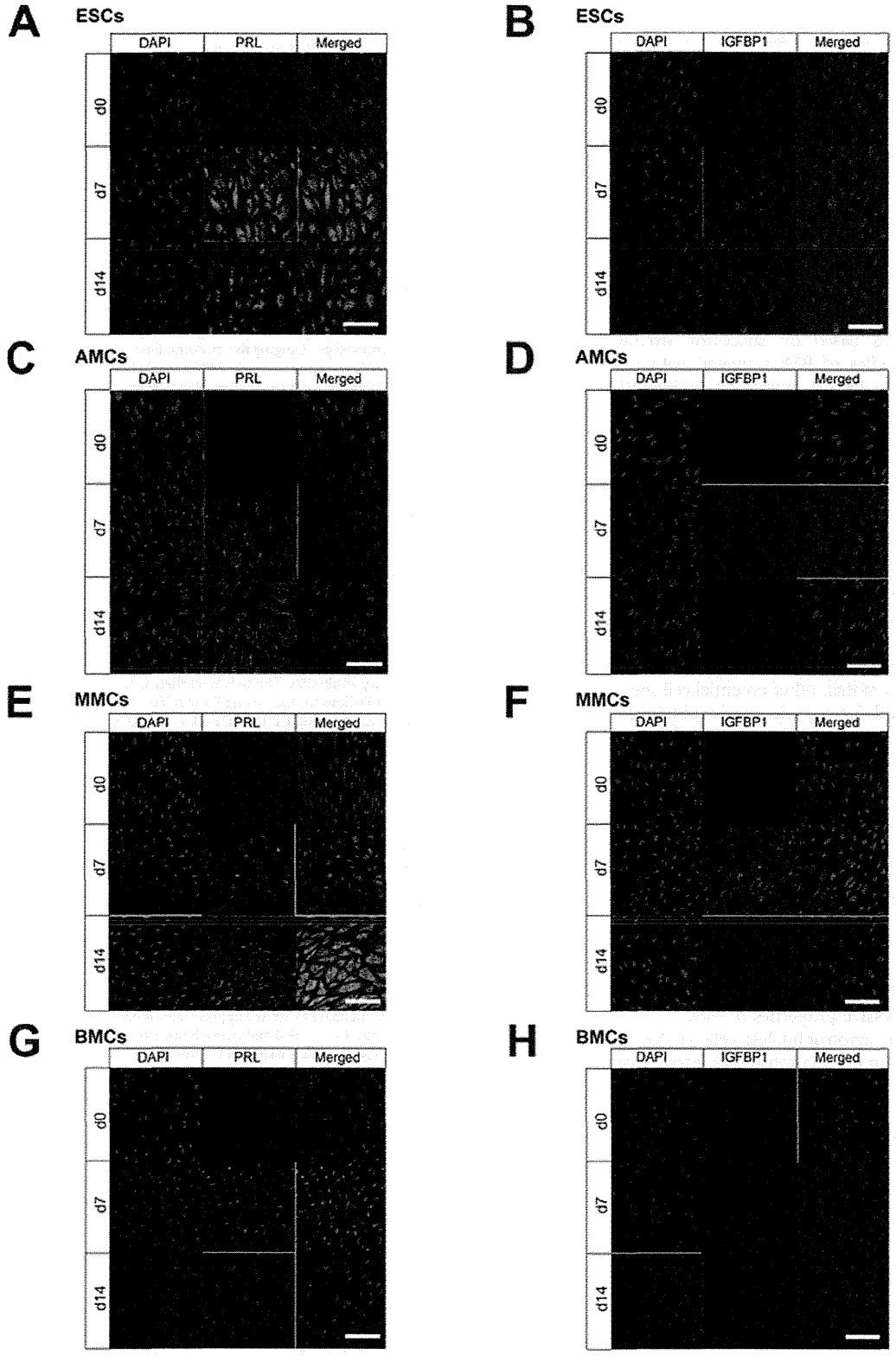


**Figure 5 | Immunofluorescence staining of decidualization markers (PRL and IGFBP1) in mesenchymal stem cells treated with cAMP.** Laser confocal microscopic view of immunofluorescence staining for PRL (green signals in A, C, E, and G) and IGFBP1 (red signals in B, D, F, and H) in endometrial stromal cells (ESCs) (A and B), amnion-derived mesenchymal stem cells (AMCs) (C and D), menstrual blood-derived mesenchymal stem cells (MMCs) (E and F), bone marrow-derived mesenchymal stem cells (BMCs) (G and H) treated with 1 mM 8-Br-cAMP for 0, 7, and 14 days. Scale bars, 200 μm.

observed in the current study may indicate immunosuppression in AMCs and MMCs that is advantageous in terms of their potential clinical application. CD105 (endoglin) is a reliable marker for highly multipotent human MSCs, with CD105-expressing MSCs showing high efficiency in repairing the infarcted heart[28]. Therefore, the disappearance of CD105 with cAMP treatment would ensure differentiation of MSCs[29,30]. Collectively, our findings indicated that administration of cAMP effectively leads to differentiation and decidualization of AMCs and MMCs.

**Advantages of AMCs and MMCs.** It has been reported that endometrial stem cells are CD45-positive cells around blood vessels and likely to be derived from BMCs[31]. A previous study also indicated that BMCs might be a source of endometrial stem/progenitor cells based on successful decidualization and gene expression profiles of BMCs treated with cAMP[19]. If BMCs are indeed a physiological source of endometrial progenitor cells, BMCs could be ideal for use in cell-based therapies for infertile women who have thinning of the endometrium. However, our findings revealed that not only BMCs, but AMCs and MMCs, also showed decidualization potential, and that all three cell types showed a similar profile of cell surface marker expression to that of ESCs. Therefore, the current study suggested that BMCs is not the only such candidate for cell therapy.

In general, the collection of bone marrow is an invasive technique performed with a biopsy needle that requires local or even general anesthesia, and occasionally raises an ethical problem. On the other hand, human amnion is non-invasively and adequately obtained at delivery. Therefore, human amnion is more simply accessed with informed consent than other potential cell sources. Human primary menstrual blood-derived cells are also obtained by a simple, safe, and painless procedure, and efficiently expanded in vitro. In particular, the MMC-derived cells can be used for autologous transplantation. Accordingly, if culture condition for MMCs could be more successfully optimized for differentiation to ESCs, MMCs would be definitely useful for eventual cell-based therapies for infertility.

Furthermore, previous studies suggested that human amnion has the potential for immunological tolerance[23,32]. AMCs are known to express less MHC compared to other MSCs, which is important in host tolerance. Cells that do not express MHC are potentially recognized by the immune system as non-self cells and accordingly, attacked by natural killer (NK) cells. A soluble factor such as the non-classic MHC class I antigen, HLA-G, is also critical for the immunosuppressive properties of MSCs[33]. HLA-G is expressed on the extravillous cytotrophoblast cells at the fetomaternal interface and plays a major role in protecting the fetus from maternal rejection by NK cells[34,35]. HLA-G thus blocks the immunological response of NK cells[36] and induces regulatory T cells[37], which is a requisite condition of immunological tolerance[35,38]. We previously showed by western blotting that HLA-G is also expressed in placenta-derived tissues including amnion[23]. Thus, AMC-derived cells have several advantages for application to cell-based therapy.

Based on the results of our current study, AMCs and MMCs in addition to BMCs show potential for decidualization, and are therefore candidate cell sources for therapy to support implantation and placentation. The current study provides novel and important information on AMCs and MMCs treated with cAMP as a model of decidualization.

## Methods

**Tissue isolation and cell culture.** All experiments involving human cells and tissues were performed according to the Tenets of the Declaration of Helsinki and were approved by the Ethics Committee of the National Institute for Child and Health Development, Tokyo. Signed informed consent was obtained from all donors, and all specimens were irreversibly de-identified. Human endometrium was obtained from a patient undergoing hysterectomy due to myoma uteri and human placenta was collected after selective caesarean section delivery.

Human amnion-derived cells were isolated using the explant culture method, in which the cells were outgrown from pieces of amnion. Briefly, the amnion was cut into pieces of approximately 2 $mm^3$ in size, which were then washed in Dulbecco's modified Eagle's medium (DMEM) (high glucose; Sigma) supplemented with 100 U/ml penicillin-streptomycin (Gibco). Some pieces were attached to the substratum in a 10-cm dish. The cells migrated out from the cut ends after approximately 20 days of incubation at 37°C in 5% $CO_2$. The migrated cells were harvested with Dulbecco's phosphate-buffered saline containing 0.1% trypsin and 0.25 mM EDTA for 5 min at 37°C and counted. The harvested cells were then re-seeded at a density of $3 \times 10^5$ cells in 10-cm dishes. Once confluent, the cell monolayers were subcultured at a 1 : 8 split ratio onto new 10-cm dishes, and the culture medium was replaced with fresh DMEM supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin every 3 or 4 days[22].

Menstrual blood samples (n = 21) were collected in DMEM containing 100 U/ml penicillin-streptomycin and 2% FBS, and processed within 24 h. The centrifuged pellets containing endometrium-derived cells were resuspended in high-glucose DMEM with 10% FBS and penicillin-streptomycin, and then maintained at 37°C in a humidified atmosphere containing 5% $CO_2$ to attach for 48 h. Non-adherent cells were removed by changing the medium. Once sub-confluent, the cells were harvested with 0.25% trypsin and 1 mM EDTA, and plated to new dishes. After 2–3 passages, the attached cells were devoid of blood cells[21].

A human bone marrow-derived mesenchymal cell line, 3F0664 was purchased from Lonza (PT-2501, Basel, Switzerland) and cultured in the mesenchymal stem-cell-basal-medium (MSCBM)-Medium-BulletKit (PT-3238, Lonza). The normal human fetal fibroblast cell line MRC5 was also passaged in DMEM supplemented with 10% (v/v) FBS and 100 U/ml penicillin-streptomycin. Cultures were maintained at 37°C with 5% $CO_2$ in a humidified atmosphere.

For decidual differentiation, human endometrial cells, amnion-derived cells, menstrual blood-derived cells, bone marrow-derived cells, and MRC5 were first cultured as described above, then trypsinized and plated in 10-cm plates. Nearly confluent cells were cultured for 0, 7, and 14 days in low-serum medium (2% FBS) supplemented with: 1) 10 nM estrogen ($E_2$) and 1 μM progesterone ($P_4$) or 2) 1 mM 8-Br-cAMP (cAMP) (Sigma-Aldrich, Saint Louis, MO, USA).

**Flow cytometric analysis.** Cells were incubated for 20 min at 4°C with primary antibodies or isotype-matched control antibodies, followed by immunofluorescent secondary antibodies. The cells were then analyzed on a FACS Aria™ IIIu Cellsorter (Becton Dickinson, Inc.) using FlowJo Ver.7 (Tree Star, Inc.). Antibodies against human CD9, CD10, CD13, CD14, CD16, CD19, CD29, CD31, CD34, CD44, CD45, CD54, CD55, CD59, CD73, CD90, CD105, CD133, CD146, CD166, HLA-ABC, and HLA-DR were purchased from Beckman Coulter, Immunotech (Marseille, France), BD Pharmingen and BioLegend (San Diego, CA, USA)[21,22]. Supplementary table S1 shows the list of CD antigens investigated in this study[39–49].

**Immunofluorescence staining.** The immunofluorescence staining was performed as previously described[50]. Briefly, cells were fixed with 4% paraformaldehyde for 10 min at 4°C, treated with 0.1% Triton X-100 (Sigma) in PBS for 15 min at RT, and then incubated for 30 min at RT in protein-blocking solution consisting of PBS supplemented with 5% FBS. The samples were then incubated overnight with the following primary antibodies diluted in PBS: an anti-prolactin mouse monoclonal antibody, (QED Bioscience, San Diego, CA, USA), anti-IGFBP1 mouse monoclonal antibody (Santa Cruz Biotechnology, Dallas, Texas, CA, USA), anti-vimentin mouse monoclonal antibody, V9, (DAKO, Tokyo, Japan), and an anti-cytokeratin mouse monoclonal antibody, AE1 + AE3, (DAKO). Tissues were washed twice for 5 min in PBS and incubated with the appropriate secondary antibodies (Alexa Fluor 488 goat anti-mouse IgG, 1 : 600 and Alexa Fluor 546 goat anti-mouse IgG, 1 : 600; Life Technologies, Grand Island, NY). Nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI; Biotium, CA, USA) for 30 min at RT followed by 10 final 5-min PBS washes before being cover slipped using mounting medium. Omission of primary antibodies served as a negative control.

**Quantitative reverse transcriptase-polymerase chain reaction (qRT- PCR) analysis.** Total cellular RNA was isolated from cells using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Total RNA (2.5 μg each) for quantitative RT-PCR was converted to cDNA using an Oligo (dT) primer with Superscript VILO™ cDNA Synthesis Kit (Life Technologies Corp., Carlsbad, CA, USA), according to the manufacturer's manual. The cDNA template was amplified by thermal cycle reactions (Quant Studio™ 12 K Flex Real-Time PCR System) using the Platinum SYBR Green qPCR SuperMix-UDG (Life Technologies Corp.) under the following reaction conditions: 40 cycles of PCR (95°C for 15 s and 60°C for 1 min) after an initial denaturation (95°C for 2 min). Fluorescence was monitored during every PCR cycle at the annealing step. The authenticity and size of the PCR products were confirmed by a melting curve analysis using software provided by Applied Biosystems. mRNA levels were normalized using *GAPDH* as a housekeeping gene. Primers used to amplify a cDNA fragment for human PRL and IGFBP1 were the following: PRL, 5′-CATCAACAGCTGCCACACTT-3′ (F) and 5′-CGTTTGGTTTGCTCCTCAAT-3′ (R); IGFBP1, 5′-CTATGATGGCTCGAAGGCTC-3′ (F) and 5′-TTCTTGTTGCAGTTTGGCAG-3′ (R).

1. Lee, T. H. *et al.* Embryo quality is more important for younger women whereas age is more important for older women with regard to in vitro fertilization outcome and multiple pregnancy. *Fertil Steril* **86**, 64–69 (2006).

2. Navot, D. *et al.* Poor oocyte quality rather than implantation failure as a cause of age-related decline in female fertility. *Lancet* **337**, 1375–1377 (1991).
3. Amir, W. *et al.* Predicting factors for endometrial thickness during treatment with assisted reproductive technology. *Fertil Steril* **87**, 799–804 (2007).
4. Senturk, L. M. & Erel, C. T. Thin endometrium in assisted reproductive technology. *Curr Opin Obstet Gynecol* **20**, 221–228 (2008).
5. Sallam, H. N. Embryo transfer: factors involved in optimizing the success. *Curr Opin Obstet Gynecol* **17**, 289–298 (2005).
6. Check, J. H. *et al.* Neither sildenafil nor vaginal estradiol improves endometrial thickness in women with thin endometria after taking oral estradiol in graduating dosages. *Clin Exp Obstet Gynecol* **31**, 99–102 (2004).
7. Landgren, B. M., Johannisson, E., Stavreus-Evers, A., Hamberger, L. & Eriksson, H. A new method to study the process of implantation of a human blastocyst in vitro. *Fertil Steril* **65**, 1067–1070 (1996).
8. Eyheremendy, V. *et al.* Beneficial effect of autologous endometrial cell coculture in patients with repeated implantation failure. *Fertil Steril* **93**, 769–773 (2010).
9. Lee, E. J. *et al.* Spherical bullet formation via E-cadherin promotes therapeutic potency of mesenchymal stem cells derived from human umbilical cord blood for myocardial infarction. *Mol Ther* **20**, 1424–1433 (2012).
10. Qiu, Y., Marquez-Curtis, L. & Janowska-Wieczorek, A. Mesenchymal stromal cells derived from umbilical cord blood migrate in response to complement C1q. *Cytotherapy* **14**, 285–295 (2012).
11. Moorefield, E. C. *et al.* Cloned, CD117 selected human amniotic fluid stem cells are capable of modulating the immune response. *PLoS One* **6**, e26535 (2011).
12. Gargett, C. E. & Healy, D. L. Generating receptive endometrium in Asherman's syndrome. *J Hum Reprod Sci* **4**, 49–52 (2011).
13. Pittenger, M. F. *et al.* Multilineage Potential of Adult Human Mesenchymal Stem Cells. *Science* **284**, 143–147 (1999).
14. Miki, T., Lehmann, T., Cai, H., Stolz, D. B. & Strom, S. C. Stem cell characteristics of amniotic epithelial cells. *Stem Cells* **23**, 1549–1559 (2005).
15. Santos, T. M. *et al.* Expression of pancreatic endocrine markers by mesenchymal stem cells from human umbilical cord vein. *Transplant Proc* **42**, 563–565 (2010).
16. Lee, K. D. *et al.* In vitro hepatic differentiation of human mesenchymal stem cells. *Hepatology* **40**, 1275–1284 (2004).
17. Pournasr, B. *et al.* In vitro differentiation of human bone marrow mesenchymal stem cells into hepatocyte-like cells. *Arc Iran Med* **14**, 244–249 (2011).
18. Dezawa, M. *et al.* Specific induction of neuronal cells from bone marrow stromal cells and application for autologous transplantation. *J Clin Invest* **113**, 1701–1710 (2004).
19. Aghajanova, L., Horcajadas, J. A., Esteban, F. J. & Giudice, L. C. The bone marrow-derived human mesenchymal stem cell: potential progenitor of the endometrial stromal fibroblast. *Biol Reprod* **82**, 1076–1087 (2010).
20. Diaz-Prado, S. *et al.* Human amniotic membrane as an alternative source of stem cells for regenerative medicine. *Differentiation* **81**, 162–171 (2011).
21. Cui, C. H. *et al.* Menstrual blood-derived cells confer human dystrophin expression in the murine model of Duchenne muscular dystrophy via cell fusion and myogenic transdifferentiation. *Mol Biol Cell* **18**, 1586–1594 (2007).
22. Kawamichi, Y. *et al.* Cells of extraembryonic mesodermal origin confer human dystrophin in the mdx model of Duchenne muscular dystrophy. *J Cell Physiol* **223**, 695–702 (2010).
23. Tsuji, H. *et al.* Xenografted human amniotic membrane-derived mesenchymal stem cells are immunologically tolerated and transdifferentiated into cardiomyocytes. *Circ Res* **106**, 1613–1623 (2010).
24. Sakai, N. *et al.* Involvement of histone acetylation in ovarian steroid-induced decidualization of human endometrial stromal cells. *J Biol Chem* **278**, 16675–16682 (2003).
25. Tamura, I. *et al.* Induction of IGFBP-1 expression by cAMP is associated with histone acetylation status of the promoter region in human endometrial stromal cells. *Endocrinology* **153**, 5612–5621 (2012).
26. Malumbres, M. & Pellicer, A. RAS pathways to cell cycle control and cell transformation. *Front Biosci* **3**, d887–912 (1998).
27. Campioni, D. *et al.* A decreased positivity for CD90 on human mesenchymal stromal cells (MSCs) is associated with a loss of immunosuppressive activity by MSCs. *Cytometry B Clin Cytom* **76**, 225–230 (2009).
28. Gaebel, R. *et al.* Cell origin of human mesenchymal stem cells determines a different healing performance in cardiac regeneration. *PLoS One* **6**, e15652 (2011).
29. Rosu-Myles, M., Fair, J., Pearce, N. & Mehic, J. Non-multipotent stroma inhibit the proliferation and differentiation of mesenchymal stromal cells in vitro. *Cytotherapy* **12**, 818–830 (2010).
30. Jin, H. J. *et al.* Down-regulation of CD105 is associated with multi-lineage differentiation in human umbilical cord blood-derived mesenchymal stem cells. *Biochem Biophys Res Commun* **381**, 676–681 (2009).
31. Bratincsák, A. *et al.* CD45-positive blood cells give rise to uterine epithelial cells in mice. *Stem Cells* **25**, 2820–2826 (2007).
32. Cui, C. H. *et al.* Dystrophin conferral using human endothelium expressing HLA-E in the non-immunosuppressive murine model of Duchenne muscular dystrophy. *Hum Mol Genet* **20**, 235–244 (2011).
33. Selmani, Z. *et al.* HLA-G is a crucial immunosuppressive molecule secreted by adult human mesenchymal stem cells. *Transplantation* **87**, S62–S66 (2009).
34. Rouas-Freiss, N., Goncalves, R. M., Menier, C., Dausset, J. & Carosella, E. D. Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytolysis. *Proc Natl Acad Sci USA* **94**, 11520–11525 (1997).
35. Fernandez, N. *et al.* A critical review of the role of the major histocompatibility complex in fertilization, preimplantation development and feto-maternal interactions. *Hum Reprod Update* **5**, 234–248 (1999).
36. Pazmany, L. *et al.* Protection from natural killer cell-mediated lysis by HLA-G expression on target cells. *Science* **274**, 792–795 (1996).
37. Selmani, Z. *et al.* Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4 + CD25highFOXP3+ regulatory T cells. *Stem Cells* **26**, 212–222 (2008).
38. Hutter, H. *et al.* Expression of HLA class I molecules in human first trimester and term placenta trophoblast. *Cell Tissue Res* **286**, 439–447 (1996).
39. Paietta, E. Expression of cell-surface antigens in acute promyelocytic leukaemia. *Best Practice & Research Clinical Haematology* **16**, 369–385 (2003).
40. Gayoso, I. *et al.* Immunosenescence of human natural killer cells. *J Innate Immun* **3**, 337–343 (2011).
41. Fujimoto, M. & Sato, S. B cell signaling and autoimmune diseases: CD19/CD22 loop as a B cell signaling device to regulate the balance of autoimmunity. *J Dermatol Sci* **46**, 1–9 (2007).
42. Mafi, P., Hindocha, S., Mafi, R., Griffin, M. & Khan, W. S. Adult mesenchymal stem cells and cell surface characterization - a systematic review of the literature. *The Open Orthopaedics Journal* **5**, 253–260 (2011).
43. Han, K. *et al.* Human amnion-derived mesenchymal stem cells are a potential source for uterine stem cell therapy. *Cell Prolif* **41**, 709–725 (2008).
44. Nery, A. A. *et al.* Human mesenchymal stem cells: from immunophenotyping by flow cytometry to clinical applications. *Cytometry Part A* **83**, 48–61 (2013).
45. Kasprzak, A., Surdacka, A., Tomczak, M. & Konkol, M. Role of high endothelial postcapillary venules and selected adhesion molecules in periodontal diseases: a review. *J Periodontal Res* **48**, 1–21 (2013).
46. Elghetany, M. T. & Lacombe, F. Physiologic variations in granulocytic surface antigen expression: impact of age, gender, pregnancy, race, and stress. *J Leukoc Biol* **75**, 157–162 (2004).
47. Crisan, M., Corselli, M., Chen, W. C. W. & Péault, B. Perivascular cells for regenerative medicine. *J Cell Mol Med* **16**, 2851–2860 (2012).
48. Alakel, N. *et al.* Direct contact with mesenchymal stromal cells affects migratory behavior and gene expression profile of CD133+ hematopoietic stem cells during ex vivo expansion. *Exp Hematol* **37**, 504–513 (2009).
49. Dvorakova, J., Hruba, A., Velebny, V. & Kubala, L. Isolation and characterization of mesenchymal stem cell population entrapped in bone marrow collection sets. *Cell Biol Int* **32**, 1116–1125 (2008).
50. Mori, T. *et al.* Combination of hTERT and bmi-1, E6, or E7 induces prolongation of the life span of bone marrow stromal cells from an elderly donor without affecting their neurogenic potential. *Mol Cell Biol* **25**, 5183–5195 (2005).


## Acknowledgments

We express our sincere thanks to Hatsune Makino, Kazumi Takanashi, Masanori Ihara, Tohru Sugawara, Yuichiro Harada, Yayoi Kajiwara, Tetsuo Maruyama, Hiroshi Uchida, Hirotaka Masuda, and other members of the National Research Institute for Child Health and Development and Keio University School of Medicine for valuable discussions and helpful advice. We also thank Kayoko Saito for secretarial work. This work was supported, in part, by a National Grant-in-Aid from the Japanese Ministry of Health, Labor, and Welfare (NCCHD24-6 to T.H. and H.A.), a Grant-in-Aid from the Japan Health Sciences Foundation (KHD1220 to T.H. and H.A.), and Grants-in-Aid from the Japan Society for the Promotion of Science (Kiban-C-23592413 to T.H. and Wakate-B-25861518 to K.S.).

## Author contributions

K.S. and T.H. contributed to the experimental design, data acquisition, analysis and interpretation, and drafting of the article. M.Y., S.K. and S.O. provided technical support and assisted with the data analysis and interpretation. N.K., H.A., K.M., Y.Y. and A.U. assisted with experimental design as well as data analysis and interpretation. All authors examined the data and approved the final manuscript.

## Additional information

**Supplementary information** accompanies this paper at http://www.nature.com/scientificreports

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Sugawara, K. *et al.* Derivation of human decidua-like cells from amnion and menstrual blood. *Sci. Rep.* **4**, 4599; DOI:10.1038/srep04599 (2014).

BASIC SCIENCE

Nanomedicine: Nanotechnology, Biology, and Medicine
10 (2014) 1165–1174

Original Article

nanomedjournal.com

# Pleiotropic functions of magnetic nanoparticles for *ex vivo* gene transfer

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Received 17 October 2013; accepted 27 March 2014

**Abstract**

Gene transfer technique has various applications, ranging from cellular biology to medical treatments for diseases. Although nonviral vectors, such as episomal vectors, have been developed, it is necessary to improve their gene transfer efficacy. Therefore, we attempted to develop a highly efficient gene delivery system combining an episomal vector with magnetic nanoparticles (MNPs). In comparison with the conventional method using transfection reagents, polyethylenimine-coated MNPs introduced episomal vectors more efficiently under a magnetic field and could express the gene in mammalian cells with higher efficiency and for longer periods. This novel *in vitro* separation method of gene-introduced cells utilizing the magnetic property of MNPs significantly facilitated the separation of cells of interest. Transplanted cells *in vivo* were detected using magnetic resonance. These results suggest that MNPs play multifunctional roles in *ex vivo* gene transfer, such as improvement of gene transfer efficacy, separation of cells, and detection of transplanted cells.

***From the Clinical Editor:*** This study convincingly demonstrates enhanced efficiency of gene transfer via magnetic nanoparticles. The method also enables magnetic sorting of cells positive for the transferred gene, and in vivo monitoring of the process with MRI.


*Key words:* Episomal vector; *Ex vivo* gene transfer; *In vitro* cell separation; Magnetic nanoparticles; Magnetic resonance imaging

Nanotechnology describes the creation and utilization of materials, devices, and systems with nanometer-sized scaffolds that are applied in fields such as physics, chemistry, biology, engineering, materials science, and medicine.[1] Nanoparticles are generally defined as particles of approximately 1-100 nm; this size range is similar to that of several proteins and nucleic acids. In particular, intensive efforts are underway to develop nanomaterials for ideal pharmaceutical agents specific to a target organ, tissue, or cell. Nanomedicine refers to the medical application of nanotechnology in the monitoring, diagnosis, prevention, and treatment of various diseases, which is currently revolutionizing modern medicine.[2] Several clinical trials using nanocarriers loaded with anticancer drugs and RNAs for gene silencing are underway to treat several types of cancer.[3]

Till date, several delivery systems using nanoparticles in combination with nucleic acids and chemical drugs have been reported.[4-6] Magnetic nanoparticles (MNPs) opened a novel avenue for integrative therapeutics and diagnostic applications, which is known as nanotheranostics.[2] The attraction of MNPs toward an external magnetic field has been exploited for purposes such as delivering genes or drugs to target sites and

We express our sincere gratitude to M. Nakata (Kyoto Prefectural University of Medicine, Kyoto, Japan) for contributing toward cell culture and FACS analysis.

Funding: This study was supported by a Grant-in-Aid for the Science for Future Molecular Systems from the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT).

Conflict of Interest: The authors report no conflicts of interest in this work. The authors have no financial interests or conflicts with regard to the subject matter discussed in this manuscript.

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http://dx.doi.org/10.1016/j.nano.2014.03.018
1549-9634/

tracing cells by magnetic resonance imaging (MRI).[7] MNPs have been used as adjuvants to improve gene transfer efficacy by up to several 100-fold under a magnetic field. Magnetic gene transfer is defined as "magnetofection".[8] Magnetofection has been combined with either nonviral[9] or viral vectors including retrovirus,[10] adenovirus,[11] and adeno-associated virus.[12] In the mechanism of magnetofection, magnetic forces may accelerate the sedimentation of the complex between MNPs and DNAs or viral vectors onto the cell surface and not enforce endocytosis into the cell.[13] Although there is a concern regarding nanotoxicity owing to the greatly enhanced reactive surface area of nanoparticles, their ability to cross cell membranes, and their resistance to biodegradation, which may result in apoptosis and genotoxicity via inflammatory responses,[14] their advantages such as improving the dose–response relationship and kinetics of nucleic acid delivery are pushing the methodology toward the clinical arena.

In our laboratory, as previously described, we have developed a gene delivery system using iron oxide ($\gamma$-$Fe_2O_3$) MNPs and magnetic force.[15,16] To efficiently increase gene delivery, we focused on cationic polymer deacylated polyethylenimine (PEI) and PEI-coated MNPs (PEI-MNPs) that are capable of interacting with nucleic acids. PEI-MNPs form complexes with nucleic acids (e.g., plasmids), called magnetoplexes, in water.[15] They are efficiently introduced into cells by a magnetic field produced by a magnetic plate under the culture dish. PEI-MNPs have been reported to show dramatically decreased cytotoxicity compared with uncoated nanoparticles,[17] and iron oxide has already been used in clinical applications as a contrast agent for MRI.[18]

However, there is still a serious concern about the genomic integration of exogenous genes, which may result in carcinogenesis and ectopic differentiation. Therefore, a transient gene transfer system is required, particularly for clinical applications. For the same reason, several protocols involving the use of episomal vector plasmids,[19,20] Sendai virus,[21] and synthesized mRNA[22] have been reported. However, these methods are much less efficient than the retrovirus method. A gene transfer system using an episomal vector was adopted as a standard protocol to generate induced pluripotent stem cells (iPSCs) for human cell banking in Japan. The episomal vector constructed by Mazda et al[19] comprised the Epstein–Barr (EB) virus antigen-1 (*EBNA-1*) gene and *OriP* sequence placed in the backbone of a construct with artificial chromosome-like characteristics such as cytoplasm-to-nuclear transport, nuclear retention, and replication and segregation of the DNA. Insertion of the *EBNA-1* gene and *OriP* sequence into recombinant plasmid DNA, the so-called episomal vector, may allow the replication and maintenance of the plasmid in mammalian cells. We accordingly examined whether the gene delivery system combined with an episomal vector and modified PEI-MNPs could reinforce the gene transfection efficacy using a novel *in vitro* cell separation procedure based on the magnetic property of PEI-MNPs themselves without any additional magnetic materials.

## Methods

### *Experimental procedures*

Details of quantitative reverse transcription PCR (qRT-PCR), flow cytometric analysis, inductively coupled plasma mass spectrometry (ICP-MS), cell viability, MRI, pathohistological analysis, and statistical analysis are provided in the Supplementary Document.

### *Materials*

MNPs ($\gamma$-$Fe_2O_3$) were purchased from CIK NanoTek (Tokyo, Japan). Deacylated PEI, PEI max linear (MW 25,000), was purchased from Polysciences, Inc. (Warrington, PA, USA). Deionized water was purchased from Life Technologies, Corp. (Carlsbad, CA, USA). Magnetic plate (1.2 T) was purchased from OZ Biosciences Inc. (Marseille, France). Episomal vector was provided by T.K. and O.M. (Supplementary Figure 1).

### *Preparation of PEI-MNPs*

PEI-MNPs were prepared as reported in previous reports[15,16] (Supplementary Figure 2). In brief, MNPs (1.0 g) were dissolved in 30 ml PEI solution (1.6 mg PEI/ml). The mixture was sonicated for 2 min at 40 W on ice, and 20 ml of deionized water was then added (final concentration, 1.0 mg PEI/ml). The ferrofluid was centrifuged at 4,100 $\times g$ for 5 min, and subsequently, the supernatant was transferred into fresh tubes. The fluids were washed twice with deionized water and dissolved into equal volumes of PEI solution (1.0 mg PEI/ml) to prevent agglomerations. MNPs in this fluid were coated with PEI and dispersed (PEI-MNPs). The diameter of PEI-MNPs was approximately 120 nm as deduced using laser light scattering method.[16]

### *Mouse embryonic fibroblast (MEF) isolation, cell culture, and MNP transfection*

Experimental procedures and protocols were approved by the Animal Experiment Ethics Committee of the Kyoto Prefectural University of Medicine. For MEF isolation, uteri removed from 13.5-day pregnant C57/B6 mice were washed using phosphate-buffered saline (PBS; Wako Pure Chemical Industries, Ltd., Osaka, Japan). The head and visceral tissues were removed from isolated embryos. The remaining bodies were washed using fresh PBS, minced with scissors, and transferred into fresh Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries, Ltd.) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (Life Technologies, Corp.). Three days after incubation, outgrowth cells were trypsinized, collected by centrifugation (200 $\times g$ for 5 min), and resuspended in fresh medium. Following the first passage, $1 \times 10^6$ cells were cultured in 150-mm dishes at 37 °C under 5% $CO_2$. In this study, we used MEFs within 3-5 passages. MEFs were cultured using DMEM containing 10% FBS and 1% penicillin and streptomycin at 37 °C under 5% $CO_2$. To perform transfection using episomal GFP vector, $1 \times 10^5$ MEFs were plated in each well of a 6-well plate a day before transfection. Immediately before transfection, cells were rinsed and supplemented with 1 ml of fresh culture medium. PEI-MNPs (7.5 μl, 0.05 mg PEI-MNPs in 1 mg PEI/ml) were mixed with 2.5 μg episomal vector (pEF.OriP9.GFP.E.) and incubated in deionized water at a final volume of 50 μl at room temperature for 15 min. The complexes were then added to the MEF culture dishes placed on a magnetic plate for 4 h. PEI (7.5 μl) solution (1 mg PEI/ml, without MNPs) was used as a control. The medium was

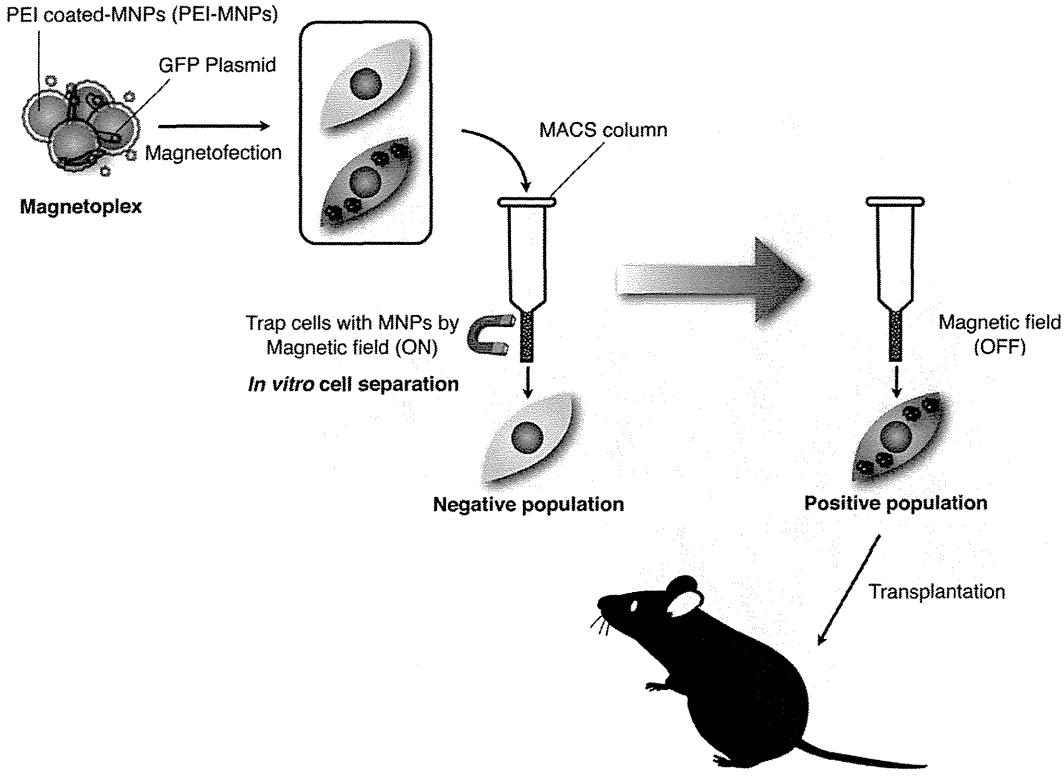


Figure 1. Schema of the experiment.First, PEI-MNPs containing the episomal plasmid (i.e., magnetoplex) were transferred into the cells. Second, *in vitro* cell separation was performed as illustrated at the center of figure. Cells containing the magnetoplex, which has a net magnetization, were enriched using an MACS column and a magnetic field. Third, the positive population was transplanted into the legs of mice.

replaced with fresh DMEM supplemented with 10% FBS 4 h after MNP transfection, and the cells were cultivated for subsequent experiments.

*In vitro cell separation by PEI-MNPs*

Two days after transfection, transfected MEFs were harvested by trypsin treatment and resuspended into 0.5 ml autoMACS running buffer (Miltenyi Biotec KK, Bergisch-Gladbach, Germany). The cell solution was loaded into a magnetic-activated cell sorting (MACS) MS column (Miltenyi Biotec KK) under a magnetic field. The column was washed thrice using autoMACS buffer to elute MEFs that did not contain PEI-MNPs; these were considered to be non-transfected and were designated as the negative population of cells (Figure 1). The cells retained in the column under the magnetic force, which were expected to be gene-transfected cells, were flushed out with autoMACS buffer using a plunger supplied with the column; these cells were designated as the positive population of cells (Figure 1). Subsequent to this *in vitro* step, the enriched cells were seeded in a 6-well culture plate for 5 days at 37 °C under 5% $CO_2$.

## Results

*Episomal vector transfection efficiency was increased by PEI-MNPs*

Episomal vector transfection efficiency in the presence of PEI-MNPs (PEI-MNPs with magnetic field group) was compared with the PEI transfection method (PEI group) or PEI-MNPs without placing on the magnetic plate (PEI-MNPs without magnetic field group). Using fluorescence microscopy, we detected more GFP-positive cells using PEI-MNPs with magnetic field than those using PEI or PEI-MNPs without magnetic field (Figure 2, *A*). To evaluate the transfection efficiency, qRT-PCR (Figure 2, *B*) and flow cytometric analysis (Figure 2, *C*) were performed at days 2, 4, and 7 after transfection. GFP expression level in the PEI-MNPs with magnetic field group was increased by approximately 6-8-fold (Figure 2, *B*), and the expression in MEFs was sustained for 7 days (Figure 2, *C*) in comparison with that in the PEI group. Both the relative GFP expression level and the GFP-positive cell ratio to the complete cell number decreased with time in the PEI-MNPs with magnetic field group. The decrease in the relative GFP expression level was consistent with that in other studies using the same episomal vector[19]; further, the levels in the PEI or PEI-MNPs without magnetic field groups were too low to allow estimation of a time trend (Figure 2, *B*). The reduction rate of GFP positive ratio in PEI, and PEI-MNPs without, or with magnetic field on day 7, compared with GFP positive cell ratio in each group on day 2, were 43.6%, 20.5%, and 42.7%, respectively, which were not significant different based on ordinary one-way ANOVA (Figure 2, *C*).

*Cell characterization after in vitro cell separation*

We successfully developed a novel *in vitro* separation method for transfected cells containing PEI-MNPs using a

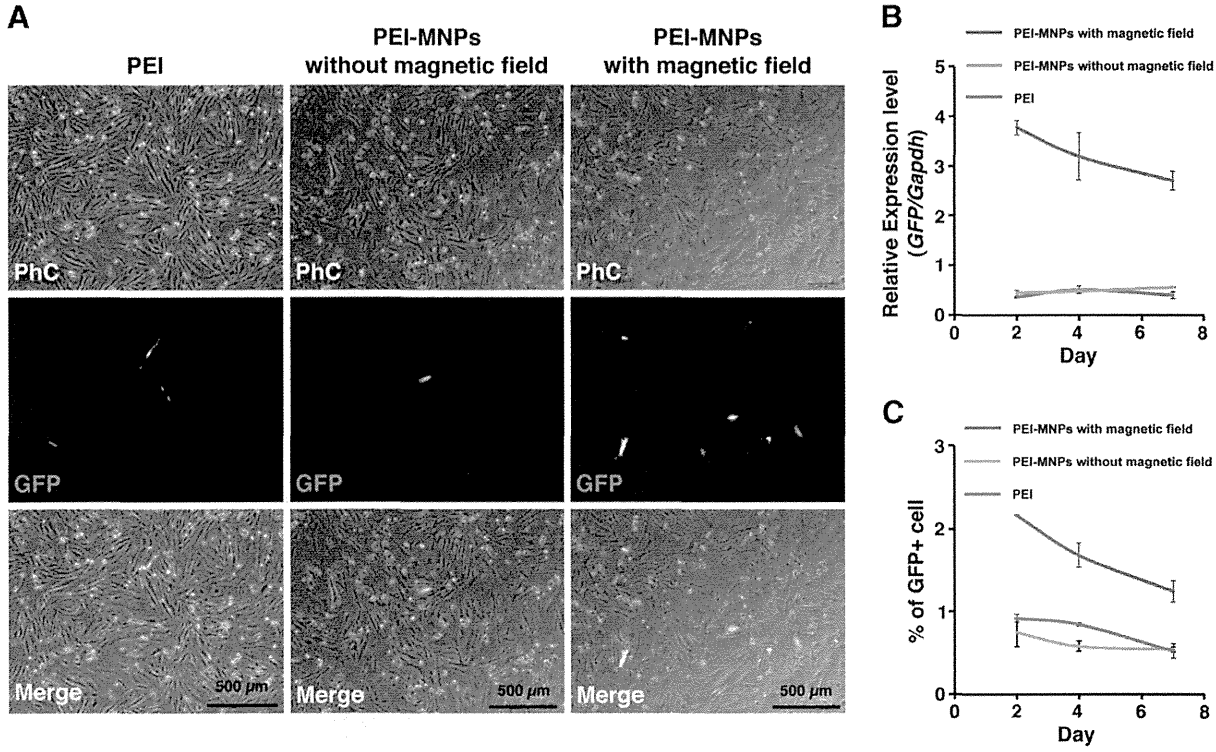


Figure 2. Efficient transfection method using magnetic nanoparticles (MNPs) and an episomal vector.**(A)** Phase contrast fluorescence microscopy images. Mouse embryonic fibroblasts (MEFs) were transfected with episomal vector pEF.OriP9.GFP.E. and polyethylenimine (PEI)-coated MNP (PEI-MNPs) with magnetic field. Transfections with PEI or with PEI-MNPs without magnetic field were used as controls. Black bar indicates 500 μm; PhC, phase contrast. **(B)** qRT-PCR of GFP expression in MEFs using PEI, PEI-MNPs with magnetic field, or PEI-MNPs without magnetic field. Individual RNA expression levels were normalized to the respective mouse *Gapdh* expression levels. pCAGGS-GFP plasmid-transfected MEFs were used as a reference. Error bars indicate SE (n = 4). **(C)** Flow cytometric analysis of GFP-positive MEFs treated with PEI, PEI-MNPs with magnetic field, or PEI-MNPs without magnetic field. All results were compared with non-transfected cells. Error bars indicate SE (n = 3).

magnetic field and MACS column (*in vitro* cell separation) (Figure 1). We evaluated the total iron content per cell, cell viability, and GFP-positive cell percentage of PEI-MNP-transfected MEFs in the time course depicted in Figure 3, *A*. First, we measured the total iron content per cell using ICP-MS. The total iron content per unenriched MEF remained approximately the same from days 2 to 14 (Figure 3, *B*). In addition, the total iron content per MEF at day 2 in the positive population was 3.12 ± 0.60 pg/cell (expressed as mean ± standard error), whereas that in the negative population was 0.14 ± 0.05 pg/cell; the total iron content per MEF before the procedure was 0.56 ± 0.10 pg/cell (Figure 3, *C*). In comparison with the negative population, the positive population exhibited an approximately 23-fold increase in the total iron content. In addition, the number of PEI-MNPs in the positive and negative populations was calculated to be $22.4 \pm 4.31 \times 10^3$ and $1.0 \pm 0.36 \times 10^3$ MNPs/cell, respectively (Figure 3, *D* and Supplementary Figure 3). We measured cell viability after separation at days 2 and 7. Although the enriched cell viability on day 2 was 38% ± 8.9%, the viability on day 7 recovered to 83% ± 8.6% (Figure 3, *E*). This poor cell viability of the positive population was attributed to excessive genetic material within the cells, because the viability of the positive population in a mock experiment, which was performed without the episomal vector, was similar to that of the control (Supplementary Figure 4). The percentage of PEI-MNP-transfected cells was too low to influence the cell viability of the entire population, and there was no significant difference between the entire cell population prior to the enrichment and the negative population with regard to cell viability (Figure 3, *E*). The absolute cell number at days 2 and 7 and the doubling times during this period for both the populations following the *in vitro* separation procedure were plotted (Figure 3, *F* and *G*). The growth properties were not significantly influenced by the separation procedure. The number of cells in the positive population was $2.9 \times 10^4$ and the doubling time was 3.28 days; the number of cells and doubling time were similar to the cells that were only transfected and to those in the negative population.

### *Efficacy of in vitro cell separation*

To evaluate the efficacy of *in vitro* cell separation, we compared the expression of GFP in the gene-transfected cells without *in vitro* cell separation (Figure 4, *A*) with that those with *in vitro* cell separation (Figure 4, *B*) using fluorescence microscopy. Because the cell viability in the positive population was approximately 38%, the cell number was less than that prior to the separation procedure. Although as per the fluorescent images, the positive population was not enriched for exogenous gene expression, the merged views reveal the sparseness of cells after the procedure, and the ratio of the positive population to the entire cell population was judged to be higher than that without the

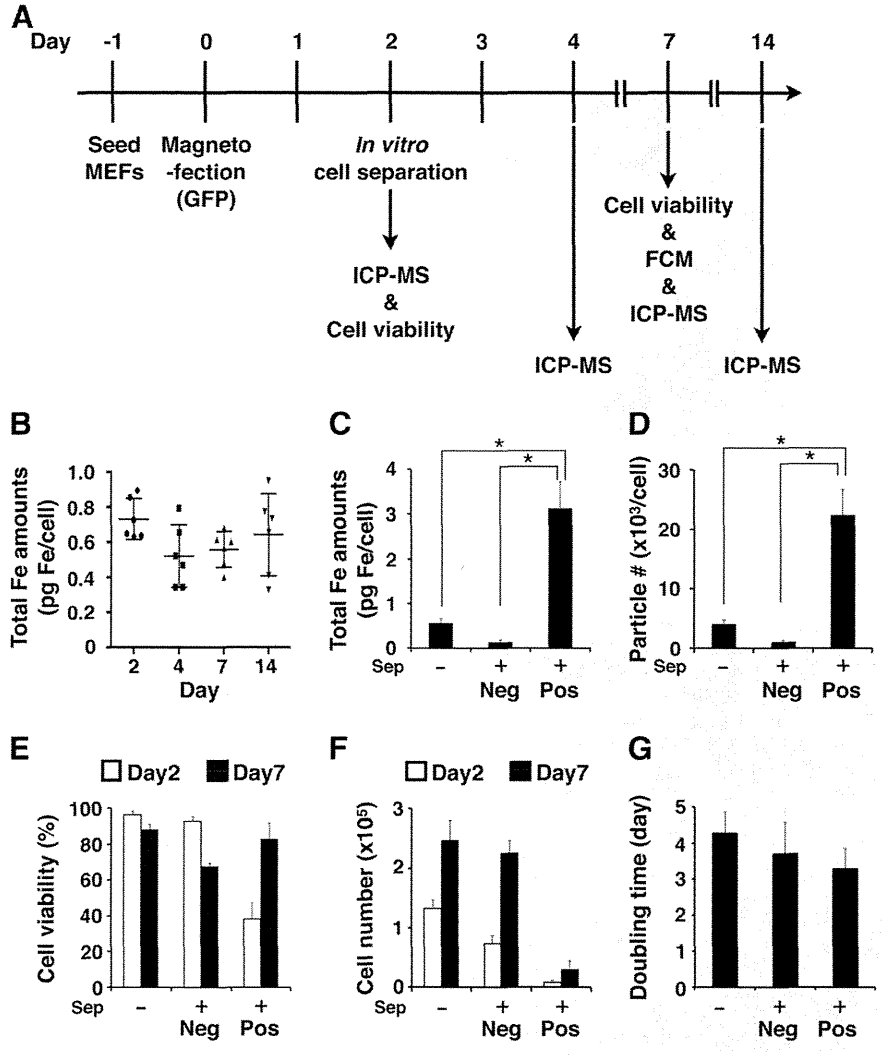


Figure 3. Evaluation of the total iron content per cell and cell viability by *in vitro* cell separation.**(A)** Time course for evaluating *in vitro* cell separation. MEFs were analyzed by ICP-MS, cell viability assay, and flow cytometric analysis. Medium was replaced every 2 or 3 days. **(B** and **C)** Inductively coupled plasma-mass spectrometer (ICP-MS) was used to measure iron uptake by MEFs. The total iron content was corrected according to cell number. Error bars indicate SE; * indicates statistical significance ($P < 0.05$). Time-dependent changes in the total iron content per unenriched MEF were approximately the same from days 2 to 14 **(B)**. Two days after transfection, MEFs containing PEI-MNPs were enriched by a magnetic field **(C)**. **(D)** The number of MNPs in MEFs was calculated (Supplementary Figure 3). **(E)** Cell viability was examined at days 2 and 7 following gene transfer. Pos and Neg represent the positive and negative populations for *in vitro* cell separation, respectively. **(F)** Cell number in both fractions after *in vitro* cell separation. **(G)** Doubling times in both the groups were approximately the same. Sep represents *in vitro* cell separation.

enrichment (Figure 4, *A* and *B*). Figure 4, *C* demonstrates the representative density plot analysis by flow cytometer. The percentages of GFP-positive cells as the average of the experimental series without and with the separation procedure were 3.7% and 8.4%, respectively; the enrichment ratio was approximately 2.3-fold (Figure 4, *D*). We calculated the total number of GFP-positive cells in both the positive and negative populations 7 days after gene transfer. The sensitivity and specificity were 82.65% ± 1.51% and 59.68% ± 0.55%, respectively. We repeated the separation procedure to determine whether the number of GFP-positive cells increased upon sequential *in vitro* cell separation. The average number of viable cells on day 2 following second enrichment decreased to $2.0 \times 10^4$ from $2.42 \times 10^4$, which were acquired following the first enrichment. The GFP-positive cell ratio on day 7 following second enrichment also decreased to 6.5% from 8.9% (Figure 4, *C*). To compare our procedure with a standard protocol involving an episomal vector for human iPSCs, electroporation was performed (Supplementary Figure 5). The total number of GFP-positive cells was $6.27 \times 10^2$ cells 7 days after electroporation, while it was $2.4 \times 10^3$ cells in the positive population 7 days after *in vitro* cell separation using PEI-MNPs (Figures 3, *F* and 4, *D* and Supplementary Figure 5).

### *MRI analysis of PEI-MNP-enriched cells in mice*

Compared with the absence of signals in the control sample (Figure 5, *A* and *B*), GFP gene-transfected MEFs could be

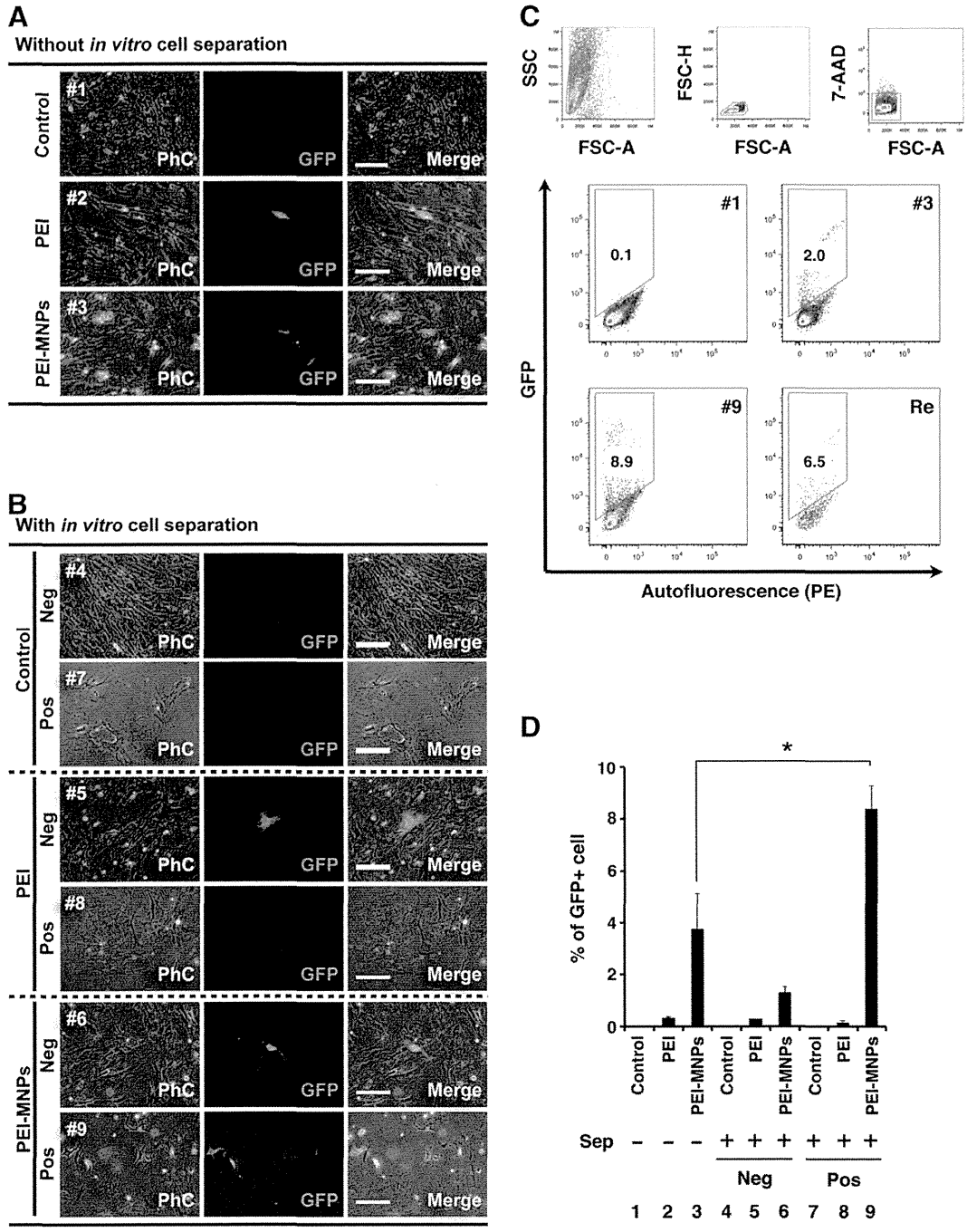


Figure 4. Efficacy of *in vitro* cell separation by fluorescent microscopy and flow cytometric analysis.**(A** and **B)** Phase contrast fluorescence microscopy images of GFP-transfected MEFs 7 days after transfection without *in vitro* cell separation **(A)** and with separation **(B)**. White bar indicates 500 μm. Numbers listed in each phase contrast image (PhC) in **(A)**, **(B)**, and **(C)** correspond to the numbers in the graph **(D)**. **(C)** Representative flow cytometric analysis of GFP-positive MEFs treated with PEI or PEI-MNPs. Three figures in the upper row (FSC-A vs. SSC, FSC-A vs. FSC-H, and FSC-A vs. 7-AAD) reveal sample gating. The remaining 4 figures [autofluorescence (PE) vs. GFP] represent each sample [the numbers correspond to those in **(A)**, **(B)**, and **(D)**]. Re indicates the sequential re-separation of MNPs. **(D)** The plot at the bottom right shows the percentage of GFP-positive MEFs in each condition analyzed by flow cytometric analysis. All results were compared with non-transfected cells as a control. Error bars indicate SE (n = 3-5); * indicates statistical significance ($P < 0.05$).

clearly detected with MRI at the point of transplantation corresponding to the injection site (Figure 5, *C*, white arrowhead) immediately after implantation on day 7. The signal intensity of the grafts slightly decreased but did not migrate to other parts of the body.

*Pathohistological analysis*

The quadriceps femoris muscle was dissected after MRI measurement on day 7 for pathohistological analyses. Prussian blue staining, which targets iron, clearly revealed positive cells

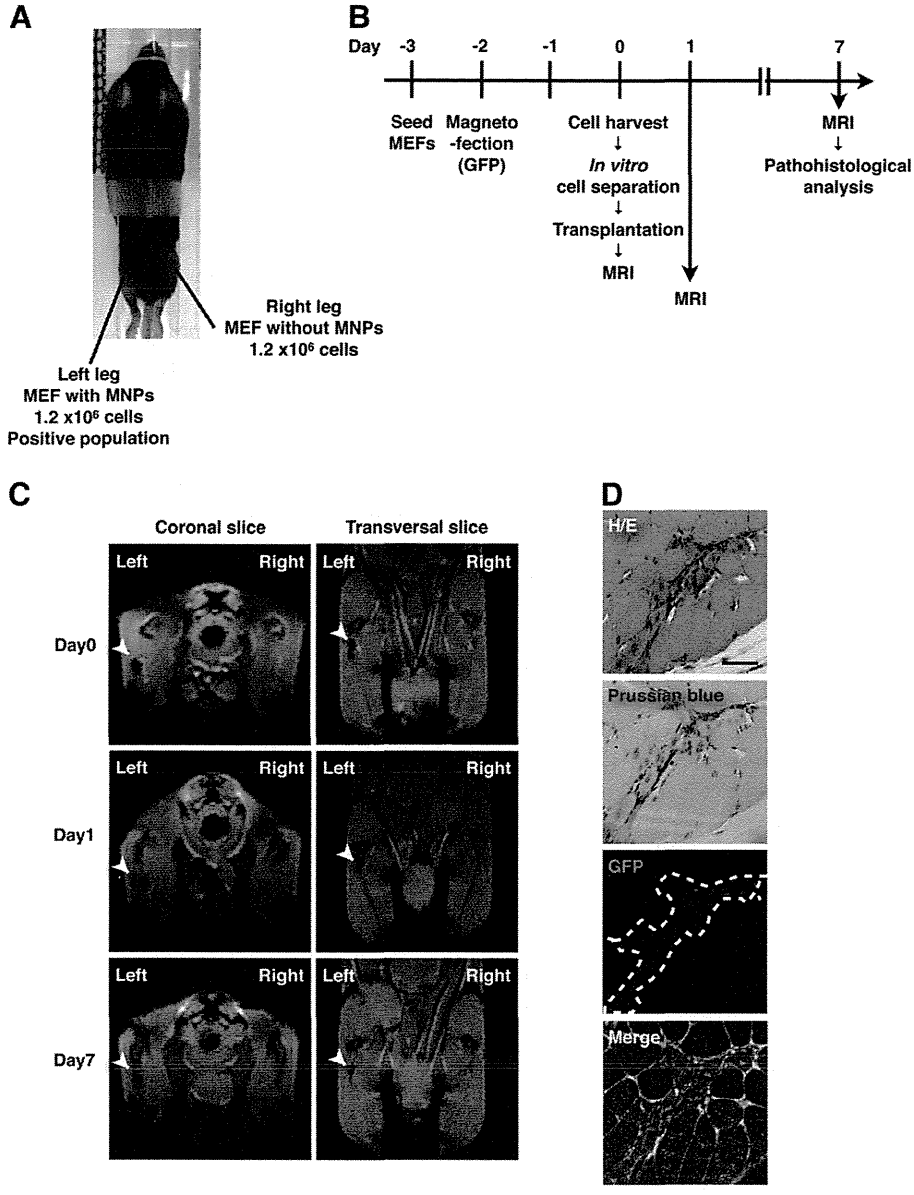


Figure 5. *In vivo* MRI measurements using MNP-transfected and -enriched MEFs. **(A)** Posture of the anesthetized mouse at MRI scans. The right leg was injected with MEFs without treatment. The left leg was injected with the positive population for *in vitro* cell separation. **(B)** Schematic illustration of the experimental design for *in vivo* MRI measurements using PEI-MNP-transfected and -enriched MEFs. **(C)** After intramuscular injection in the legs of C57/B6 mice, sequences of 4.7-T magnetic resonance images were obtained at days 0, 1, and 7 in the coronal and transversal planes. The GFP-transfected MEFs were injected into the left leg, whereas the untreated MEFs were injected into the right leg. **(D)** Pathohistological analysis of transplantation of GFP-transfected MEFs at day 7 in the injected leg by hematoxylin–eosin (H&E) and Prussian blue staining and immunohistochemical staining with anti-GFP. Black bar indicates 50 μm.

corresponding to MRI-positive sites (Figure 5, *D*). Using immunofluorescence staining with GFP antibody, GFP-positive cells in tissue sections indicated that PEI-MNP-transfected cells were viable. No inflammatory cells were identified in the equivalent areas of the histology specimen.

## Discussion

Here we report that in addition to their previously reported dual roles in aiding gene transfer and cell tracing, PEI-MNPs coupled with an episomal vector can successfully enable *in vitro* cell separation.

Gene delivery techniques enable the introduction of a gene of interest in host cells to express the corresponding encoded protein either *in vivo* or *in vitro*. At present, there are 3 primary gene delivery systems that employ viral vectors (e.g., retroviruses and adenoviruses), nucleic acid electroporation, and nucleic acid transfection. Their efficacy and cytotoxicity depend on their machinery for transferring genes into target cells. Although gene delivery by viral vectors generally exhibits high efficiency, these methods have some drawbacks, such as