Multipotent Vasculogenic Pericytes from Human Pluripotent Stem Cells

Promote Recovery of Murine Ischemic Limb

Running title: Dar et al.; Human pluripotent stem cell-derived pericytes

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Abstract:

**Background** - Pericytes represent a unique subtype of microvessel-residing perivascular cells with diverse angiogenic functions and multilineage developmental features of mesenchymal stem cells. Although various protocols for derivation of endothelial and/or smooth muscle cells from human pluripotent stem cells (hPSC, either embryonic or induced) have been described, the emergence of pericytes in the course of hPSC maturation was not elucidated yet.

**Methods and Results** - We found that during hPSC development, spontaneously differentiating embryoid bodies give rise to CD105<sup>+</sup>CD90<sup>+</sup>CD73<sup>+</sup>CD31<sup>−</sup> multipotent clonogenic mesodermal precursors, which can be isolated and efficiently expanded. Isolated and propagated cells expressed characteristic pericytic markers, including CD146, NG2 and PDGFR-β but not the SMC marker, α-smooth muscle actin. Co-implantation of hPSC-derived endothelial cells with pericytes resulted in functional and rapid anastomosis to the murine vasculature. Administration of pericytes into immuneodeficient mice with limb ischemia promoted significant vascular and muscle regeneration. At day 21 after transplantation recruited hPSC-pericytes were found incorporated into recovered muscle and vasculature.

**Conclusions** - Derivation of vasculogenic and multipotent pericytes from hPSC can be used for the development of vasculogenic models using multiple vasculogenic cell types for basic research and drug screening and can contribute to angiogenic regenerative medicine.

**Key words:** human pluripotent stem cells, ischemia, mesenchymal stem cells, pericytes, vasculature.
Introduction

In adult tissues, the majority of blood vessels are composed of three layers including a luminal inner monolayer of endothelial cells (EC), tunica intima, an intermediate muscular layer, tunica media, of smooth muscle cells (SMC) and an outer layer of fibroblast-like adventitial cells and connective tissue components, tunica adventitia. Microvessels, including capillaries, precapillary arterioles, postcapillary venues and collecting venules are composed of internal endothelial layer surrounded by outer coverage of pericytes (also known as Rouget cells or mural cells).1 Both perivascular SMC and pericytes have been shown to function as critical regulators of vascular development, stabilization, maturation, and remodeling mediated by transforming growth factor-β (TGF-β), platelet-derived growth factor-B (PDGF-B), sphingosine-1-phosphate (S1P) or angiopoietin-1 (Ang1).2,3 Although related in function and anatomical localization pericytes can be distinguished from SMC based on their characteristic morphology, and specific cell marker expression: While SMC form a separate layer of the tunica media in blood vessels, pericytes are physically embedded within the endothelial basement membrane to promote mutual communication with the underlying endothelium.4 In addition, SMC and the majority of pericytes in multiple human and murine tissue types express α-smooth muscle actin (α-SMA), which is involved in regulation of vessel contractility.5 Although specific markers of pericytes are not defined yet, the majority co-express α-SMA, CD133, NG2, CD146, alkaline phosphatase and PDGFR-β, with minor subsets co-expressing CD346 or do not express α-SMA7 and assumed to represent more primitive pericytic precursors.

Besides angiogenic features, subsets of blood vessel residing cells are defined as stem cells, which are capable of multiple developmental ability, including osteogenic, chondrogenic, adipogenic and myogenic differentiation capabilities5,6.
During early stages of vertebrate embryogenesis and similarly in embryonic stem cell differentiation, EC arise from mesodermal angioblasts/hemangioblasts precursors.\textsuperscript{8-10} In comparison, the ontogeny of pericytes is more complex, share similarities with SMC, and related to their deposition in different regions of the body.\textsuperscript{11} In adult tissue the saphenous vein was shown to contain a novel subset of CD34\textsuperscript{+}CD31\textsuperscript{−} pericytic precursors\textsuperscript{6}. In vitro, the pluripotent differentiation capability of human embryonic stem cells (hESC),\textsuperscript{12} and more recently the rapidly growing platform of human induced pluripotent stem cells (hIPSC),\textsuperscript{13} provides a powerful model system to study vasculogenic development. Several studies have described protocols for the derivation of endothelial and SMC from progenitor cells\textsuperscript{14-17}. However, derivation of multipotent vasculogenic pericyte population from hPSC was not documented until now. Therefore, we used differentiating hPSC as a model to determine whether pericytes emerge alongside with development of endothelial and SMC based on common (CD105, \(\alpha\)-SMA) and specific (CD31, VE-Cadherin, UEA-1) vascular cell markers. We have identified a novel population of cells, positive for recognized markers of pericytes, including NG2, PDGFR-\(\beta\) and CD146 but not \(\alpha\)-SMA and characteristic markers of mesenchymal stem cells (MSC). Herein we describe a protocol for co-isolation of vasculogenic derivatives from a single source of hPSC including, pericytes, SMC and EC. In addition we identified the conditions for long-term cultivation of clonogenic hPSC-pericytes, which steadily maintained perivascular and multi-lineage characteristics in vitro and in vivo, and were proved useful in the treatment of severe ischemic tissue.

**Methods**

An expanded Supplementary Methods section is available in the online-only Data Supplement.
Pericyte and Endothelial Progenitor Cell Isolation

At indicated time points of spontaneous differentiation in EBs, single-cell suspensions were made from differentiated hPSC including, human ESC H9.2\textsuperscript{18} and I6\textsuperscript{19}; hPSC human foreskin fibroblasts–derived C3\textsuperscript{20} and human hair follicle keratinocyte-derived KTR13\textsuperscript{21}. CD31\textsuperscript{+} EC or CD105\textsuperscript{+} cells were isolated from differentiated hPSC using MACS MicroBeads (Miltenyi Biotec), according to the manufacturer’s instructions. As determined by flow cytometry, the purity of isolated CD31\textsuperscript{+} or CD105\textsuperscript{+} cells was generally 60-70% at a single column, and > 96% after the second column. Dissociated EBs-derived CD105\textsuperscript{+}CD31\textsuperscript{+} or CD105\textsuperscript{+}CD31\textsuperscript{-} subpopulations were isolated by fluorescence-activated cell sorting (Becton Dickinson Immunocytometry Systems). In some experiments, CD31\textsuperscript{+} EC were isolated from cultured hPSC-derived CD105\textsuperscript{+} cells for further expansion (purity of CD31\textsuperscript{+} >97%) of either CD105\textsuperscript{-}CD31\textsuperscript{+} EC or CD105\textsuperscript{+}CD31\textsuperscript{-} pericytes.

Matrigel Implants

EC (HUVEC or hPSC-derived EC) or hPSC-pericytes were re-suspended in 250 μl phenol-red free Matrigel (BD Biosciences), either alone (3-5x10\textsuperscript{5} EC, 6-8x10\textsuperscript{5} pericytes) or mixed (1:1). Matrigel mixture was then injected subcutaneously into 6-8 weeks old NOD/SCID mice. Implants were removed at the indicated time points, fixed in 4% formalin, sectioned and stained with Hematoxylin/Eosin.

Hind Limb Ischemia Model
Male 8 weeks old CD1 nude mice were subjected to hind limb ischemia by femoral artery ligation and transection. After surgery mice received of $2 \times 10^6$ hPSC-pericytes (H9.2, I6 or C3) or unconditioned M-199 medium (200 µl) intramuscularly into 4 sites of the gracilis muscle in the medial thigh. Limb perfusion was assessed with a non contact laser Doppler at days 0, 7, 14 and 21 after treatment.

**Statistical Analyses**

T-test was used for comparisons between 2 groups (analyses were preformed with Excel 2007). Repeated measures ANOVA (analyses were preformed with IBM SPSS statistic 19) was used for analyses of blood flow recovery, in which measurements were repeated for the same subject at the same time point (P<0.05 was inferred to denote statistical significance) and followed by Bonferroni post hoc test.

**Results**

**Emergence of Vasculogenic Progenitor Cells in Developing EBs.**

The emergence of vasculogenic populations from hPSC was examined in spontaneously differentiating EBs. For characterization of vasculogenic precursors, developing EBs were analyzed for the expression of CD105 and CD73, which have been reported to be expressed on both endothelial and mural cells in blood vessels.\(^5\) Quantitative-PCR analyses of differentiating EBs demonstrated that CD105 and CD73 expression was detected between days 4-26 in culture, upregulated between days 10-14 and decreased between days 19-26 (Figure 1A). The expression of the EC marker, VE-Cadherin, was increased during 14 days of differentiation with low to no detectable expression thereafter (Figure 1A). Flow cytometry analyses for the expression of an
additional EC marker CD31 with CD105 revealed that two distinguished cell subsets contributed to the overall increase in CD105 mRNA expression as follows: until day 14 the majority of CD105+ cells were composed of CD105+CD31+ EC, with a small subset of non-endothelial CD105+CD31- population (Figure 1B). From day 19 onward the percentage of CD105+CD31+ EC as well as CD31 mRNA expression declined progressively coinciding with an increase in the percentage of CD105+CD31- or CD73+CD31- non-endothelial subsets (Figures 1B-1D). Of notice, in comparison to published data,22 our optimized dissociation protocol of EBs and cell isolation, significantly increased (from 1-1.5% to 3-7% CD31+ cells) the apparent percentage of dissociated CD105+CD31+ cells (H9.2 n=30, I6 n=8, C3 n=12,) and significantly increased the yield of isolated cells with 95% cell viability (Figure 1B and Figure S1 in the online-only Data Supplement). These results (Figures 1C and 1D) imply for the emergence of a CD105+ and/or CD73+CD31- non-endothelial mural subsets during the onset of vasculogenesis in hPSC-derived EBs. Therefore, 14 day-old dissociated EBs were analyzed by flow cytometry using triple labeling with antibodies against CD105 and CD31 with either CD146 or CD73 (Figure 2A). Within CD105+CD31- populations, about 25% co-expressed CD146 and 22% CD73 (Figure 2A), which are also expressed by perivascular cells of blood vessels.5 In addition, CD90+CD31- clusters were observed in 17 day-old EBs, with CD90+CD31- in proximity and aligning the CD31+ vascular network (Figure 2B). The majority of CD90+ cells co-expressed calponin, which is another characteristic marker of perivascular cells (Figure 2C and movie I in the online-only Data Supplement). Of notice, CD73 and CD105 are expressed also by subsets of blood-derived CD45+ hematopoietic cells. However, it is well established that in the course of EBs spontaneous differentiation only very rare events (<0.02%) of CD45+ hematopoietic mature cells are detected10 (Figure S2 in the online-only Data Supplement) and the induction of hematopoietic
lineages differentiation requires further maturation of CD34+/ACE+ progenitor cells on specific cell feeders. Altogether these results demonstrate that a subset of non-endothelial/non-hematopoietic CD105−CD31− spontaneously emerge in the course of vasculogenesis in differentiating hPSC and may represent a perivascular progenitor cell population with potential vasculogenic properties.

Characterization of Pericyte-Like Progenitor Cells

Next, CD105+ cells were isolated from 13-19 day-old EBs for further cultivation. Within 1-2 passages, CD105+CD31− subset dominated the mixed CD105+ vasculogenic cultures (Figure S3 in the online-only Data Supplement) exhibiting loosen cell-cell contacts in a multilayered hill-and-valley morphology (Figure S3 in the online-only Data Supplement), which is typical of cultured pericytes as well as SMC. Both hESC and hIPSC-pericytes exhibited similar growth potential in long term cultures up to 8 passages for 8 weeks, with a population doubling time (PDT) of 108 hours between weeks 1-2 and a faster growth rate between weeks 3-5, at which the PDT was about 60 hr. After 7 weeks of culture, pericytes gradually entered hypertrophic senescence (Figure 2D). Contact inhibition was not observable and adherent spherical clusters of multilayered cells were detached from the plastic at high densities (data not shown).

CD105−CD31− pericyte-like cells could be efficiently expanded: up to 2900, 5000 and 4200-fold increase for H9.2, I6 and C3 respectively (Figure 2E). To examine their clonogenic potential, sorted CD105−CD31− cells were cultured, pre-labeled with the CellTracker CM-DiI (Figure 2F), cloned (passages 2-4) by limiting dilution in Terasaki plates and scored 24 hours post seeding for the efficiency of single cell seeding (Figures 2F and 2G). One week later, 16.5±1.1% of H9.2, 18.5±2.7% of I6 and 16.8±2.7% of C3 single adhesive pericytes formed colonies (Figure 2H).
with similar pericytic morphology (Figure 2F). Long-term cultured pericytes exhibited a uniform and high expression of a comprehensive set of markers characteristic of perivascular and MSC including maintenance of CD105 expression in long-term culture, as well as CD73, CD29, CD44, CD90 and CD146 (Figure 3A). From the first passage to senescence the majority of pericytes were negative for α-SMA, with only 6%-8.5% α-SMA+ cells throughout the culture period (Figure 3B). Similar to adult-derived pericytes and MSC, isolated and cultured CD105+CD31- cells did not express CD56 and E-cadherin or specific markers of endothelial (CD34, CD31, vW Factor, VE-Cadherin) or hematopoietic (CD45, CD14) cells (Figure 3A and data not shown). In addition, PCR analyses of sorted CD105+CD31- and CD105+CD31+ subpopulations, endogenous placenta-derived pericytes and hPSC-vascular derivatives at early and late passages, demonstrated co-expression of markers indicative of perivascular/MSC phenotype in isolated and cultured CD105+CD31- cells, with CD31 expression only in endothelial hPSC-derivatives (Figure 3C). In accordance with their perivascular cell-like appearance, and similarly to human placenta CD146+CD31- pericytes, hPSC-pericytes were immuno-labeled for recognized pericytic markers including NG2, PDGFR-β, Calponin and nestin (Figure 3D). Furthermore cultivated pericytes expressed Tei-2 (Figures 3D-X and 3D-XI) and fibroblast specific protein-1 (fsp-1) (Figure 3D-XII), which is expressed by myofibroblasts and cardiac mesenchymal derivatives of trans-differentiating EC.25,26

**CD105+CD31- hPSC-Pericytes and Endothelial Cells Assemble to Generate Human Vascular Network**

The vasculogenic potential of the pericytes was initially evaluated in vitro. Although tube formation on Matrigel is a common feature of EC, it has been previously demonstrated that fetal
tissue pericytes can actively create tubular networks. In accordance, hPSC-pericytes formed short, intricate tubular structures on Matrigel within 2 hours (Figure 4A), while HUVEC (data not shown) or hPSC-derived CD31+ EC re-arranged in linear, asymmetric tube networks (Figure 4A). When CFSE-labeled pericytes were co-cultured with H9.2 hESC CD31+ EC on Matrigel, both cell-types were assembled to form thicker tubular networks (Figure 4B). It has been previously demonstrated that the presence of murine or human adult tissue derived mesenchymal precursors is essential for de novo generation and stabilization of vascular networks using immunodeficient murine experimental models. Therefore, the vasculogenic potential of CD105+CD31- pericytes was studied in vivo by the Matrigel implant assay. Pericytes and EC, either HUVEC or hPSC-EC, were mixed in Matrigel and injected subcutaneously into immunodeficient NOD/SCID mice. In accordance with previous studies, Hematoxylin/Eosin staining revealed that empty control Matrigel implants (Figure 4C-I), implants with HUVEC (data not shown) or hPSC-CD31+ cells only (Figure 4C-II), did not contain microvessels. Implants with only pericytes induced some infiltration of murine vessels (Figure 4C-III). However, when pericytes were mixed (1:1) with either HUVEC (Figure 4C-IV) or hPSC-EC (Figure 4C-V), and implanted, a robust generation of evenly distributed vascular network was detected inside the implant (Figures 4C-4E). The presence of luminal murine erythrocytes and nucleated leukocytes (Figures 4C-IV, 4C-V and 4D) suggests that the newly formed human blood vessels anatomized with the host vasculature and were perfused with murine blood within 1 week. Human MHC Class I+CD31- pericytes (Figure 4D and Figure S5 in the online-only Data Supplement) assembled into luminal structures with human endothelial CD31+ or CD34+ EC (specificity of anti-human CD31, CD34 and MHC Class I antibodies is shown in Figure S5 in the online-only Data Supplement), which co-expressed vW factor (Figure 4D-IV and Figure S5 in
the online-only Data Supplement). Pericytes were identified in proximity and aligning CD31+/CD34+ human vascular structures (Figure 4D). Of notice, efficient generation of chimeric human vasculogenic structures was detected even when the implants were devoid of administration of angiogenic factors such as vascular endothelial growth factor (VEGF) or/and bFGF. In addition, implantation within Matrigel, induced α-SMA expression by implanted pericytes, demonstrating their ability to further differentiate in vivo toward SMC-like perivascular cells and implying for their more their immature phenotype in culture as SMC precursors (Figure 4F). Altogether these findings support the notion that pericytes and SMC share a common progenitor as was previously demonstrated using quail-to-chick-transplantation11 and mouse experimental models.31 The ability of pericytes to recruit murine blood vessels and to support the formation as well as stabilization of the newly formed vascular network can be attributed by part to the secretion of VEGF, which is a key regulator of angiogenesis and vasculogenesis as well as EC survival.32 In conformity, secreted VEGF165 was detected by ELISA in the conditioned medium of cultivated pericytes (Figure 4G).

**Durability of hPSC-Pericyte Based Vasculature**

To further evaluate the engineered vasculature, luciferase-labeled hPSC-pericytes were implanted in the presence or absence of HUVEC in Matrigel implants. Perfusion of the implants was then monitored at days 1, 7 and 14 using luciferase-based live imaging system. While strong bioluminescence signal was detected in all implants at day 1 (Figure 5A), pericytes survived up to 14 days post implantation only in implants in which HUVEC were co-implanted (Figure 5A). Matched histological data (Figure 5B) confirmed that the survival of luciferase+-pericyte in vivo correlated with significant and durable perfusion of the implants by de novo formation of human
blood vessels (Figure 5B), which were stabilized by implanted pericytes. It appears that pericytes undergo a process of remodeling, characterized by reduction in total cellularity and viability of the pericytes, which are not incorporated into engineered blood vessels. In addition, functionality of CM-DiI pre-labeled hESC I6 vascular derivatives (Figure 5C) in Matrigel implants and anastomosis with murine circulation was examined after 7 days by intravenous administration of the human specific EC binding lectin, UEA-1-FITC (Figure 5D), which was incorporated into the human blood vessels (Figure 5E). Confocal microscopy slicing through blood vessel structures revealed nestin⁺ pericytes (Figure 5F) partially aligning CM-Dil⁺ (Figure 5G) UEA-1-FITC⁺ (Figure 5H and 5I and movie II in the online-only Data Supplement) hPSC-EC.

Altogether these results demonstrate the hPSC-pericytes are crucial for rapid perfusion of the implants via a progressive process of vascular remodeling.

**Human hPSC-Pericytes are Multipotent Clonogenic MSC**

A perivascular origin for MSC was previously demonstrated in multiple human organs, which harbor a reserve of multipotent progenitor cells. This notion, together with the expression of recognized MSC markers (Figure 3) and the vasculogenic functionality of hPSC-pericytes (Figures 4 and 5) imply that these cells might have a developmental potential equivalent to MSC. Therefore, cultured pericytes were analyzed for their ability to form mesenchymal tissues, including bone, fat, cartilage and muscle. Upon appropriate inductive experimental conditions, hPSC-pericytes (H9.2 and C3) exhibited uniform and efficient osteogenic appearance, with robust mineral and calcium depositions (Figures 6A and 6B). Following short (3 days) or long (14 days) osteogenic stimulation in vitro, differentiated pericytes were subcutaneously implanted within Matrigel into NOD/SCID mice for assessment of ectopic bone formation. Examination of
the implants 2 weeks later by staining for Hematoxylin/Eosin or Alizarin red revealed the presence of mineral deposits produced by 3 days stimulated hIPSC-pericytes (Figures 6C and 6D) with dramatic increase in mineral deposit-content in implants of 14 days stimulated pericytes (Figures 6E and 6F). In addition, more mature bone-like structures and erythrocyte containing blood vessels could be seen in implants of 14 days stimulated pericytes (Figure 6E). Furthermore, pericytes were capable of adipogenic (Figures 6G and 6H) and chondrogenic (Figures 6I and 6J) differentiation in vitro. High efficiency (>95%) adipogenic differentiation and whole cell osteogenic differentiation (Figure S6 in the online-only Data Supplement) were documented from passage 1 to passage 8 until senescence, designating that our culture conditions were sufficient for stabilization and maintenance of the multilineage features of expanded hPSC-CD105+/CD73+CD31- pericytic-precursors. After myogenic induction in vitro, skeletal myotubes could be detected (Figure 6K and 6L and Figure S7 in the online-only Data Supplement), with the fraction of differentiated hPSC-pericytes, expressing characteristic skeletal markers similarly to human-derived skeletal muscle (Figures 6K and 6M). Expandable single-cell derived clones (Figure 6N), were further sub-divided to give rise to tripotent osteogenic-chondrogenic-adipogenic pericytes (Figure 6O), of which 16±0.5, 14±0.5 and 11±3 of H9.2, I6 and C3 respectively, exhibiting myogenic differentiation (Figures 6N and 6O).

Transplantation of hPSC-Pericytes Promotes Vascular and Muscle Recovery in Ischemic Limbs of Transplanted Mice

To determine the effectiveness of hPSC-pericytes on vascular and muscle recovery, 2x10^6 hPSC-pericytes (H9.2, I6 and C3) or unconditioned medium were injected intramuscularly into ischemic hind limb of immunodeficient CD1 nude mice. Representative laser Doppler perfusion
images are shown weekly to document the recovery of perfusion (Figure 7A). Within 21 days untreated control mice showed about 2 fold endogenous recovery (Figure 7B), which was sufficient to prevent untoward morbidity or limb loss. In comparison, a significantly higher (about 4 fold) and accelerated recovery of foot perfusion was measured in pericytes treated group (Figure 7B and Figure S8 in the online-only Data Supplement). Histology of ischemic, non-ischemic and healthy muscle from mice, which were not subjected to limb ischemia, demonstrated that perfused blood vessel density was significantly (p<0.001) higher in pericyte treated ischemic mice compared to control group and compared to healthy muscle (Figure 7C and Figure S8 in the online-only Data Supplement). At day 21 post injection, CM-DiI labeled pericytes were still detected incorporated into recovered muscle in ischemic limb but not in non-ischemic or untreated ischemic limb (Figure 7D). Increased positive immunostaining for desmin revealed that muscle regeneration was more pronounced in hPSC-pericytes treated group compared to control group (Figures 7D and 7E). In addition, double staining of CM-DiI+ desmin+ of myotube incorporated pericytes demonstrate their ability to contribute to myogenesis via direct myogenic differentiation (Figure 7F). Recruited CM-DiI+ pericytes maintained their perivascular features in ischemic tissues and were also found incorporated into murine blood vessels (Figure 7G). These data confirm that transplanted hPSC-pericytes directly incorporate into host ischemic muscle and recovered vasculature to promote improvement in perfusion and muscle recovery.

Discussion

We have demonstrated that pericytic-progenitor cells can be isolated from hPSC, including hESC and hIPSC, to be further efficiently expanded in long-term cultures, maintaining stable
perivascular features and multilineage potential. Our protocol is based on 3D scalable (either hESC\textsuperscript{33} or EBs\textsuperscript{34}) feeder free differentiation platforms. In addition, we show that co-implantation of a single source-derived EC and pericytic-progenitor cells in Matrigel into immunodeficient mice resulted in rapid formation of robust vascular network, which anatomized the host circulation. Pericytes, immediately adjacent to the vascular endothelium of both arteries and veins, are not in themselves SMC. They do, however, share angiogenic and regulation of blood vessel properties.\textsuperscript{3,35} Similarly, we have demonstrated here that hPSC-pericytes were found to reside around engineered blood vessels and were essential for the de novo formation of these blood vessels. These findings are consistent with previous reports showing that interactions between EC and murine perivascular cells are essential for development of vascular networks in vivo. In previous models, either murine embryonic cells line 10T1/2\textsuperscript{14,36} or murine myofibroblasts\textsuperscript{22} were used as perivascular cells. However, neither source is useful for clinical applications. Although protocols for generation of perivascular hESC-derived SMC, with therapeutic effectiveness in limb and cardiac ischemic experimental models were previously described\textsuperscript{9,22,37} it is not clear whether sufficient numbers of SMC with stable phenotype can be achieved in long term culture as a fundamental need for potential usage in regenerative medicine. In addition, generation of mature functional SMC on murine OP9 feeder, in 2D differentiation system, was previously described\textsuperscript{15}, however it is not clear whether OP9 induced differentiation result in generation of multipotent pericyte-like perivascular cells or directly induce the production of terminally differentiated SMC via another mechanism of cell commitment. Furthermore OP9 feeder also induces the differentiation of hematopoietic CD105\textsuperscript{+}CD45\textsuperscript{+} or CD73\textsuperscript{+}CD45\textsuperscript{+} from hPSC-derived progenitor cells\textsuperscript{23,38}, making this and similar 2D differentiation protocols unsuitable for our CD105/CD73 based isolation approach.
In our cultures, pericytes were rapidly and robustly expanded and could further gain SMC-phenotype in vivo, when implanted together with EC in Matrigel, while uniformly maintaining multi-lineage potential throughout the whole culture period. Similarly, human bone marrow-derived adult MSC gained mature SMC phenotype when implanted with cord blood-derived EC and could be efficiently expanded, however the differentiation potential of expanded MSC is significantly reduced with increasing cell passages and is donor-age restricted, due to heterogeneous deterministic hierarchy and acquired in vitro cues in long term cultures.

Protocols for induction of differentiation in serum free cultures and sequential activation by various growth factors enable the generation of either mesodermal progenitor cell or EC. Further studies are required to evaluate whether culture conditions, in particular differentiation in 2D induced and defined cultures, which favors the emergence and stabilization of particular derivatives affect the specification of pericytes in developing hPSC. For example, while blockade of TGF-β signaling at initial developmental stages of hPSC induce neural conversion inhibition of TGF-β signaling following specification of mesodermal progenitor cells contributes to generation of EC. However, TGF-β signaling interfere with the maturation as well as stabilization of vascular phenotype of hPSC-endothelial progenitor cells and was shown to be involved in cell differentiation toward α-SMA positive SMC. Interestingly, CD34^+NG2^+PDGFR-β^+CD31^ perivascular progenitor cells were detected in human saphenous veins. Following isolation, CD34^+ cells differentiated in vitro into multipotent pericytes, while losing CD34 expression. These findings coincide with alternative pathway for derivation of perivascular EC or SMC from hESC, in which CD34^+ progenitor cells are further induced to differentiate into these vascular derivatives. Saphenous vein-derived CD34^+ cells may therefore represent progenitor intermediates of differentiating CD34^+ during induced differentiation.
commitment toward the perivascular lineage in native tissue and in the course of hESC differentiation. In view of the fact that implantation of either HUVEC, blood-derived EC or as demonstrated here, hPSC-derived EC is insufficient for the formation of vascular network in the absence of supporting perivascular cells, the effort to achieve large numbers of EC from hPSC should be accompanied with the ability to gain sufficient numbers of perivascular cells, including SMC or pericytes.

Previous attempts to generate perivascular cells from hESC resulted in generation of \( \alpha \)-SMA positive SMC.\( ^{9,14,17,22,37} \) Intact SMC can be distinguished from pericytes in fetal or adult tissues based on a combination of cell markers, cell morphology and the size of the blood vessels in their tissue of origin. However, in differentiating hPSC, visualization of pericytes cannot be based on correlation between their anatomical localization in physiological association with vascular endothelium together with expression of a combination of cell markers (e.g. CD146, NG2, CD90) and the absence of others (e.g. CD31, CD56)\(^5\) due to non-organized immature-vascular appearance in developing hPSC.\(^9\) In addition, MSC and pericytes share common features of multilineage progenitors,\(^7\) but exhibit distinct characteristics as well. Therefore, a comprehensive set of pericytic markers, morphology in culture as well as perivascular and MSC features should be demonstrated in order to define the hPSC-pericyte equivalents. Generation of MSC from hESC was previously demonstrated using two distinguished protocols involving either neural crest progenitor cell CD73\(^+\) progeny\(^{45}\) or CD73\(^+\) mesoendodermal precursors,\(^{46}\) with the neural crest progenitor-derived CD73\(^+\) precursors differing from the mesodermal intermediates in their inability to give rise to skeletal muscle progeny.\(^{45,46}\) However, several reports demonstrate that cranial and cephalic neural crest give rise to pericytes and smooth and skeletal muscle cells in vivo.\(^{31,47}\) We report here that hPSC-pericytes exhibited myogenic
differentiation in vitro and in vivo as well as characteristic perivascular morphology in cultures, with hill-and-valley morphology and no contact inhibition. In addition the lack of \( \alpha \)-SMA expression distinguishes the hPSC-pericytes from the \( \alpha \)-SMA positive mesodermal progenitor cells, which were derived by different protocols,\(^{46,48} \) and suggest that these intermediates represent a more primitive perivascular precursors, with MSC definitive characteristics. In addition, the type and the time course of emergence of hPSC-cell intermediates are largely depend on the dynamic or induced environmental cues. For example, paraxial mesodermal-derived CD73\(^+\) MSC were generated by long-term differentiation of hESC in 2D culture,\(^{46} \) wherein alternative protocol and experimental conditions was used to generate CD73\(^+\) MSC, this time from isolated neural crest progenitor cells.\(^{45} \) Furthermore, in the course of vertebrate valvulogenesis in the developing heart, endocardial cells undergo endothelial to mesenchymal transition in a VEGF, Notch and TGF-\( \beta \) dependent manner.\(^{49} \) NFATc1\(^+\) endocardial subset was also identified in differentiating murine EBs\(^{50} \) implying that endothelial intermediates in differentiating hPSC can serve as alternative source for pericytes. Supporting this notion, transcripts and protein expression of the endothelial to mesenchymal transition marker, fsp-1,\(^{25,26} \) were detected in cultured hPSC-pericytes. It would be interesting in future studies to identify the developmental origin of hPSC-pericytes based on germ line specific markers and cell tracing in developing hPSC.

We demonstrate here that transplantation of hPSC-pericytes significantly attenuated critical limb ischemia. A physiological role of hPSC-pericytes in various injury and regeneration models remains, however, to be further demonstrated. Currently, the endothelial and perivascular derivatives, which are co-derived from a single source of hPSC, can be utilized by in vitro models for drug screening, biological studies of pericyte-endothelial interactions and for
engineering of 3D-vascular grafts. Importantly, given that our protocol allows efficient generation of pericytes from hIPSC, it can be applied for isolation of pericytic-precursors from hIPSC with inherited genetic mutations for evaluation of a variety of pericyte degree of plasticity or dysfunctions. Considering the shortage in sources for uniform cultures of pericytes, the complex ontogeny of pericytes in animal experimental models and the elusive origin of human pericytes, the usage of developing hPSC as a human in vitro model for pericytes identification, isolation and expansion can highly contribute to studies of development and origin of pericytes and can be essential for improvement of differentiation protocols, generation of vascular constructs, disease modeling and development of strategies in regenerative medicine.

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**Conflict of Interest Disclosures:** None.

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**Figure Legends:**

**Figure 1.** Expression of vasculogenic markers on developing hPSC-derived EBs. (A) Quantitative-PCR gene expression analyses of CD105, CD73 and VE-Cadherin in developing H9.2 and C3-derived EBs. The average expression normalized to GAPDH is shown. (B) Representative flow cytometric analyses for CD105 and CD31 expression pattern in different aged hPSC-derived EBs. (C-D) Time course of emergence of CD105+//-CD31+ (C) or CD73+/CD31+ (D) endothelial populations and non-endothelial CD105-CD31- (C) CD73-CD31- (D) subsets in developing EBs. Data are mean±S.D. of at least 3 independent experiments per time point.

**Figure 2.** Identification and characterization of emerging perivascular populations. (A-B) Representative flow cytometry analyses implying for the spontaneous emergence of CD105-CD31-CD73+/CD146+ pericytic population in 14 days old, I6-derived EBs. (B-C) Immunostaining of 17 days old EBs (hPSC, KTR13). (B) Vascular-like network formation by CD31+ (red) EC is accompanied by the appearance of CD90+ (green) cell clusters. (C) CD90 (green) and calponin (red) expression display similar patterns with co-expression at single cell level. (D) Expansion of hPSC-CD105+ pericytes during 8 weeks of culture. (E) Fold increase in hPSC-pericytes numbers until senescence relative to 50,000 pericytes input at day 0. Data are mean ± s.e.m. of at least 3 independent experiments. (F-H) Clonogenic assay and clonal efficiency of sorted CD31-CD105+ hPSC-pericytes (passage 2-4). (F) Pericytes were detected
with CellTracker-CM-DiI (red) during limiting dilution. A representative fluorescence image and corresponding phase-contrast image of single adhesive pericyte, giving rise to pericytic-clone. (G) Efficiency of plating clones 24 hours post cell seeding. (H) Colony forming efficiency of single adherent pericyte 7 days post seeding. Data are mean±SEM of n=7-8 microplates of 3 independent experiments per each hPSC line.

**Figure 3.** Characteristics of long-term cultivated hPSC-pericytes. (A) Flow cytometry analyses of marker expression profiles of pericytes. Filled histograms: isotype-matched IgG controls. ND=not detected. (B) Representative dot plot analyses of α-SMA expression in cultivated hPSC-pericytes. (H9.2: passage 4, I6: passage 5, C3: passage 7). (C) PCR analyses for characteristic messengers of vasculogenic and MSC of sorted CD105 expressing subpopulations and pericytes. (D) Immunofluorescence stainings of full term human placenta or hPSC-pericytes. Nuclear staining by DAPI (blue). Data are representative of cultured hPSC-pericytes between passages 1-8 (until senescence).

**Figure 4.** Formation of vascular networks by hPSC-pericytes and EC. (A) Tube formation on Matrigel by hPSC-pericytes (upper panel) or hPSC-EC (lower panel). (B) A mixture (1:1) of hPSC-EC (CD31+, red) and pericytes (CFSE-labeled, green) assemble to form thick tube network on Matrigel. Nuclei were labeled by DAPI (blue). (C) Hematoxylin/Eosin staining of Matrigel implants (n=4 mice per group) after 7 days. (C-I) Control empty Matrigel, (C-II) hPSC-pericytes induced infiltration of few murine blood vessels (arrows). (C-III) hPSC-EC rearranged in elongated empty cord structures. (C-IV-C-V) Robust vascular network formation, in the presence of hPSC-pericytes. (D) Specific localization of hPSC-EC and pericytes in the vascular
network. Immunolabeling of cultured pericytes with anti-human MHC Class I (red, D-I) or implanted hPSC-pericytes (green, D-II, D-III, D-V). (D-II-V) Human vasculature composed of hPSC-pericytes and CD31⁺ or CD34⁺ human EC (red). (D-IV-D-V) Representative higher magnification image of newly formed human blood vessel. CD31⁺MHC Class I⁺ (green), pericytes tightly cover underlying CD31⁺MHC Class I⁺, (red and green spotted) EC (D-V, inset). Unnucleated erythrocytes are packed within blood vessel lumens (D-IV-D-V, asterisk) indicating that human blood vessels anatomized the murine circulation. (E) Quantification of human CD31⁺ (H9.2 and C3) or UEA-1⁺ (I6), erythrocytes containing blood vessels. Each bar represents 4 implants at day 7 post implantation. (F) hPSC-pericytes (H9.2, passage 6) in Matrigel implant further differentiate to express α-SMA⁺ (red) within one week. (G) Concentrations of VEGF₁₆₅ in conditioned medium of cultivated hPSC-pericytes.

**Figure 5.** Anastomosis and durability of the human vascular network. (A-B) Matrigel implants containing either luciferase labeled hPSC-pericytes (Luc⁺) alone or mixed with HUVEC (1:1). Mice were imaged using an IVIS Imaging System. Bioluminescence was recorded 30 minutes after intraperitoneal injection of luciferin 1 day post implantation and retained up to 2 weeks only in mixed implants (A). Matched Hematoxylin/Eosin staining reveals that viability of Luc⁺ pericytes was correlated with blood vessel density (BV/mm²). ND=not detected. Bioluminescence was not detected in mice with empty Matrigel implants or implants containing unlabeled hPSC-pericytes or HUVEC. N=4 implants per time point. (C-I) Representative images of Matrigel implants containing hESC-I6 derived, CM-DiI-labeled (red, C, G) EC and pericytes. Human specific endothelial binding UEA-1-FITC lectin (green, D, H) was injected i.v. just before death at day 7 and denotes for functional vessels (yellow, E, I). Confocal microscope
images of nestin expressing pericytes (white, F. Pink arrows, I) aligning UEA-1 perfused human vessels (yellow arrows, I). Nuclear staining by DAPI (blue, I).

**Figure 6.** Multilineage differentiation of clonogenic hPSC-pericytes. (A) Osteogenic differentiation is seen accompanied by mineral production (black granules). (B) Calcium production is detected by alizarin red staining. (C-F) Implantation of osteogenic pericytes stimulated for 3 (C, D) or 14 days (E, F), in Matrigel into NOD/SCID mice. (C, E) Hematoxylin/Eosin. (D, F) Alizarin red. (E, arrows) Murine blood vessels. Newly formed bone-like matrix (E, Inset). (G-J) Induced adipogenic (G, H, Oil) and chondrogenic (I, J, Alcian blue) differentiation of hESC (G, I) and hPSC- (H, J) pericytes. (K-M) Multinucleated myotubes of myogenically induced hPSC-pericytes (K-L) are positive for characteristic skeletal markers (K: immunofluorescence, M: Quantitative-PCR). (N-O) Expandable single cell-derived pericytic clones (N) are all tripotent osteo-adipo-chondrogenic (O) with part exhibiting also myogenic capacity (N, O).

**Figure 7.** Transplanted hPSC-pericytes improved perfusion, blood vessel density and muscle regeneration in ischemic limb. (A) Representative laser Doppler images following femoral artery ligation and intra-muscle injection of unconditioned M-199 medium or 2x10^6 hPSC-pericytes (H9.2, I6 and C3, n=7 mice) monitored at the indicated time points. (B-C) Significant improvement in blood perfusion (B, *P<0.05, **P<0.001 compared to control mice at the same time point. #P<0.05 and ##P<0.0005 compared to Day 0) and in density of perfused blood vessels at day 21 (C, *P<0.05 and **P<0.001 compared to treated mice, #P<0.001 compared to non-ischemic mice) was measured for all hPSC-pericytes treated mice in comparison to control.
mice. Data are mean±SEM. (D-G) hPSC-pericytes are recruited to the recovered ischemic muscle. Representative images of myogenesis evaluated with desmin stain (green, D-F) at day 21 in either control ischemic muscle (D, low expression) and ischemic muscle treated with CM-DiI (red, E-G) labeled hPSC-pericytes (E, F, high expression), which are seen incorporated into recovered myofibers. (G) CM-DiI (red) labeled pericytes aligning (arrowheads and inset) vWF-Factor⁺ (green) EC of perfused blood vessel or incorporated into the ischemic muscle. Nuclear staining by DAPI (blue).
Multipotent Vasculogenic Pericytes from Human Pluripotent Stem Cells Promote Recovery of Murine Ischemic Limb

Ayelet Dar, Hagit Domev, Oren Ben-Yosef, Maty Tzukerman, Naama Zeevi-Levin, Atara Novak, Igal Germanguz, Michal Amit and Joseph Itskovitz-Eldor

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SUPPLEMENTAL MATERIAL

Supplementary Methods

Culture and Differentiation of hPSC

Human ESC H9.2 (passages 29+36-60), I6 (passages 50-71) or human foreskin fibroblasts-derived IPSC clone C3 (passages 17-38) and human hair follicle keratinocyte-derived IPSC clone KTR13 (passages 30-49) were grown on mitomycin C (1 mg/ml) inactivated mouse embryonic fibroblasts (MEF) in hESC culture medium consisting of advanced DMEM/F12 (Biological Industries, Biet HaEmek, Israel) supplemented with 20% knockout serum replacement (GIBCO™ Knockout™ Serum Replacement), 1× non-essential amino acids (Gibco), 1× l-glutamine (Invitrogen), 1× β-mercaptoethanol (Gibco) and 4 ng/ml FGF-2 (R&D systems) without antibiotics.

To induce spontaneous EBs formation and differentiation, hPSC were removed from MEF feeder by 0.2% Collagenase IV (Warthington) and were suspended in low attachment culture dishes in differentiating medium consisting of DMEM, 20% FBS, 1× non-essential amino acids, 1× l-glutamine, 1× β-mercaptoethanol without antibiotics. Culture medium was changed every 3 days.

EB Dissociation Protocols

Single cell suspensions were achieved by incubation of EBs for 20 minutes at 37°C on shaker with either (1) 0.5% Trypsin/EDTA (Sigma), (2) non-enzymatic solution (Sigma) or (3) 1 mg/ml collagenase B (Roche) and 150 U/ml DNAsce I (Roche), followed by addition of Trypsin-EDTA (0.05%) for another 5 minutes. To minimize cell aggregation the dissociated EBs were then passed several times through a 25-Gauge needle and filtered through PBS/0.5% FBS pre-washed 0.45µm cell-strainers (BD Biosciences). Cell viability was determined by Trypan Blue assay. The
percentages of live CD31⁺ endothelial progenitor cells (Figure I in the online-only Data Supplement) or CD105⁺ cells were analyzed by flow cytometry upon dissociation with Colagenase B (≥95% viability).

**Cell Cultures**

Pericyte cultures were generated either by depletion of CD31⁺CD105⁺ endothelial cells at passage 0-1, domination of pericytes in culture within 1-2 passages or selection of pericytic colonies by cloning rings. CD31⁺CD105⁺ isolated endothelial progenitor cells or CD105⁺CD31⁻ pericytes were seeded on human fibronectin (Millipore, Billerica, MA) coated culture dishes and cultured in either EBM-2 (Lonza) or EC M-199 media containing 20% inactivated-FBS (HyClone, Utah, USA), 1% Pen-Strep, 1% l-glutamine, 1mM HEPES (Biological Industries) 20 U/ml heparin (Sigma-Aldrich) and 50 µg/ml endothelial cell growth supplement (ECGS) (Biomedical Technologies Inc., Stoughton, MA). All hPSC-derived pericytes in this study are of polyclonal origin. Isolated CD31⁺ cells were either grown in EC M-199 media or in EBM-2 with supplements (Lonza, Walkerville, MD, USA) on 0.1% gelatin or human fibronectin (25 µg/ml) coated dishes. In some experiments 10µM SB431542 (Tocris Biosciences, Bristol, UK) was added to EC M-199 growth media for expansion of endothelial cells. EB-derived CD105⁺ cells were grown on human fibronectin (25 µg/ml) coated dishes only at passage 0 (immediately post isolation to passage 1).

**Isolation and Cultivation of Placenta-Derived Pericytes**

Full term human placenta tissues were obtained from healthy donors following informed consent (n=6). Tissue villi fragments were dissociated with collagenase type
I, IV and V and dispase (1 mg/ml HBSS of each) for 30 minutes at 37°C on rotating device. Trypsin was then added for additional 10 minutes. Dissociated cells and tissue fragments were washed and seeded onto gelatin coated flasks in M-199 supplemented with 20% FBS, 20 U/ml heparin (Sigma-Aldrich) and 50 µg/ml endothelial cell growth supplement (ECGS) (Biomedical Technologies Inc., Stoughton, MA). Confluent monolayers were removed by Trypsin and CD146⁺ pericytes were then isolated by magnetic bead separation (Miltenyi Biotec) and further expanded in M-199 growth medium.

**Flow Cytometry**

At indicated time points, differentiated EBs were dissociated with Collagenase B and DNAse I as described above, washed in PBS and labeled with indicated combinations of coupled antibodies. Cultured hPSC-derived pericytes were labeled between passages 0-8. Antibodies were used according to the manufacturer instructions and are listed in Supplemental Table S1. Analyses were carried out using Facscalibur flow cytometer and CELLQuest program (Becton Dickinson).

**Immunocytochemistry and Immunohistochemistry**

For fluorescence microscopy, cells or EBs were fixed with 4% paraformaldehyde, washed 3 times with PBS and permeabilized with PBS 0.1% Triton X-100 (Sigma) for 10 minutes and immuno-labeled. For evaluations of implanted cells, at the indicated time points Matrigel implants were harvested and fixed in 4% formalin (BioLab LTD). Sections were labeled with primary antibodies O.N. at 4°C and with secondary antibodies for 1 hour at R.T. Nuclei labeling with DAPI (40, 6-diamino-2-phenylindole dihydrochloride, Molecular Probes, 1:1000) for 5 min at RT, and an
isotype-matched negative control or irrelevant isotype-matched antibody (e.g. mouse anti-human CD45, Dako) was performed with each immunostaining. See supplemental Table S1 for a list of antibodies. FITC-conjugated Ulex europaeus lectin (UEA-1-FITC, Sigma, 1:200,) and labeled Dil-Ac-LDL (Biomedical Technologies Inc, 5 µg/ml) were used to identify of endothelial cells. CFSE and CM-DiI (Molecular Probes) labeling was performed according to the manufacturer instruction with no effect on pericyte viability and proliferation.

**Pericyte Single Cell Cloning**

To detect single cells during cloning, cultures of CD105⁺CD31⁻ sorted pericytes (passages 2-4) were pre-labeled with CellTracker CM-DiI. After trypsinization, pericytic clones were generated by limiting dilution into a 60-well Terasaki microplates coated with human fibronectin (25µg/ml in PBS), containing 50 µl/well of fresh M-199 growth medium and 75 µl/well of pericyte conditioned medium (conditioned by pericytes for 48 hours and sterile-filtered to remove any suspended cells). Each well was examined with a fluorescent microscope to determine the plating efficiency as the percentage of wells inoculated with a single cell. On day 7 colony forming efficiency was calculated as the percentage of pericytic colonies originating from a single cell, divided by the number of total single adhesive cells at day 1. For differentiation assays proliferating clones were further cultivated until day 10 and then sub-divided (1:5) into gelatin coated 96-well dish and allowed to grow for additional 5-7 days. Single cell-derived pericytic sub-divided clones were then analyzed for osteo-adipo-chondro-myogenic differentiation (up to 14 days of differentiation) compared to non-induced matched control.
**Microvessel Density Analysis**

Microvessels were quantified by evaluation of 10 randomly selected fields of H&E stained, immuno-labeled, or CM-DiI labeled blood vessels in sections taken from various parts of the implants. Microvessels were identified as lumenal structures containing red blood cells and counted. Microvessels density was reported as the average number of microvessels from the fields analyzed and expressed as blood vessels/mm². Values reported for each experimental condition correspond to the average values ± s.e.m obtained from at least four individual mice.

**Quantification of Human VEGF**

Conditioned medium of cultivated hPSC-derived pericytes was collected at intervals of 3-4 days and pericyte numbers were determined. Human VEGF₁₆₅ in conditioned medium was detected by ELISA kit (Peprotech) according to the manufacturer instructions.

**Luciferase Assay**

Luciferase expressing hESC-derived perivascular progenitor cell population was established using the lentiviral infection approach for transgene insertion that enables in vivo vital and longitudinal cell tracking. For this purpose cells were seeded to 80% confluency on gelatin coated plates and infected 3 times using the HIV-based pGreenFire1 Reporter Vector (SBI, CA, USA) that expresses green fluorescent protein and Firefly luciferase from the constitutive CMV promoter and carries the self-cleaving T2A peptide. A mixture of Matrigel and Luciferase-labeled hPSC-derived pericytes (same batch and passage) was prepared and divided into two parts. HUVECs were added to one part. Exactly 300 μl from each mixture were
subcutaneously implanted on the back of 8 weeks old NOD/SCID mice (1 implant/mouse). At various intervals post implantation mice were anesthetized, received intraperitoneal injection of the substrate luciferine and bioluminescence was recorded using IVIS200 imaging system (Xenogen Corporation, Alameda, CA). Collected data analyzed with Live Image 3.0 (Xenogen Corporation).

**Functional Assessment of hESC I6-Derived Vasculature**

To determine angiogenic potential of hESC I6 vascular derivatives in vivo, CD105+ population was isolated from 14-day old EBs. At passage 1, CD31+ endothelial subpopulation was further isolated by magnetic separation (Miltenyi Biotec) for further expansion in EBM-2 with supplements (Lonza, Walkerville, MD, USA). CD105+CD31- pericytes were expanded in M-199 growth medium. At passage 4, I6-derived endothelial and pericytes were pre-labeled with CellTracker CM-DiI, washed thoroughly, mixed with Matrigel, and implanted subcutaneously into 8-weeks old NOD-SCID mice (350 μl). After 1 week, mice were inoculated intravenously with 100 μg/250 μl PBS of UEA-1-FITC (Sigma–Aldrich) to detect the incorporation of human endothelial cells. After 5 minutes, mice were killed and implants were removed for fixation in 4% formalin for 24 hours and then equilibrated for 48h in 30% sucrose, and frozen in OCT. To assess for functional CM-DiI+ human vessels, sections were incubated with DAPI and examined by confocal microscopy (Zeiss LSM 510 Meta) for the presence of vessels co-stained for CM-DiI and UEA-1-FITC. No staining was detected in adjacent murine muscle and adipose tissues, which served as controls.
**Adipogenic Differentiation**

Pericytes were seeded (2x10^5 cell/cm²) on 12-well multidish, in the presence of 0.5mM IBMX, 10µg/ml Insulin, 10^-6M Dexamethasone, and 0.1mM Indomethacin in DMEM/F12 medium containing 10% FBS for up to 4 weeks. Adipogenic differentiation was assessed by Oil Red O staining of lipids.

**Osteogenic Differentiation**

Pericytes were seeded (2x10^4-3x10^4 cell/cm²) on 12-well dish, in the presence of 10mM β-glycerol-phosphate and 0.1µM Dexamethasone in GMEM BHK-21 medium, containing 10% FBS for up to 4 weeks. Cell cultures were assayed for calcium content by Alizarin red staining.

**Chondrogenic Differentiation**

For chondrogenic differentiation, 2x10^5 pericytes were centrifuged (300g) to form a cell pellet, which was grown in the presence of 10ng/ml TGF-β3 in DMEM medium for up to 9 weeks. Pellets were fixed with 4% PFA and embedded in low melting point agarose (1.5%). Sections were stained for Alcian blue.

**Myogenic Differentiation**

Human PSC-derived pericytes were incubated with DMEM supplemented with 2% horse serum for 7 days or expanded in skeletal muscle media (PromoCell, Heidelberg, Germany) and further induced to differentiate into skeletal muscle in differentiation media (PromoCell). Fixed cells were labeled with antibodies against myosin heavy chain, or desmin and MyoD for evaluation of myotubes formation. The mRNA for Calponin, MyoD and NCAM/CD56 was determined by PCR. Relative gene
expression of MyoD, MYF5, Myogenin and GAPDH was measured by Quantitative PCR in comparison to human skeletal muscle mRNA (FirstChoice® Human Total RNA Survey Panel, AM6000, AMBION) and served as positive control.

Supplemental Tables

Table S1. List of antibodies and sources.
Table S2. List of human primers for PCR.

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Total RNAs were isolated using Trizol/Tri-reagent (Invitrogen) and reverse transcribed by the iScript™ cDNA synthesis kit (BIO-RAD). PCR was performed DreamTaq™ Green Master Mix (Fermentas, Ontario, Canada). Primer sequences and product sizes are provided in Table S2.

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Table S3. List of human primers for quantitative PCR (QPCR).
Total RNAs were isolated using Trizol/Tri-reagent (Invitrogen). QPCR analysis was performed in triplicates and normalized by the internal endogenous GAPDH gene expression. The reaction was performed in an ABI Prism 7000 (Applied Biosystems, Warrington, UK) with Power SYBR® Green Master Mix (Applied Biosystems). Analysis was conducted using the Relative Quantification (RQ) study in the Sequence Detection Software (V. 1.2; Applied Biosystems). Primer sequences and product sizes are provided in Table S3.

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<th>Gene Name</th>
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<td>GGCAACAATATCCACTTACCAG</td>
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Figure S1. EBs dissociation with Collagenase B and DNase I increase cell viability and CD31⁺ cell yield. To optimize the dissociation step for obtaining single cell suspensions, three methods of dissociating EBs were tested. These included incubation of EBs for 20 minutes at 37°C on shaker with either (1) 0.5% Trypsin/EDTA (Sigma), (2) non-enzymatic solution (Sigma, catalogue No. C5914) or (3) 1 mg/ml collagenase B and 150 U/ml DNase I (Roche), followed by addition of Trypsin-EDTA (0.05%) for another 5 minutes. To minimize cell aggregation the dissociated EBs were then passed several times through a 20-Gauge needle and filtered through PBS/0.5% FBS pre-washed 0.45 cell-strainers (BD Biosciences). Cell viability of total dissociated cells was determined by Trypan Blue with 50%-60% cell viability by Trypsin or non enzymatic solution dissociation and 95% cell viability by Collagenase B and DNase I dissociation. The percentages of CD31⁺ endothelial progenitor cells (EPC) or CD105⁺ cells were analyzed by flow cytometry upon dissociation.
Figure S2. Differentiation kinetics of c-kit and CD45 on developing human EBs.

Representative flow cytometry dot plot analyses of the expression of c-kit and the mature hematopoietic cell marker CD45 expression at days 4, 8 and 14 of EB spontaneous differentiation. Rare populations of CD45 positive cells are detected at 8 and 14-days old EBs.
Figure S3. Pericytes dominate cultures of isolated EB–derived CD105⁺ cells. CD105⁺ cells were isolated/sorted from dissociated EBs (13-17 days old) and cultured in EC growth media. (A) Gated events represent the percentage of CD31⁺ endothelial cells at the end of the indicated passage. N=5 and 23 independent experiments for H9.2 and C3 respectively. (B) Two distinguished populations are identified at passages 1-2 of expanded isolated CD105⁺ cells: CD31⁺CD105⁺ endothelial cells and CD105⁺CD31⁻ cells. (C) Morphology of cultures post pericyte domination, occurring between passages 2-3.
Figure S4. Cluster formation by hPSC-Derived isolated CD105\(^+\) cells. Sparsely plated isolated CD105\(^+\) cells were scored for colony/cluster formation and expansion at the indicated time points. (A) Colony with cobblestone-like morphology of endothelial cells, capable of Dil-ac-LDL uptake (red). (B) Mixed colony of CD31\(^+\) (red) endothelial cells, CD31-\(\alpha\)-SMA\(^+\) (green) SMC and CD31\(^+\)\(\alpha\)-SMA\(^+\) cells. (C) Pericyte-like colony exhibiting a typical hill-and-valley morphology, with only few \(\alpha\)-SMA\(^+\) cells (red). N=12 independent experiments for H9.2 hESC and n=4 independent experiments for hiPSC C3. Nuclear staining by DAPI (blue).
Figure S5. Specificity of anti-human antibodies for detection of implanted human cells in murine tissues. Matrigel implants were removed after 1 week, sectioned and labeled with either (A) mouse anti-human CD31 and rabbit anti-human MHC class I (B) mouse anti-human CD34 and rabbit anti-human MHC class I (C) mouse anti-human CD31 and rabbit anti-human and mouse vW factor. Antibodies against CD31, CD34 (red, A, B) and MHC class I (green, A, B) specifically labeled only human cells within the implant (broken white line defines the edge of MHC class I positive implant) border and not murine blood vessels and adjacent tissues (A, B) while anti-vW factor labeled both human and murine vascular structures within the implants (C). BV=blood vessel. Nuclear staining by DAPI (blue).
Figure S6. Differentiation potential of long-term cultured hPSC-derived pericytes. Cultivated hPSC-derived pericytes were cultured in either adipogenic medium (B-D) or osteogenic medium (E-H) at passage 1 (B, F), passage 5 (C, G) and before entering senescence at passage 8 (D, H). Efficient adipogenic differentiation is indicated by positive staining of lipids by Oil Red (B-D) and robust osteogenic differentiation is seen by Alizarin-red positive calcium deposits (F-H). Representative images of matched controls (A and E for adipogenic and osteogenic differentiation in accordance) consisted of pericytes cultures in differentiating medium without supplements.
Figure S7. Characterization of hPSC-derived skeletal myotubes. (A) Representative PCR analysis for myogenic genes of hPSC-derived pericytes. The PCR product represent the fraction of skeletal differentiated pericytes out of total cells after myogenic induction in vitro (2% horse serum containing media). (B) Immunofluorescent staining of multinucleated myotubes (left panel: hESC H9.2-derived. Right panel: hIPSC C3-derived) for smooth muscle myosin heavy chain (red). Nuclear staining by DAPI (blue).
Figure S8. Improvement of perfusion and blood vessel density in murine ischemic limb by transplanted pericytes derived from all PSC lines. (A-C) Significant improvement in blood perfusion (*P<0.05, **P<0.001 compared to control mice at the same time point. #P<0.05 and ##P<0.01 compared to Day 0) and (D) in density of perfused blood vessels at day 21 (*P<0.05 and **P<0.001 compared to treated mice, #P<0.001 compared to non-ischemic mice) was measured for all hPSC-derived pericytes treated mice in comparison to control mice. Data are mean±SEM.
**Supplemental movie I: Three-dimensional reconstruction of CD90\(^+\)Calponin\(^+\) pericytes in a developing EB.** Z-stack of confocal images of CD90\(^+\) (green) Calponin\(^+\) (red) pericytes in hIPSC KTR13 17-days old EB. Co-expression of CD90 and Calponin is indicated by yellow. Nuclear staining by DAPI (blue).

**Supplemental movie II: Three-dimensional reconstruction of an engineered human blood vessel from vasculogenic derivatives of hESC I6 in Matrigel implant.** Z-stack of confocal images of a segment of the vasculature formed by I6-derived pericytes and endothelial cells in Matrigel implant were taken and reconstructed. The software elaboration created a rotating 3D structure of the blood vessel composed of CM-DiI pre-labeled hESC I6-derived pericytes and endothelial cells (red). Nestin\(^+\) pericytes (white and pink) overlay the endothelial cells, which incorporated intravenous administered UEA-1-FITC (green and yellow). Nuclear staining by DAPI (blue).
Stem Cell에서 유래되는 혈관형성 Pericyte들은 하지허혈을 회복시킨다

한 기 훈 교수 서울아산병원 심장내과

Summary

배경
Pericyte들은 고유의 소혈관(microvessel)에 상주하는 신혈관형성을 유도하는 세포들이며, 다양한 발전양상을 나타내는 mesenchymal stem cell들이다. Human pluripotent stem cell(hPSC, either embryonic or induced)들로부터 혈관내피세포 또는 평활근세포를 분화시키는 다양한 protocol들이 있지만 pericyte들이 hPSC의 분화 성숙을 통하여 형성되는 지에 대한 규명은 이루어지지 않았다.

방법 및 결과
hPSC의 발전단계에서 저절로 분화되는 embryoid body들이 CD105/CD90/CD73 양성, CD31 음성 multipotent clonogenic mesodermal precursor들로 나타나며, 이들은 용이하게 분리되며 효과적으로 증식한다. 분화된 세포들은 pericyte에 특이적인 세포표지자를 표현하며(CD146, NG2, platelet-derived growth factor receptor β), 평활근세포의 표지자들은 발현하지 않는다(α-smooth muscle actin). hPSC에서 유래한 내피세포들을 pericyte들과 같이 이식하면 기능적인 접합이 이루어져 혈관계의 형성이 이루어진다. Pericyte들을 immunodeficient mice들의 허혈성 사지에 도입하면 혈관과 근육의 재생이 이루어지며, 이식 21일 후 recruited hPSC pericyte들이 회복되는 근육과 혈관계에서 관찰된다.

결론
hPSC로부터 pericyte의 형성은 신혈관생성을 유도하며, 이는 기초연구 및 신약개발을 위한 다양한 혈관형성 세포가 관여하는 혈관형성 모델에 이용될 수 있으며, 또한 신혈관생성을 통한 재생의학에 사용될 수 있을 것이 다.
Commentary

Pericyte란 무엇인가?

Pericyte란 어의는 그대로 주변세포라는 것으로, 장기에 따라서 매우 다양한 ‘pericyte’들이 있다. 가령 지방세포의 pericyte는 흔히 지방세포 사이에 존재하는 macrophage-like cell들을 의미하기도 한다. 본 논문에 언급된 pericyte는 엄밀히 소혈관에 존재하는 것으로서 microvascular pericyte라고 정하는 것이 더욱 정확하다고 할 수 있다. 이들의 기능은 주로 endothelial cell layer의 형성, 유지, 재생에 기본적으로 backbone으로서 작용하는 데 의미를 가지고 있다고 생각되며, 주로 기저층(basement membrane) 내에 존재하는 것으로 기술된다. 따라서 소혈관의 신형성에 기여하는 것은 당연하다고 할 수 있으며, 이에 더하여 소혈관의 긴축도를 유지하는 기능을 할 것으로 추측된다.

Pericyte의 특이한 cell marker는 본문의 서론에 잘 기술되어 있다. 이 중 pericyte들이 직접 혈관의 긴축을 유지하는(즉, 수축기능이 있는) 세포인지 아니면, 이를 유도하는 세포인지가 논란이 있는 것처럼 같은 부위에 존재할 수 있는 α-smooth muscle cell들의 cell marker도 중첩되는 측면이 있다. 본 논문은 이러한 점을 구분하여 접근하고 있으며, 결국 pericyte의 원류를 독창적으로 기술하고 있으므로 두 세포 간의 형질차이 및 원류의 차이가 존재하는 것으로 보인다.

Microvascular Pericytes의 기능/조절 정리

이들에는 많은 매개물질들이 관여하고 있으나, platelet-derived growth factor receptor β(PDGF-β), transforming growth factor β(TGF-β), vascular endothelial growth factor(VEGF), angiopoietin(Angs), signaling pathways involving Notch and ephrins 등에 의한 조절기능들이 보고되고 있다. 이들을 간략히 정리하면 다음과 같다.

1. 혈관내피세포에서 분비되는 PDGF-β는 pericyte precursor cell들의 recruitment를 유도한다. Pericyte 에는 이에 반응하는 PDGFR-β가 발현됨이 보고된다. 홍미로운 사실은 이미 발현된 VEGF는 VEGF-R2의 발현을 촉진시키고, 이와 PDGFR-β 중합체의 형성을 유도하여 PDG-β의 기능을 저해하는 것으로 기술된다.

2. TGF-β는 모여든 pericyte precursor cell들의 분화를 유도한다. TGF-β 또는 이의 수용체인 endoglin의 결핍에서는 pericyte의 숫자뿐 아니라, 평활근세포 종국에는 혈관내피세포의 숫자들이 감소하는 현상을 보인다.

3. 저산소상태에서 분비되는 VEGF는 간접적으로 NO의 생성을 통하여 pericyte의 recruitment를 도울 수 있다. 역으로 pericyte에서는 VEGF가 분비되어 혈관내피세포의 생명연장에 도움을 준다.

4. Pericyte(그리고 혈관평활근세포)에서 분비되는 Ang-1은 혈관내피세포의 Tie-1을 활성화시켜 leading edge의 활성을 증가시켜 혈관형성을 숙성시킨 역할을 하며, 이는 아마도 PDGF-β 기능의 중요한 하부신호 체계인 것으로 판단되고 있다. Ang-2는 이와 반대의 작용을 하는 아형으로서 Tie-1에 붙어 작용을 방해한다. 이는 혈관을 불안정화시켜 VEGF의 역할을 중화시킨다.

5. NG2 proteoglycan은 미성숙된 pericyte에 발현하여 galectin-3 및 α3β1 integrin과 결합하여 상호세포 간의 교통을 가능하게 한다. Pericyte에는 Notch-3가 발현하는데, 이가 결핍되었을 시 pericyte들이 자취를 감추고 소혈관이 약해지며 늘어나는 현상을 보이게 된다.

본 논문의 새로운 점
Pericyte들이 hPSC로부터 유래하였다는 점을 증명하였 다. 또한, 이들에 대한 안정적인 배양을 이루었으며, 충분한 수의 pericyte를 확보할 수 있었다. 이를 이용하여 사지 허혈상태를 개선하였다.

논문의 주요 Figure 중 요점
1. Figure 1: CD105+/CD31-가 nonendothelial mural cell이다(B). 이중 CD73+, CD105- 아형이 많이 늘어난다(C, D).
2. Figure 2: CD105+ 세포 중 많은 수가 perivascular and MSC(mesenchymal stem cell)의 표지자인 CD146+, CD73+ 세포임을 알 수 있다.
3. Figure 4: EC(endothelial cell) progenitor cell과의 공동배양으로 혈관이 생성됨을 보여준다.
4. Figure 7: 하지허혈 동물에서의 허혈 개선효과를 보여주며, pericyte의 존재를 증명한다.

Reference
Multipotent Vasculogenic Pericytes From Human Pluripotent Stem Cells Promote Recovery of Murine Ischemic Limb

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Background—Pericytes represent a unique subtype of microvessel-residing perivascular cells with diverse angiogenic functions and multilineage developmental features of mesenchymal stem cells. Although various protocols for derivation of endothelial and/or smooth muscle cells from human pluripotent stem cells (hPSC, either embryonic or induced) have been described, the emergence of pericytes in the course of hPSC maturation has not yet been elucidated.

Methods and Results—We found that during hPSC development, spontaneously differentiating embryoid bodies give rise to CD105+CD90+CD73+CD31+ multipotent clonogenic mesodermal precursors, which can be isolated and efficiently expanded. Isolated and propagated cells expressed characteristic pericytic markers, including CD146, NG2, and platelet-derived growth factor receptor β, but not the smooth muscle cell marker α-smooth muscle actin. Coimplantation of hPSC-derived endothelial cells with pericytes resulted in functional and rapid anastomosis to the murine vasculature. Administration of pericytes into immunodeficient mice with limb ischemia promoted significant vascular and muscle regeneration. At day 21 after transplantation, recruited hPSC pericytes were found incorporated into recovered muscle and vasculature.

Conclusions—Derivation of vasculogenic and multipotent pericytes from hPSC can be used for the development of vasculogenic models using multiple vasculogenic cell types for basic research and drug screening and can contribute to angiogenic regenerative medicine. (Circulation. 2012;125:87-99.)

Key Words: pluripotent stem cells • ischemia • mesenchymal stem cells • pericytes • vasculature

In adult tissues, the majority of blood vessels are composed of 3 layers including a luminal inner monolayer of endothelial cells (EC), tunica intima, an intermediate muscular layer, tunica media, of smooth muscle cells (SMC) and an outer layer of fibroblast-like adventitial cells and connective tissue components, tunica adventitia. Microvessels, including capillaries, precapillary arterioles, postcapillary venules, and collecting venules are composed of internal endothelial layer surrounded by outer coverage of pericytes (also known as Rouget cells or mural cells).1 Both perivascular SMC and pericytes have been shown to function as critical regulators of vascular development, stabilization, maturation, and remodeling mediated by transforming growth factor β (TGF-β), platelet-derived growth factor B, or angioptotin-1.2,3 Although related in function and anatomic localization, pericytes can be distinguished from SMC on the basis of their characteristic morphology and specific cell marker expression: Whereas SMC form a separate layer of the tunica media in blood vessels, pericytes are physically embedded within the endothelial basement membrane to promote mutual communication with the underlying endothelium.4 In addition, SMC and the majority of pericytes in multiple human and murine tissue types express α-smooth muscle actin (α-SMA), which is involved in regulation of vessel contractility.5 Although specific markers of pericytes are not defined yet, the majority coexpress α-SMA, CD133, NG2, CD146, alkaline phosphatase and platelet-derived growth factor receptor β (PDGFR-β), with minor subsets coexpressing CD346 or do not express α-SMA5 and minor subsets of pericytes assumed to represent more primitive pericytic precursors.

Clinical Perspective on p 39

Besides possessing angiogenic features, subsets of blood vessel–residing cells are defined as stem cells, which are capable of multiple developmental paths, including osteogenic, chondrogenic, adipogenic, and myogenic differentia-
tion.\textsuperscript{5,6} During early stages of vertebrate embryogenesis, and similarly in embryonic stem cell differentiation, EC arise from mesodermal angioblast/hemangioblast precursors.\textsuperscript{8–10} In comparison, the ontogeny of pericytes is more complex, shares similarities with SMC, and is related to their disposition in different regions of the body.\textsuperscript{11} In adult tissue, the saphenous vein was shown to contain a novel subset of CD34\textsuperscript{+}CD31\textsuperscript{+} pericytic precursors.\textsuperscript{6} In vitro, the pluripotent differentiation capability of human embryonic stem cells (hESC),\textsuperscript{12} and more recently the rapidly growing platform of human-induced pluripotent stem cells (hPSC),\textsuperscript{13} provides a powerful model system to study vasculogenic development. Several studies have described protocols for the derivation of endothelial and SMC from progenitor cells.\textsuperscript{14–17} However, derivation of multipotent vasculogenic pericyte population from hPSC was not documented until now. Therefore, we used differentiating hPSC as a model to determine whether pericytes emerge alongside with development of endothelial and SMC on the basis of common (CD105, \(\alpha\)-SMA) and specific (CD31, vascular endothelial cadherin, europaeus agglutinin-I [UEA-1]) vascular cell markers. We have identified a novel population of cells, positive for recognized markers of pericytes, including NG2, PDGFR-\(\beta\), and CD146, but not \(\alpha\)-SMA and characteristic markers of mesenchymal stem cells (MSC). Herein we describe a protocol for coisolation of vasculogenic derivatives from a single source of hPSC, including pericytes, SMC, and EC. In addition, we identify the conditions for long-term cultivation of clonogenic hPSC pericytes, which steadily maintained perivascular and multilineage characteristics in vitro and in vivo and were proved useful in the treatment of severely ischemic tissue.

**Methods**

An expanded online-only Data Supplement Methods section is available in the online-only Data Supplement.

**Pericite and Endothelial Progenitor Cell Isolation**

At indicated time points of spontaneous differentiation in embryoid bodies (EBs), single-cell suspensions were made from differentiated hPSC, including human ESC H9,\textsuperscript{21,22} and I6,\textsuperscript{16,19} hPSC human foreskin fibroblast–derived C3,\textsuperscript{20} and human hair keratinocyte–derived KTR13.\textsuperscript{20} CD105\textsuperscript{+} or CD105\textsuperscript{–} cells were isolated from differentiated hPSC using MACS MicroBeads (Miltenyi Biotec) according to the manufacturer’s instructions. As determined by flow cytometry, the purity of isolated CD31\textsuperscript{+} or CD105\textsuperscript{+} cells was generally 60% to 70% at a single column, and >96% after the second column. Dissociated EB-derived CD105\textsuperscript{+}CD31\textsuperscript{+} or CD105\textsuperscript{–}CD31\textsuperscript{+} subpopulations were isolated by fluorescence-activated cell sorting (Becton Dickinson Immunocytometry Systems). In some experiments, CD31\textsuperscript{+} EC were isolated from cultured hPSC-derived CD105\textsuperscript{+} cells for further expansion (purity of CD31\textsuperscript{+} >97%) of either CD105\textsuperscript{+} CD31\textsuperscript{+} or CD105\textsuperscript{–} CD31\textsuperscript{–} pericytes.

**Matrigel Implants**

Endothelial cells (human umbilical vein EC [HUVEC] or hPSC-derived EC) or hPSC pericytes were resuspended in 250 L phenol-red free Matrigel (BD Biosciences), either alone (3–5 \(\times\) 10\textsuperscript{5} EC, 6–8 \(\times\) 10\textsuperscript{6} pericytes) or mixed (1:1). Matrigel mixture was then injected subcutaneously into 6- to 8-week-old nonobese diabetic (NOD)/severe combined immunity-deficiency (SCID) mice. Implants were removed at the indicated time points, fixed in 4% formalin, sectioned, and stained with Hematoxylin/Eosin.

**Hind Limb Ischemia Model**

Male 8-week-old CD1 nude mice were subjected to hind limb ischemia by femoral artery ligation and transection. After surgery, mice received of 2 \(\times\) 10\textsuperscript{6} hPSC pericytes (H9.2, I6, or C3) or unconditioned M-199 medium (200 \(\mu\) L) intramuscularly into 4 sites of the gracilis muscle in the medial thigh. Limb perfusion was assessed with a noncontact laser Doppler at days 0, 7, 14, and 21 after treatment.

**Statistical Analyses**

A \(t\) test was used for comparisons between 2 groups (analyses were performed with Excel 2007). Repeated measures ANOVA (analyses were performed with IBM SPSS statistic 19) was used for analyses of blood flow recovery, in which measurements were repeated for the same subject at the same time point (\(P<0.05\) was inferred to denote statistical significance) and followed by Bonferroni post hoc test.

**Results**

**Emergence of Vasculogenic Progenitor Cells in Developing EBs**

The emergence of vasculogenic populations from hPSC was examined in spontaneously differentiating EBs. For characterization of vasculogenic precursors, developing EBs were analyzed for the expression of CD105 and CD73, which have been reported to be expressed on both endothelial and mural cells in blood vessels.\textsuperscript{5} Quantitative polymerase chain reaction (PCR) analyses of differentiating EBs demonstrated that CD105 and CD73 expression was detected between days 4 and 26 in culture, upregulated between days 10 and 14, and decreased between days 19 and 26 (Figure 1A). The expression of the EC marker vascular endothelial cadherin was increased during 14 days of differentiation with low to undetectable expression thereafter (Figure 1A). Flow cytometry analyses for the expression of an additional EC marker CD31 with CD105 revealed that 2 distinguished cell subsets contributed to the overall increase in CD105 messenger RNA expression as follows: Until day 14 the majority of CD105\textsuperscript{+} cells were composed of CD105\textsuperscript{+}CD31\textsuperscript{+} EC with a small subset of nonendothelial CD105\textsuperscript{+}CD31\textsuperscript{–} population (Figure 1B). From day 19 onward the percentage of CD105\textsuperscript{+}CD31\textsuperscript{+} EC as well as CD31 messenger RNA expression declined progressively coinciding with an increase in the percentage of CD105\textsuperscript{+}CD31\textsuperscript{–} or CD73\textsuperscript{–}CD31\textsuperscript{–} nonendothelial subsets (Figure 1B–1D). Of note: In comparison to published data,\textsuperscript{22} our optimized dissociation protocol of EBs and cell isolation significantly increased (from 1%–1.5% to 3%–7% CD105\textsuperscript{+} cells) the apparent percentage of dissociated CD105\textsuperscript{+}CD31\textsuperscript{–} cells (H9.2 n = 30, I6 n = 8, and C3 n = 12,) and significantly increased the yield of isolated cells with 95% cell viability (Figure 1B and Figure I in the online-only Data Supplement). These results (Figure 1C and 1D) support the emergence of a CD105\textsuperscript{+} and/or CD73\textsuperscript{–}CD31\textsuperscript{–} nonendothelial mural subsets during the onset of vasculogenesis in hPSC-derived EBs. Therefore, 14-day-old dissociated EBs were analyzed by flow cytometry using triple labeling with antibodies against CD105 and CD31 with either CD146 or CD73 (Figure 2A). Within CD105\textsuperscript{+}CD31\textsuperscript{–} populations, \(\approx 25\%\) coexpressed CD146 and 22% CD73 (Figure 2A), which are also expressed by perivascular cells of blood vessels.\textsuperscript{5} In addition, CD90\textsuperscript{–}CD31\textsuperscript{–} clusters were observed in 17-day-old EBs,
Figure 1. Expression of vasculogenic markers on developing hPSC-derived EBs. A, Quantitative PCR gene expression analyses of CD105, CD73, and vascular endothelial cadherin in developing H9.2- and C3-derived EBs. The average expression normalized to GAPDH is shown. B, Representative flow cytometric analyses for CD105 and CD31 expression pattern in different aged hPSC-derived EBs. C through D, Time course of emergence of CD105−CD31− (C) or CD73−CD31− (D) endothelial populations and nonendothelial CD105−CD31− (C) CD73−CD31− (D) subsets in developing EBs. Data are mean ± SD of at least 3 independent experiments per time point. hESC indicates human embryonic stem cells; VE-Cadherin, vascular endothelial cadherin; iPSC, induced pluripotent stem cells; ESC, embryonic stem cells; and EBs, embryoid bodies.
with CD90^CD31^ in proximity and aligning the CD31^ vascular network (Figure 2B). The majority of CD90^ cells coexpressed calponin, which is another characteristic marker of perivascular cells (Figure 2C and Movie I in the online-only Data Supplement). Of note, CD73 and CD105 are expressed also by subsets of blood-derived CD45^-hematopoietic cells. However, it is well established that in the course of the spontaneous differentiation of EBs only very rare events (<0.02%) of CD45^-hematopoietic mature cells are detected^{10} (Figure II in the online-only Data Supplement), and the induction of differentiation of hematopoietic lineages requires further maturation of CD34^-angiotensin-converting enzyme^+-positive progenitor cells on specific cell feeders.^{10,23}

Altogether, these results demonstrate that a subset of nonendothelial/nonhematopoietic CD105^-CD31^- spontaneously emerge in the course of vasculogenesis in differentiating hPSC and may represent a perivascular progenitor cell population with potential vasculogenic properties.

**Characterization of Pericyte-Like Progenitor Cells**

Next, CD105^- cells were isolated from 13- to 19-day-old EBs for further cultivation. Within 1 to 2 passages, the CD105^-CD31^- subset dominated the mixed CD105^-vasculogenic cultures (Figure III in the online-only Data Supplement), exhibiting loosened cell-cell contacts in a multilayered hill-and-valley morphology^{24} (Figure III in the online-only Data Supplement), which is typical of cultured pericytes as well as SMC. Both hESC and hiPSC pericytes exhibited similar growth potential in long-term cultures up to 8 passages for 8 weeks, with a population doubling time of 108 hours between weeks 1 and 2 and a faster growth rate between weeks 3 and 5, at which point the population...
CD105\(^{+}\)CD31\(^{-}\) hPSC Pericytes and EC Assemble to Generate Human Vascular Network

The vasculogenic potential of the pericytes was initially evaluated in vitro. Although tube formation on Matrigel is a common feature of EC, it has been previously demonstrated that fetal tissue pericytes can actively create tubular networks.\(^{27}\) In accordance with this finding, hPSC pericytes formed short intricate tubular structures on Matrigel within 2 hours (Figure 4A) whereas HUVEC (data not shown) or hPSC-derived CD31\(^{+}\) EC rearranged into linear asymmetrical tube networks (Figure 4A). When CFSE-labeled pericytes were cocultured with H9.2 hESC CD31\(^{-}\) EC on Matrigel, both cell types were assembled to form thicker tubular networks (Figure 4B). It has been previously demonstrated that the presence of murine or human adult tissue–derived mesenchymal precursors is essential for de novo generation and stabilization of vascular networks using immunodeficient murine experimental models.\(^{14,28,29}\) Therefore, the vasculogenic potential of CD105\(^{+}\)CD31\(^{-}\) pericytes was studied in vivo by the Matrigel implant assay. Pericytes and EC, either HUVEC or hPSC EC, were mixed in Matrigel and injected subcutaneously into immunodeficient NOD/SCID mice. In accordance with previous studies,\(^{30}\) Hematoxylin/Eosin staining revealed that empty control Matrigel implants (Figure 4C-I), implants with HUVEC (data not shown), or hPSC-CD31\(^{+}\) cells only (Figure 4C-II) did not contain microvessels. Implants with only pericytes induced some infiltration of murine vessels (Figure 4C-III). However, when pericytes were mixed (1:1) with either HUVEC (Figure 4C-IV) or hPSC-EC (Figure 4C-V) and implanted, a robust generation of evenly distributed vascular network was detected inside the implant (Figure 4C–4E). The presence of luminal murine erythrocytes and nucleated leukocytes (Figure 4C-IV, 4C-V, and 4D) suggests that the newly formed human blood vessels anastomized with the host vasculature and were perfused with murine blood within 1 week. Human MHC Class I\(^{+}\)CD31\(^{-}\) pericytes (Figure 4D and Figure V in the online-only Data Supplement) assembled into luminal structures with human endothelial CD31\(^{+}\) or CD34\(^{+}\) EC (specificity of antihuman CD31, CD34, and MHC Class I antibodies is shown in Figure V in the online-only Data Supplement), which coexpressed von Willebrand factor (Figure 4D-IV and Figure V in the online-only Data Supplement). Pericytes were identified in proximity and aligning CD31\(^{+}$/CD34\(^{+}\) human vascular structures (Figure 4D). Of note, efficient generation of chimeric human vasculogenic structures was detected even when the implants were devoid of administration of angiogenic factors such as vascular endothelial growth factor (VEGF) and/or basic fibroblast growth factor. In addition, implantation within Matrigel induced \(\alpha\)-SMA expression by implanted pericytes, demonstrating their ability to further differentiate in vivo toward SMC-like perivascular cells and implying for their more their immature phenotype in culture as SMC precursors (Figure 4F). Altogether, these findings support the notion that pericytes and SMC share a common progenitor, as was previously demonstrated using quail-to-chick transplantation\(^{31}\) and mouse experimental models.\(^{31}\) The ability of pericytes to recruit murine blood vessels and to support the formation as well as stabilization of the newly formed vascular network can be attributed in part to the secretion of VEGF, which is a key regulator of angiogenesis and vasculogenesis, as well as of EC survival.\(^{32}\) In conformity with this suggestion, secreted VEGF\(_{165}\) was detected by ELISA in the conditioned medium of cultivated pericytes (Figure 4G).

Durability of hPSC Pericyte–Based Vasculature

To further evaluate the engineered vasculature, luciferase-labeled hPSC pericytes were implanted in the presence or absence of HUVEC in Matrigel implants. Perfusion of the implants was then monitored at days 1, 7, and 14 using a luciferase-based live imaging system. Whereas strong bioluminescence signal was detected in all implants at day 1 (Figure 5A), pericytes survived up to 14 days postimplanta-
Figure 3. Characteristics of long-term cultivated hPSC pericytes. **A**, Flow cytometry analyses of marker expression profiles of pericytes. Filled histograms: isotype-matched immunoglobulin G controls. **B**, Representative dot plot analyses of α-SMA expression in cultivated hPSC pericytes (H9.2: passage 4, I6: passage 5, C3: passage 7). **C**, Polymerase chain reaction analyses for characteristic messengers of vasculogenic and MSC of sorted CD105 expressing subpopulations and pericytes. **D**, Immunofluorescence stainings of full-term human placenta or hPSC pericytes. Nuclear staining by DAPI (blue). Data are representative of cultured hPSC pericytes between passages 1 and 8 (until senescence). ND indicates not detected; EC, endothelial cells; iPSC, induced pluripotent stem cells; hESC, human endothelial stem cells; and PDGFR-β, platelet-derived growth factor receptor β.
tion only in implants in which HUVEC were coimplanted (Figure 5A). Matched histological data (Figure 5B) confirmed that the survival of luciferase+ pericyte in vivo correlated with significant and durable perfusion of the implants by de novo formation of human blood vessels (Figure 5B), which were stabilized by implanted pericytes. It appears that pericytes undergo a process of remodeling characterized by reduction in total cellularity and viability of
the pericytes, which are not incorporated into engineered blood vessels. In addition, functionality of CM-DiI prelabeled hESC I6 vascular derivatives (Figure 5C) in Matrigel implants and anastomosis with murine circulation was examined after 7 days by intravenous administration of the human-specific endothelial-binding UEA-1 FITC lectin (green, D and H) which was incorporated into the human blood vessels (Figure 5E). Confocal microscopy slicing through blood vessel structures revealed nestin+ pericytes (Figure 5F) partially aligning CM-DiI+ (Figure 5G) UEA-1 FITC+ (Figure 5H and 5I and Movie II in the online-only Data Supplement) hPSC EC. Altogether, these results demonstrate that hPSC pericytes are crucial for rapid perfusion of the implants via a progressive process of vascular remodeling.

**Human hPSC Pericytes Are Multipotent Clonogenic MSC**

A perivascular origin for MSC was previously demonstrated in multiple human organs, which harbor a reserve of multipotent progenitor cells. This notion, together with the expression of recognized MSC markers (Figure 3) and the vasculogenic functionality of hPSC pericytes (Figures 4 and 5) imply that these cells might have a developmental potential equivalent to MSC. Therefore, cultured pericytes were analyzed for their ability to form mesenchymal tissues, including bone, fat, cartilage, and muscle. On appropriate inductive experimental conditions, hPSC-pericytes (H9.2 and C3) exhibited uniform and efficient osteogenic appearance, with robust mineral and calcium depositions (Figure 6A and 6B). After short (3 days) or long (14 days) osteogenic stimulation in vitro, differentiated pericytes were subcutaneously implanted within Matrigel into NOD/SCID mice for assessment of ectopic bone formation. Examination of the implants 2 weeks later by staining for Hematoxylin/Eosin or Alizarin red revealed the presence of mineral deposits produced by 3 days–stimulated hIPSC pericytes (Figure 6C and 6D) with a dramatic increase in mineral deposit content in implants of 14 days–stimulated pericytes (Figure 6E and 6F). In addition, more mature bone-like structures and erythrocyte-containing blood vessels could be seen in implants of 14 days–stimulated pericytes (Figure 6E). Furthermore, pericytes were capable of adipogenic (Figure 6G and 6H) and chondrogenic (Figure 6I and 6J) differentiation in vitro. High-efficiency (>95%) adipogenic differentiation and whole-cell osteogenic differentiation (Figure VI in the online-only Data Supplement) were documented from passage 1 to passage 8 until senescence, designating that our culture conditions were sufficient for stabilization and maintenance of the multilineage features of expanded hPSC-CD105−/CD73−/CD31− pericytic precursors. After myogenic induction in vitro, skeletal myotubes could be detected (Figure 6K and 6L and Figure VII in the

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*Figure 5. Anastomosis and durability of the human vascular network. A and B, Matrigel implants containing either luciferase-labeled hPSC pericytes (Luc+) alone or mixed with HUVEC (1:1). Mice were imaged using an IVIS Imaging System. Bioluminescence was recorded 30 minutes after intraperitoneal injection of luciferin 1 day after implantation and retained up to 2 weeks only in mixed implants (A). Matched Hematoxylin/Eosin staining reveals that viability of Luc+ pericytes was correlated with blood vessel density (BV/mm²). Bioluminescence was not detected in mice with empty Matrigel implants or implants containing unlabeled hPSC pericytes or HUVEC. N=4 implants per time point. C-I, Representative images of Matrigel implants containing hESC I6–derived CM-DiI–labeled (red, C and G) EC and pericytes. Human-specific endothelial-binding UEA-1 FITC lectin (green, D and H) was injected i.v. just before death at day 7 and denotes functional vessels (yellow, E and I). Confocal microscope images of nestin-expressing pericytes (white, F; pink arrows, I) aligning UEA-1–perfused human vessels (yellow arrows, I). Nuclear staining by DAPI (blue, I). HUVEC indicates human umbilical vein endothelial cells; Luc+, luciferase labeled; ND, not detected; UEA-1, europaeus agglutinin-1; and BV, blood vessel.*
online-only Data Supplement), with the fraction of differentiated hPSC pericytes expressing characteristic skeletal markers similar to that expressed by human-derived skeletal muscle (Figure 6K and 6M). Expandable single-cell–derived pericytic clones (Figure 6N) were further subdivided to give rise to tripotent osteo-adipo-chondrogenic (O) with part exhibiting also myogenic capacity (N and O). hESC indicates human embryonic stem cells; iPSC, induced pluripotent stem cells; and hSkeletal, human skeletal.

Figure 6. Multilineage differentiation of clonogenic hPSC pericytes. A, Osteogenic differentiation is seen accompanied by mineral production (black granules). B, Calcium production is detected by alizarin red staining. C through F, Implantation of osteogenic pericytes stimulated for 3 (G and D) or 14 days (E and F) in Matrigel into NOD/SCID mice. C and E, Hematoxylin/Eosin. D and F, Alizarin red. E, Murine blood vessels (arrows). Newly formed bone-like matrix (E, Inset). G through J, Induced adipogenic (G and H, Oil) and chondrogenic (I and J, Alcian blue) differentiation of hESC (G and I) and iPSC (H and J) pericytes. K through M, Multinucleated myotubes of myogenically induced hPSC pericytes (K through L) are positive for characteristic skeletal markers (K, Immunofluorescence; M, Quantitative PCR). N through O, Expandable single cell–derived pericytic clones (N) are all tripotent osteo-adipo-chondrogenic (O) with part exhibiting also myogenic capacity (N and O). hESC indicates human embryonic stem cells; iPSC, induced pluripotent stem cells; and hSkeletal, human skeletal.

Transplantation of hPSC Pericytes Promotes Vascular and Muscle Recovery in Ischemic Limbs of Transplanted Mice

To determine the effectiveness of hPSC pericytes on vascular and muscle recovery, 2×10⁶ hPSC pericytes (H9.2, I6, and C3) or unconditioned medium were injected intramuscularly into the ischemic hind limb of immunodeficient CD1 nude mice. Representative laser Doppler perfusion images are shown weekly to document the recovery of perfusion (Figure 7A). Within 21 days, untreated control mice showed ~2-fold endogenous recovery (Figure 7B), which was sufficient to prevent untoward morbidity or limb loss. In comparison, a significantly higher (~4-fold) and accelerated recovery of foot perfusion was measured in the pericyte-treated group (Figure 7B and Figure VIII in the online-only Data Supplement). Histology of ischemic, nonischemic, and healthy muscle from mice that were not subjected to limb ischemia demonstrated that perfused blood vessel density was significantly (P<0.001) higher in pericyte-treated ischemic mice.
compared with the control group and compared with healthy muscle (Figure 7C and Figure VIII in the online-only Data Supplement). At day 21 postinjection, CM-DiI–labeled pericytes were still detected incorporated into recovered muscle in ischemic limb but not in nonischemic or untreated ischemic limb (Figure 7D). Increased positive immunostaining for desmin revealed that muscle regeneration was more pronounced in the hPSC pericyte–treated group compared with the control group (Figure 7D and 7E). In addition, double staining of CM-DiI and desmin of myotube-incorporated pericytes demonstrate their ability to contribute to myogenesis via direct myogenic differentiation (Figure 7F). Recruited CM-DiI pericytes maintained their perivascular features in ischemic tissues and were also found incorporated into murine blood vessels (Figure 7G). These data confirm that transplanted hPSC pericytes directly incorporate into host ischemic muscle and recovered vasculature to promote improvement in perfusion and muscle recovery.

Discussion

We have demonstrated that pericytic progenitor cells can be isolated from hPSC, including hESC and hIPSC, to be further efficiently expanded in long-term cultures, maintaining stable perivascular features and multilineage potential. Our protocol is based on 3-dimensional scalable (either hESC or EBs) feeder-free differentiation platforms. In addition, we show that coimplantation of a single source–derived EC and pericytic progenitor cells in Matrigel into immunodeficient mice resulted in rapid formation of a robust vascular network, which anatomized the host circulation. Pericytes, immediately adjacent to the vascular endothelium of both arteries and veins, are not in themselves SMC. They do, however, share angiogenic properties and properties related to regulation of blood vessels. Similarly, we have demonstrated here that hPSC pericytes were found to reside around engineered blood vessels and were essential for the de novo formation of these
blood vessels. These findings are consistent with previous reports showing that interactions between EC and murine perivascular cells are essential for development of vascular networks in vivo. In previous models, either murine embryonic cell line 10T1/2 or murine myofibroblasts were used as perivascular cells. However, neither source is useful for clinical applications. Although protocols for generation of perivascular hESC-derived SMC, with therapeutic effectiveness in limb and cardiac ischemic experimental models were previously described, it is not clear whether sufficient numbers of SMC with stable phenotype can be achieved in long-term culture as a fundamental need for potential usage in numbers of SMC with stable phenotype can be achieved in long-term culture as a fundamental need for potential usage in regenerative medicine. In addition, generation of mature functional SMC on murine OP9 feeder, in a 2-dimensional (2D) differentiation system, was previously described. However, it is not clear whether OP9-induced differentiation results in generation of multipotent pericyte-like perivascular cells or directly induces the production of terminally differentiated SMC via another mechanism of cell commitment. Furthermore, OP9 feeder also induces the differentiation of hematopoietic CD105+CD45+ or CD73+CD45+ from hPSC-derived progenitor cells, making this and similar 2D differentiation protocols unsuitable for our CD105/CD73-based isolation approach.

In our cultures, pericytes were rapidly and robustly expanded and could further gain SMC phenotype in vivo when implanted together with EC in Matrigel while uniformly maintaining multilineage potential throughout the whole culture period. Similarly, human bone marrow-derived adult MSC gained mature SMC phenotype when implanted with cord blood–derived EC and could be efficiently expanded. However, the differentiation potential of expanded MSC is significantly reduced with increasing cell passages and is donor-age restricted because of heterogeneous deterministic hierarchy and acquired in vitro cues in long-term cultures. Protocols for induction of differentiation in serum-free cultures and sequential activation by various growth factors enable the generation of either mesodermal progenitor cells or EC. Further studies are required to evaluate whether culture conditions, in particular differentiation in 2D-induced and defined cultures, which favors the emergence and stabilization of particular derivatives that affect the specification of pericytes in developing hPSC. For example, although blockade of TGF-β signaling at initial developmental stages of hPSC induce neural conversion, inhibition of TGF-β signaling after specification of mesodermal progenitor cells contributes to generation of EC. However, TGF-β signaling interferes with the maturation as well as stabilization of vascular phenotypes of hPSC endothelial progenitor cells and was shown to be involved in cell differentiation toward α-SMA-positive SMC. Interestingly, CD34+NG2+PDGF-β+CD31− perivascular progenitor cells were detected in human saphenous veins. After isolation, CD34+ cells differentiated in vitro into multipotent pericytes while losing CD34 expression. These findings coincide with an alternative pathway for derivation of perivascular EC or SMC from hESC, in which CD34+ progenitor cells are further induced to differentiate into these vascular derivatives. Saphenous vein–derived CD34+ cells may therefore represent progenitor intermediates of differentiating CD34+ during induced commitment toward the perivascular lineage in native tissue and in the course of hESC differentiation. In view of the fact that implantation of either HUVEC, blood-derived EC, or—as demonstrated here—hPSC-derived EC is insufficient for the formation of vascular network in the absence of supporting perivascular cells, the effort to achieve large numbers of EC from hPSC should be accompanied by the ability to gain sufficient numbers of perivascular cells, including SMC or pericytes.

Previous attempts to generate perivascular cells from hESC resulted in the generation of α-SMA–positive SMC. Intact SMC can be distinguished from pericytes in fetal or adult tissues based on the basis of a combination of cell markers, cell morphology, and the size of the blood vessels in their tissue of origin. However, in differentiating hPSC, visualization of pericytes cannot be based on correlation between their anatomic localization in physiological association with vascular endothelium together with expression of a combination of cell markers (eg, CD116, NG2, CD90) and the absence of others (eg, CD31, CD56) because of nonorganized immature vascular appearance in developing hPSC. In addition, MSC and pericytes share the common features of multilineage progenitors but exhibit distinct characteristics, as well. Therefore, a comprehensive set of pericytic markers, morphology in culture, and perivascular and MSC features should be demonstrated in order to define the hPSC pericyte equivalents. Generation of MSC from hESC was previously demonstrated using 2 distinguished protocols involving either neural crest progenitor cell CD73+ progeny or CD73+ mesoendodermal precursors, with the neural crest progenitor–derived CD73+ precursors differing from the mesodermal intermediates in their inability to give rise to skeletal muscle progeny. However, several reports demonstrate that cranial and cephalic neural crest connective tissue and smooth and skeletal muscle cells in vivo. We report here that hPSC pericytes exhibited myogenic differentiation in vitro and in vivo as well as characteristic perivascular morphology in cultures, with hill-and-valley morphology and no contact inhibition. In addition, the lack of α-SMA expression distinguishes the hPSC pericytes from the α-SMA–positive mesodermal progenitor cells, which were derived by different protocols and suggest that these intermediates represent more primitive perivascular precursors, with MSC-definitive characteristics. In addition, the type and the time course of emergence of hPSC-cell intermediates are largely dependent on the dynamic or induced environmental cues. For example, paraxial mesodermal-derived CD73+ MSC were generated by long-term differentiation of hESC in 2D culture wherein alternative protocol and experimental conditions were used to generate CD73+ MSC, this time from isolated neural crest progenitor cells. Furthermore, in the course of vertebrate valvulogenesis in the developing heart, endocardial cells undergo endothelial to mesenchymal transition in a VEGF-, Notch-, and TGF-β–dependent manner. The nuclear factor of activated T cells–positive endocardial subset was also identified in differentiating murine EBs, implying that endothelial intermediates in differentiating hPSC can serve as an alternative source for pericytes.
Supporting this notion, transcripts and protein expression of the endothelial to mesenchymal transition marker and fibroblast-specific protein 1 \(25,26\) were detected in cultured hPSC pericytes. It would be interesting in future studies to identify the developmental origin of hPSC pericytes on the basis of germ line–specific markers and cell tracking in developing hPSC.

We demonstrate here that transplantation of hPSC pericytes significantly attenuated critical limb ischemia. A physiological role of hPSC pericytes in various injury and regeneration models remains, however, to be further demonstrated. Currently, the endothelial and perivascular derivatives, which are derived from a single source of hPSC, can be used by in vitro models for drug screening, biological studies of pericycle-endothelial interactions, and engineering of 3-dimensional vascular grafts. Importantly, given that our protocol allows efficient generation of pericytes from hPSC, it can be applied for isolation of pericytic precursors from hPSC with inherited genetic mutations for evaluation of a variety of pericyte degree of plasticity or dysfunctions. Considering the shortage in sources for uniform cultures of pericytes, the complex ontology of pericytes in animal experimental models, and the elusive origin of human pericytes, the use of developing hPSC as a human in vitro model for pericyte identification, isolation, and expansion can strongly contribute to studies of development and origin of pericytes and can be essential for improving differentiation protocols, generation of vascular constructs, disease modeling, and development of strategies in regenerative medicine.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

In this study, we have generated for the first time a novel population of vasculogenic pericytes with multipotent mesodermal features, which is derived from human pluripotent embryonic and induced stem cells. The scalability of human pluripotent stem cells together with the stable phenotype of the highly expandable pericytic derivatives fulfills the demand for sufficient cell quantities for potential autologous clinical transplantation. Our findings support the notion that human pluripotent stem cell pericytes can be generated alongside with vascular endothelial cells from the same source of human pluripotent stem cells in a batch-to-batch consistent manner to treat ischemic diseases and potentially cardiovascular disorders, as well. Additional possible therapeutic applications include syndromes with use of pericytes derived from normal or disease-carrying human pluripotent stem cells to study disease modeling and drug screening, pericyte abnormalities such as hemangiopericytoma, diabetic retinopathy, and Alzheimer disease.