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Journal

Cell Research, 23(10)

ISSN

Authors

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Publication Date

2013-10-01

DOI

Copyright Information

Peer reviewed

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ORIGINAL ARTICLE

Driving vascular endothelial cell fate of human multipotent Isl1+ heart progenitors with VEGF modified mRNA

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Distinct families of multipotent heart progenitors play a central role in the generation of diverse cardiac, smooth muscle and endothelial cell lineages during mammalian cardiogenesis. The identification of precise paracrine signals that drive the cell-fate decision of these multipotent progenitors, and the development of novel approaches to deliver these signals *in vivo*, are critical steps towards unlocking their regenerative therapeutic potential. Herein, we have identified a family of human cardiac endothelial intermediates located in outflow tract of the early human fetal hearts (OFT-ECs), characterized by coexpression of Isl1 and CD144/vWF. By comparing angiocrine factors expressed by the human OFT-ECs and non-cardiac ECs, vascular endothelial growth factor (VEGF)-A was identified as the most abundantly expressed factor, and clonal assays documented its ability to drive endothelial specification of human embryonic stem cell (ESC)-derived Isl1+ progenitors in a VEGF receptor-dependent manner. Human Isl1-ECs (endothelial cells differentiated from hESC-derived ISL1+ progenitors) resemble OFT-ECs in terms of expression of the cardiac endothelial progenitor- and endocardial cell-specific genes, confirming their organ specificity. To determine whether VEGF-A might serve as an *in vivo* cell-fate switch for human ESC-derived Isl1-ECs, we established a novel approach using chemically modified mRNA as a platform for transient, yet highly efficient expression of paracrine factors in cardiovascular progenitors. Overexpression of VEGF-A promotes not only the endothelial specification but also engraftment, proliferation and survival (reduced apoptosis) of the human Isl1+ progenitors *in vivo*. The large-scale derivation of cardiac-specific human Isl1-ECs from human pluripotent stem cells, coupled with the ability to drive endothelial specification, engraftment, and survival following transplantation, suggest a novel strategy for vascular regeneration in the heart.

Keywords: cardiovascular progenitors; endothelial cells; Isl1; mRNA; VEGF-A
Cell Research (2013) **23**:1172-1186. doi:10.1038/cr.2013.112; published online 10 September 2013

Introduction

Unraveling the cardiogenic cell-fate map and the sig-

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naling pathways that control the formation of diverse cardiac muscle, smooth muscle and endothelial lineages is central to our understanding of the etiology of adult and congenital heart disease, as well as in the search for novel regenerative therapeutic strategies. Although significant progress has been made toward elucidating the pathways that drive cardiomyocyte (CM) lineages (reviewed in [1]), relatively less is known about the origins of the distinct endothelial cell (EC) lineages that constitute the coronary arterial, venous and endocardial compartments

of the heart. Cumulative studies from the zebrafish [2, 3], chick [4, 5], mouse [6-8] and human [9] models have revealed a heterogeneous origin of the endothelial lineages in the heart, and have recently indicated that a subset of these ECs are derived from a distinct origin of multipotent heart progenitors located in various specific regions of the early heart field [10, 11].

During vertebrate gastrulation, cardiovascular progenitors located in the anterior region of the primitive streak migrate anteriorlaterally to form bilateral heart fields, also known as the cardiac crescent [12]. As the embryo folds ventrally, the two heart fields coalesce into a linear heart tube. The second heart field (SHF) contains progenitors initially located outside the heart within the pharyngeal mesoderm, which then migrate into the heart and give rise to the endocardium, myocardium, pacemaker and smooth muscle lineages (reviewed in [13]). In the lineage tracing Isl1-cre;R26R mouse model, it has been demonstrated that Isl1 marks cell populations that contribute to subsets of ECs in myocardium and aortic endothelium [11, 14]. Coexpression of Isl1 and CD31 in cells can be observed within the outflow tract at E11.5 [15]. Moreover, using the genetic fate-mapping studies in both murine [11] and human [16] embryonic stem cells (ESCs), the Isl1+ progenitors can be isolated and clonally amplified, which resemble the developmental cardiovascular progenitors in the murine embryonic and human fetal hearts, respectively. The murine or human ESC-derived Isl1+ progenitors give rise to three distinct cardiovascular cell lineages, namely CMs, smooth muscle cells (SMCs) and ECs. These studies, primarily performed *in vitro*, further support that Isl1+ progenitors can differentiate toward an EC fate, and raise the necessity to identify the precise paracrine signals that drive this decision.

In this study, we have examined the specific developmental stimulus that supports the endothelial specification of the human Isl1+ progenitors *in vitro* and *in vivo*. By screening angiocrine factors expressed in ECs derived from the outflow tract (OFT-ECs) of human fetal hearts compared with that in outgrowth ECs (OECs) derived from human cord blood, vascular endothelial growth factor (VEGF)-A (165) was found to be the most abundantly expressed angiocrine factor that could direct the differentiation of the human ESC-derived Isl1+ progenitors toward an EC fate and away from the more spontaneously differentiated SMC lineage. Moreover, we have also identified the developmental counterpart of the Isl1-ECs in human fetal hearts. The Isl1+ endothelial intermediates, marked as Isl1+CD144+ or Isl1+vWF+, were identified in the outflow tract (OFT) region of human fetal hearts at gestation week 9. To address the longstanding question regarding cell-fate specification of the human

ESC-derived Isl1+ progenitors *in vivo*, we have employed a novel approach to overexpress VEGF-A in the Isl1+ progenitors via chemically modified mRNA (modRNA). We have demonstrated that VEGF-A promotes an EC fate decision of the human Isl1+ progenitors *in vitro* and *in vivo*, away from the SMC lineage, as well as facilitating the proliferation and survival (reduced apoptosis) of the Isl1+ progenitors. These studies suggest that VEGF-A is a switch that controls the cell-fate specification of multipotent human heart progenitors, and that *in vitro* transfection of heart progenitors prior to transplantation can enhance their engraftment and survival, adding a new potential role of VEGF-A modRNA in addition to recent studies showing its ability in driving heart regeneration following myocardial infarction (MI) [17].

Results

Human Isl1+ endothelial progenitors, found in the outflow tract region of the early human fetal hearts, express VEGF receptors 1 and 2

Our laboratory has reported previously that Isl1-ECs can be found in aorta/OFT region of embryonic hearts of the Isl1-cre;R26R;LacZ mice [11]. To evaluate whether Isl1-ECs can be found in human hearts, frozen sections of human fetal hearts at gestation week 9 were co-stained for Isl1 and EC-specific markers CD144 or vWF (Figure 1A). The Isl1+CD144+ and Isl1+vWF+ cells, found in the lower portion of the OFT septum, may represent the Isl1+ endothelial intermediates as described previously [18]. Moreover, the Isl1+ cells were also found positive for the VEGF-A receptors, VEGFR1 (Flt1) and KDR (Figure 1A). Using the lineage-tracing human Isl1-cre eGFP ESC line, in which CRE has been knocked into the Isl1 locus, one can trace the cell fate as the daughter cells of the Isl1+ lineage are marked as eGFP+ (Figure 1B), and the human ESC-derived Isl1+ progenitors (eGFP+) can also be purified following direct differentiation of ESCs using BMP4, Activin A and FGF2 (Figure 1C). Intriguingly, not only the Isl1+ cells of human fetal hearts, but also the Isl1+ progenitors derived from hESCs also expressed both Flt1 and KDR (Figure 1D). Approximately 98% (4.5% out of 4.6% total eGFP+ cells) and 9% (0.5% out of 5.3% total eGFP+ cells) of the human ESC-derived Isl1+ progenitors expressed Flt1 and KDR, respectively, on day 7 of differentiation (Figure 1D). Our result is in line with a previous report that identified low expression level of KDR but higher expression level of Flt1 in endocardial ECs [19]. Furthermore, expression of the *Isl1* gene could be found in the Flt1+ or KDR+ cells during human ESC differentiation (Supplementary information, Figure S1A). Since Isl1 is also known to be expressed
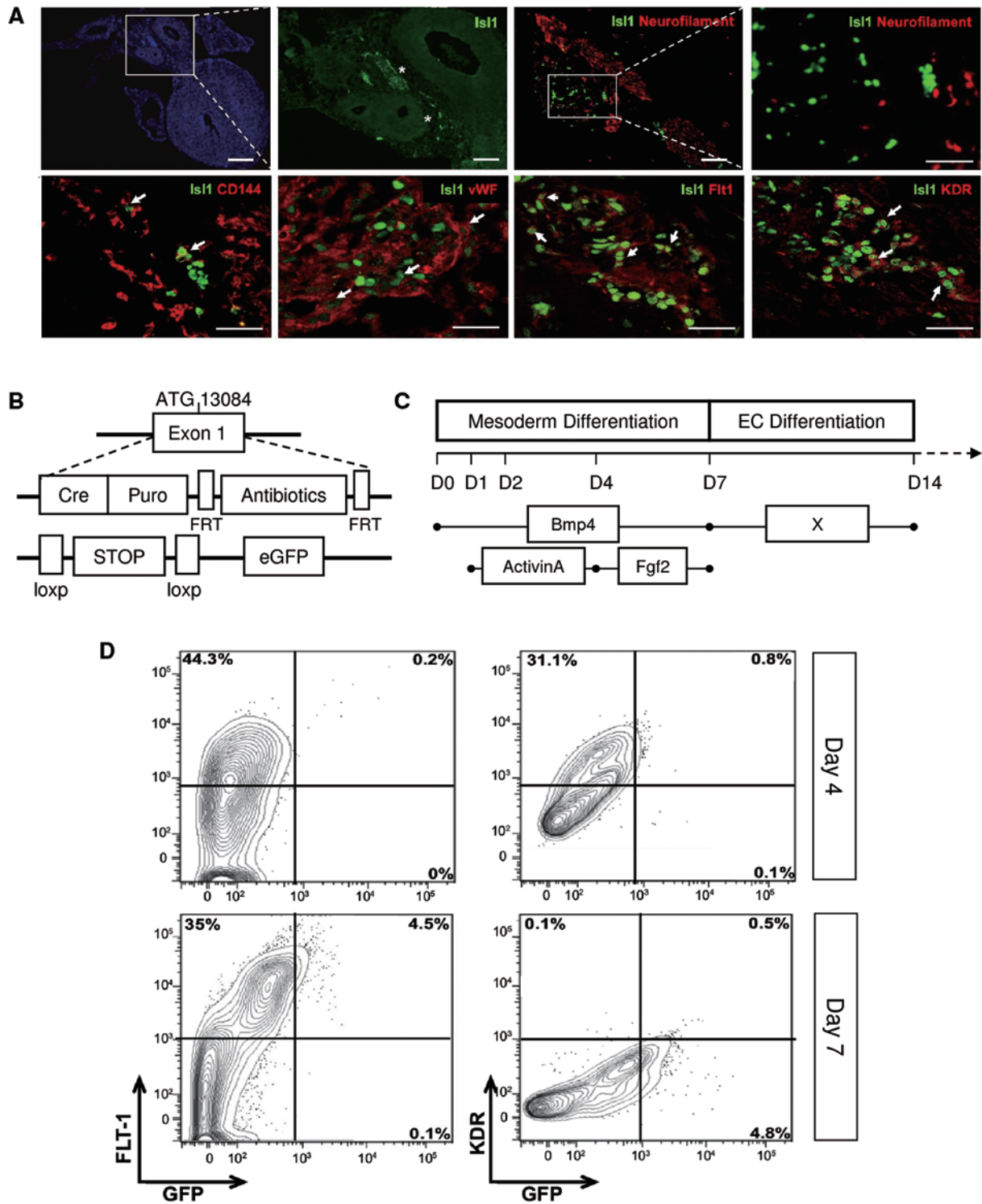
Figure 1 Expression of VEGF receptors in the human Isl1^{+} progenitors. **(A)** Frozen sections from a human fetal heart at gestation week 9 were stained for DAPI (scale bar = 500 μm), Isl1, endothelial cell-specific markers: CD144, vWF, VEGF-A receptor 1 (Flt1) or 2 (KDR), or neurofilament (scale bars = 50 μm and 10 μm). Isl1^{+} cells are indicated by white asterisks (scale bar = 100 μm) and colocalization of Isl1 and EC markers are indicated by white arrows (scale bar = 10 μM). **(B)** Schematic diagram showing the Isl1 lineage-tracing construct in human ESCs. **(C)** Differentiation protocol used to derive the Isl1^{+} progenitors from human ESCs and to examine, which angiocrine factor (X) is responsible for endothelial differentiation of the progenitors. **(D)** FACS analyses showing expression of VEGFR1 or VEGFR2 in day-4 or day-7 human Isl1^{+} progenitors.

in cardiac ganglia [15], co-staining of Isl1 and neurofilament was also performed (Figure 1A). Our result indicated that the Isl1$^+$ cells, and, therefore, the Isl1$^+$ endothelial intermediates identified in the same OFT region of human fetal hearts were negative for neurofilament.

VEGF-A is the most abundantly expressed angiocrine factor by cardiac ECs and is sufficient to drive endothelial differentiation of the human Isl1$^+$ progenitors

To elucidate the candidate angiocrine factor responsible for endothelial specification of the human Isl1$^+$ progenitors, quantitative PCR (qPCR) arrays were performed to compare the gene expression levels of angiocrine factors between the human cardiac OFT-ECs and human noncardiac EPCs such as OECs. In general, OFT-ECs express more angiocrine factors than OECs (Figure 2A and Supplementary information, Figure S2). VEGF-A was not only the most abundantly expressed angiocrine factor by OFT-ECs among the 82 factors tested (Figure 2A), but also the most abundantly expressed ligand of the VEGF family by ECs derived from different subdomains of human fetal hearts at gestation weeks 10 (Supplementary information, Figure S1B) and 20 (Supplementary information, Figure S1C), whereas PLGF was the most abundantly expressed ligand of the VEGF family by noncardiac OECs (Supplementary information, Figure S1B and S1C).

By transplanting the human Isl1-cre eGFP$^+$ embryoid bodies (EBs) under the kidney capsule of nonobese (NOD)/severe combined immunodeficient (SCID) mice, it appears that the Isl1$^+$ progenitors spontaneously differentiated into eGFP$^+$SMMHC$^+$ smooth muscle-like and eGFP$^+$cTNT$^+$ cardiac muscle-like lineages *in vivo* (Supplementary information, Figure S3A); however, eGFP$^+$CD31$^+$ endothelial-like cells were not observed in any of the recipients ($n = 5$, data not shown). Therefore, exogenous signal(s) seem to be needed in directing differentiation of the human Isl1$^+$ progenitors toward the endothelial lineage and away from the more spontaneously differentiated smooth muscle and cardiac muscle lineages. To examine which angiocrine factor might be responsible for endothelial specification of the human Isl1$^+$ progenitors, angiocrine factors that were highly expressed by OFT-ECs were employed as "X" in the endothelial differentiation protocol (Figure 1C). Among the top eight angiocrine factors that were highly expressed by OFT-ECs, VEGF-A was the most abundantly expressed one that could drive differentiation of the day-7 purified eGFP$^+$ cells (Isl1$^+$ progenitors) into eGFP$^+$CD31$^+$CD144$^+$ cells (Isl1-ECs, Figure 2B). To confirm the tri-lineage differentiation potential of hESC-derived Isl1$^+$ progenitors by VEGF-A, day-7 purified eGFP$^+$ cells were replated as single cells with or without VEGF-A for 10 days (Supplementary information, Figure S3B). eGFP$^+$SMMHC$^+$ smooth muscle-like and eGFP$^+$cTNT$^+$ cardiac muscle-like cells were found in both groups, whereas eGFP$^+$CD31$^+$ endothelial-like cells were found in the VEGF-A-treated group only. Furthermore, up-regulation of the *Cd31* gene, but downregulation of the *Smmhc* and *cTnt* genes was observed following treatment with VEGF-A compared with the untreated control group (Supplementary information, Figure S3C). Taken together, our results from the *in vivo* transplantation and *in vitro* cell clonal assays support that the day-7 purified eGFP$^+$ cells can differentiate into SMs, CMs or ECs; and VEGF-A is needed to drive endothelial differentiation of the human Isl1$^+$ progenitors. Furthermore, in addition to our previous report that showed coexpression of Isl1 and CD31 in human fetal hearts at gestation week 11 [16], our results show that CD144 seems to be an earlier and more readily expressed EC marker for Isl1$^+$ endothelial intermediates compared to CD31. Expression of CD144 was detected as early as on day 7 in the differentiated human ESC-derived Isl1$^+$ progenitors (eGFP$^+$) (Supplementary information, Figure S3C, top right panel). Using both CD31 and CD144, we could purify a more discrete population of ECs from the ESC differentiation cultures.

Endothelial differentiation of the human Isl1$^+$ progenitors is KDR dependent

In addition to its capability of directing endothelial differentiation, VEGF-A could also expand the eGFP$^+$ progenitors from 35.6% to 43.8% following treatment from day 4-7 in the differentiation cultures (Figure 2C top panels). In general, directing differentiation of the day-7 purified eGFP$^+$ progenitors by VEGF-A can yield approximately 8-10% eGFP$^+$CD31$^+$CD144$^+$ cells in 7 days of differentiation (D14, $n = 25$, Figure 2C bottom panels, Supplementary information, Figure S3C). As demonstrated in a cell clonal assay (Figure 2D), culturing the day-7 purified eGFP$^+$ progenitors on irradiated murine embryonic fibroblasts (MEF), a condition maintaining the self-renewing capacity of the multipotent Isl1$^+$ progenitors [16], in the presence of VEGF-A for 5 days upregulated expression of the SHF progenitor gene, *Isl1* ($P < 0.01$), cardiac EC gene [20], *Wt1* (Wilm's tumor gene-1, $P < 0.05$), and EC-specific gene, *Cd31* ($P < 0.005$), compared with the untreated control ($n = 33$). To examine whether the effect of VEGF-A on endothelial differentiation of the human Isl1$^+$ progenitors is dependent on its receptor VEGFR2 (KDR), a KDR inhibitor (SU5614) was used in addition to VEGF-A in differentiating the day-7 purified eGFP$^+$ progenitors from day 7-14 (Figure 2C bottom panels). As SU5614 is known to in-
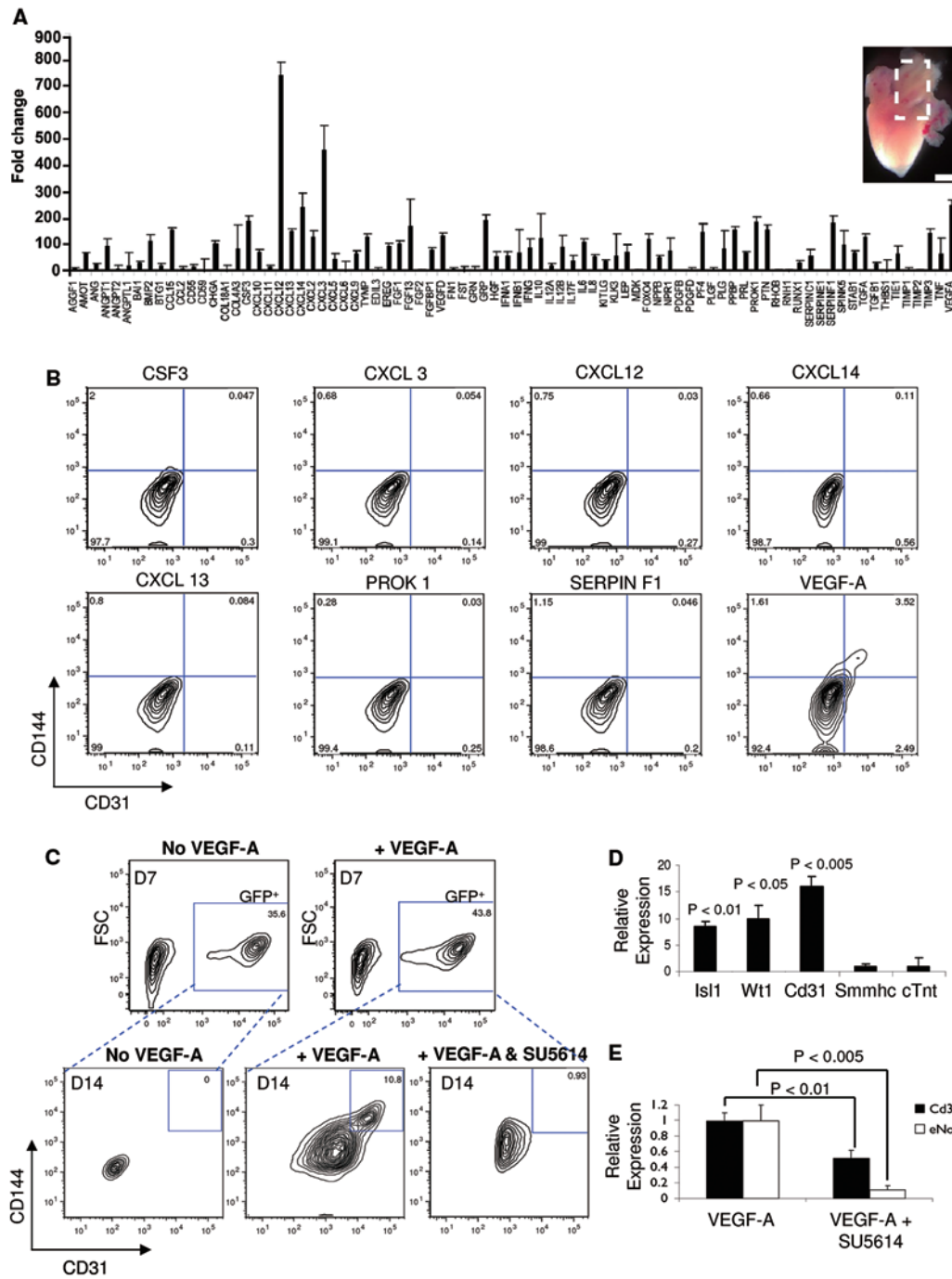
Figure 2 VEGF-A is the most abundantly expressed angiocrine factor in human fetal hearts and endothelial differentiation of the human Isl1^{+} progenitors is KDR dependent. **(A)** qPCR profiling showing expression levels of angiocrine factors derived from CD144^{+}CD31^{+} ECs purified from outflow tract of the 10-week human fetal hearts, expression levels were compared with those of the noncardiac, human cord blood-derived outgrowth endothelial cells (OECs, value on y axis = 1). **(B)** FACS analyses to determine the endothelial differentiation efficiency by factor X following treatment from day 7-12. **(C)** FACS analyses on the proliferation rate of the human Isl1-cre eGFP^{+} cells following VEGF-A treatment from day 4-7, and the endothelial differentiation efficiency without VEGF-A, with VEGF-A or with VEGF-A+KDR inhibitor (SU5614) from day 7-14. **(D)** Clonal assay was performed by culturing the day-7 human Isl1-cre eGFP^{+} cells on MEFs with VEGF-A (n = 33) for 3 days. Result was normalized to cells without VEGF-A treatment. **(E)** qPCR data showing endothelial differentiation of the day-7 human Isl1-cre eGFP^{+} cells in the presence of VEGF-A or VEGF-A+SU5614 for 7 days. Result was normalized to cells with VEGF-A alone (value on y axis =1).

duce apoptosis, only PI⁻ viable cells were taken into account during analyses. Treatment of SU5614 reduced the eGFP⁺CD31⁺CD144⁺ population from 10.8% (VEGF-A alone) to 0.93% (VEGF-A + SU5614), which was comparable to the untreated control (0%). Moreover, SU5614 downregulated the gene expression of *Cd31* ($P < 0.01$) and endothelial-specific nitric oxide synthase (*eNos*, $P < 0.005$), compared with the VEGF-A alone group (expression level = 1, Figure 2E).

Human Isl1-ECs resemble cardiac endothelial progenitors

To confirm an endothelial phenotype of the purified eGFP⁺CD31⁺CD144⁺ cells, FACS analyses were performed to demonstrate their surface expression of the endothelial markers (Figure 3A), including CD34 (99.5%), CD105 (67.9%), CD117 (89.2%), FLT1 (71.6%), KDR (67.6%) and FLT4 (99.2%). A small population of the eGFP⁺CD31⁺CD144⁺ cells also expressed CD133 and CXCR4. Moreover, qPCR was performed to evaluate expression levels of transcription factors specific for EPCs or endocardial ECs. Our result shows that OECs, OFT-ECs (gestation week 10 or 20) and eGFP⁺CD31⁺CD144⁺ cells (Isl1-ECs D14 or D28) expressed higher levels of endothelial progenitor genes, including *Etv2*, *Foxc1*, *Hoxa9* and *Id1*, compared with the adult human cardiac microvascular ECs (HMVEC-c) (Supplementary information, Figure S4A). Moreover, OFT-ECs (gestation week 10 or 20) and eGFP⁺CD31⁺CD144⁺ cells (Isl1-ECs D14 or D28), but not the day-7 purified eGFP⁺ cells (Isl1 progenitors), expressed the endocardial EC genes such as *Nfatc-1*, *Connexin 43* and *Connexin 45* (Supplementary information, Figure S4B).

To confirm the cardiac origin of the purified eGFP⁺CD31⁺CD144⁺ cells, qPCR was performed to evaluate expression levels of the cardiac progenitor genes compared with those of the non-cardiac OECs. Our result shows that both the OFT-ECs (gestation week 10 or 20) and eGFP⁺CD31⁺CD144⁺ cells (D14 or D28) expressed higher levels of *Isl1*, *Tbx20*, *Nkx2.5*, *Gata4*, *Tbx1*, *Tbx5* and *Mef2c* genes compared with the noncardiac OECs (Supplementary information, Figure S4C and S4D). Furthermore, expression patterns of angiocrine genes were compared among day-7 puified eGFP⁺ progenitors, day-14 purified eGFP⁺CD31⁺CD144⁺ cells (Isl1-ECs), OFT-ECs (gestation week 10) and OECs by qPCR arrays. Our results show that the expression pattern of angiocrine genes between the Isl1 progenitors and Isl1-ECs was comparable (Figure 3B and 3C); and the expression pattern of angiocrine genes of Isl1-ECs was more similar to that of the cardiac OFT-ECs (34 in common, Figure 3B) than that of the noncardiac OECs (8 in common, Figure

3C). To evaluate EC functions, tube formation and secretion of nitric oxide (NO) were evaluated *in vitro*. Our results show that the purified eGFP⁺CD31⁺CD144⁺ cells formed 3-dimentional tube structures in matrigel (Supplementary information, Figure S5A and S5B). Moreover, expression of eNOS was found in all of the purified eGFP⁺CD31⁺CD144⁺ cells (Supplementary information, Figure S5C), and secretion of NO was also detected at a comparable level between the eGFP⁺CD31⁺CD144⁺ cells and OECs. (Supplementary information, Figure S5D). To evaluate the potential of endothelial-to-mesenchymal transition (EndoMT) of the eGFP⁺CD31⁺CD144⁺ cells, TGF-β was added in EC cultures as described previously [21, 22]. Upregulation of mesenchymal cell-associated genes (α-SMA and Sm22) was detected in the TGF-β-treated-eGFP⁺CD31⁺CD144⁺ cells compared with the untreated controls (Supplementary information, Figure S5E).

VEGF-A modRNA can drive endothelial differentiation of the human Isl1⁺ progenitors

To further examine the role of VEGF-A in driving endothelial differentiation of the human Isl1⁺ progenitors, a novel modRNA approach [23, 24] was utilized to mediate highly efficient expression of autocrine proteins in these progenitors. We first examined the expression kinetics of a reporter modRNA transfected into the human Isl1⁺ progenitors *in vitro*. mCherry modRNA (1 μg/10⁶ cells) was transfected into the day-7 purified eGFP⁺ progenitors, and expression of the mCherry protein was evaluated at 3-150 h post-transfection. Our result shows that the expression level of mCherry peaked at 18-24 h with approximately 90% cells being transfected (Figure 4A and Supplementary information, Figure S6), and then returned to the basal level 140 h post-transfection (Figure 4B). Similarly, a time-dependent response was observed following transfection of eGFP⁺ cells with the VEGF-A modRNA (Figure 4C). To quantify protein secretion and survival of the transfected eGFP⁺ cells, supernatants and cells were assayed, respectively, at different time points following transfection of 10⁶ cells with 0.5, 1, 2, 3 or 5 μg of VEGF-A modRNA. A dose-dependent response was observed (Figure 4C) and > 80% survival could be found following *in vitro* transfection with ≤ 1 μg of VEGF-A modRNA (Figure 4D); however, > 30% cell death was observed following transfection with ≥ 2 μg of VEGF-A modRNA (Figure 4D). To evaluate whether repeated transfections with the VEGF-A modRNA can drive endothelial differentiation of the human Isl1⁺ progenitors as efficiently as the use of recombinant protein *in vitro*, protein secretion by the transfected eGFP⁺ cells was assayed at different time points following transfec-
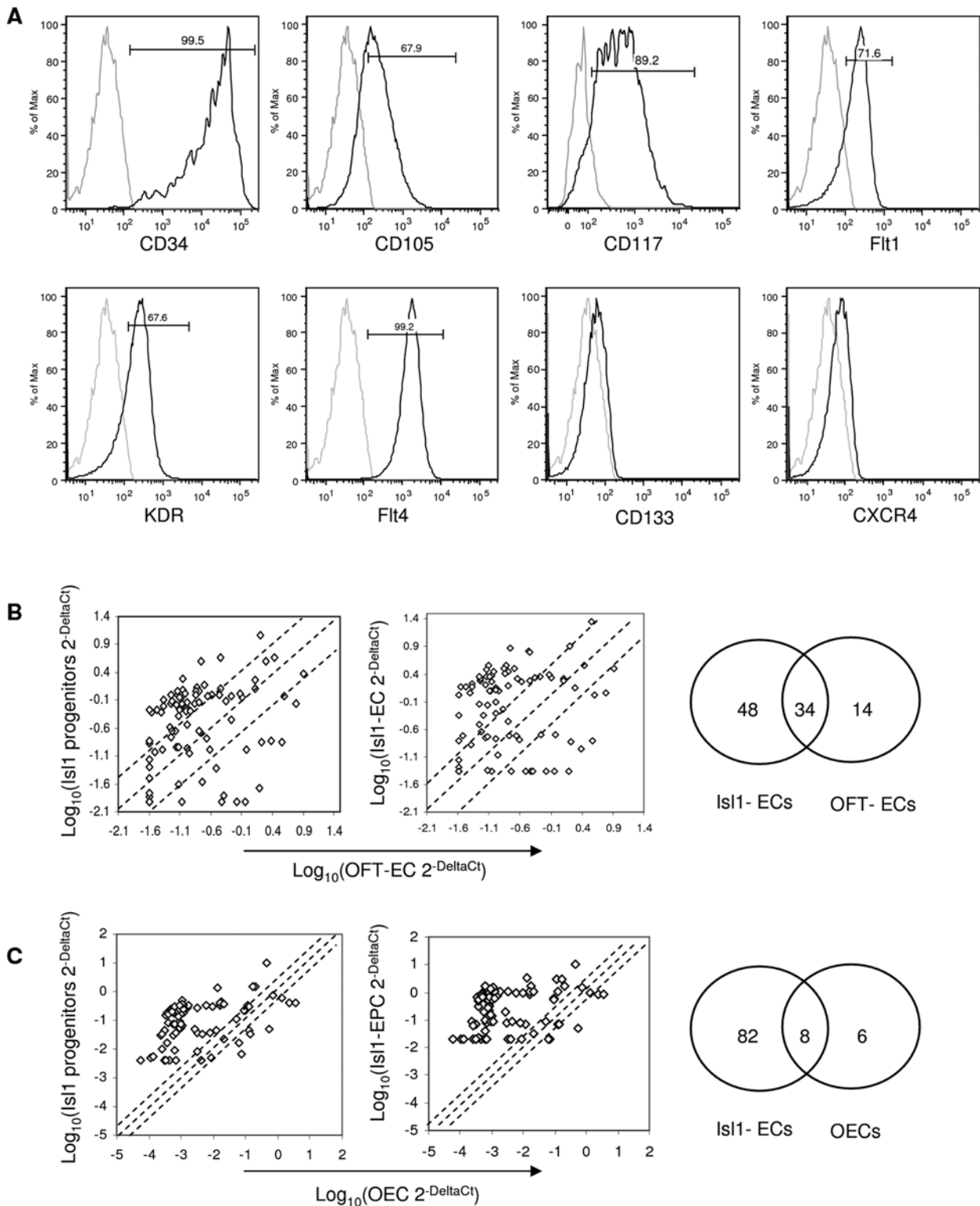
Figure 3 VEGF-A-treated human Isl1^{+} progenitors express EC markers and secrete angiocrine factors in a similar pattern to the human outflow tract-derived ECs. **(A)** FACS analyses on the purified CD144^{+}CD31^{+} ECs from the human Isl1-cre eGFP^{+} cells (Isl1-ECs) in the presence of VEGF-A from day 7-21. **(B**, **C)** Correlation of angiocrine gene expression between qPCR data obtained from the day-7 purified human Isl1-cre eGFP^{+} cells (Isl1 progenitors) or Isl1-ECs in the presence of VEGF-A from day 7-21 and **(B)** the purified CD144^{+}CD31^{+} ECs from the outflow tract of human fetal hearts at 10 weeks of gestation (OFT-ECs) or **(C)** the noncardiac, human cord blood-derived outgrowth endothelial cells (OECs) (*n* = 5).
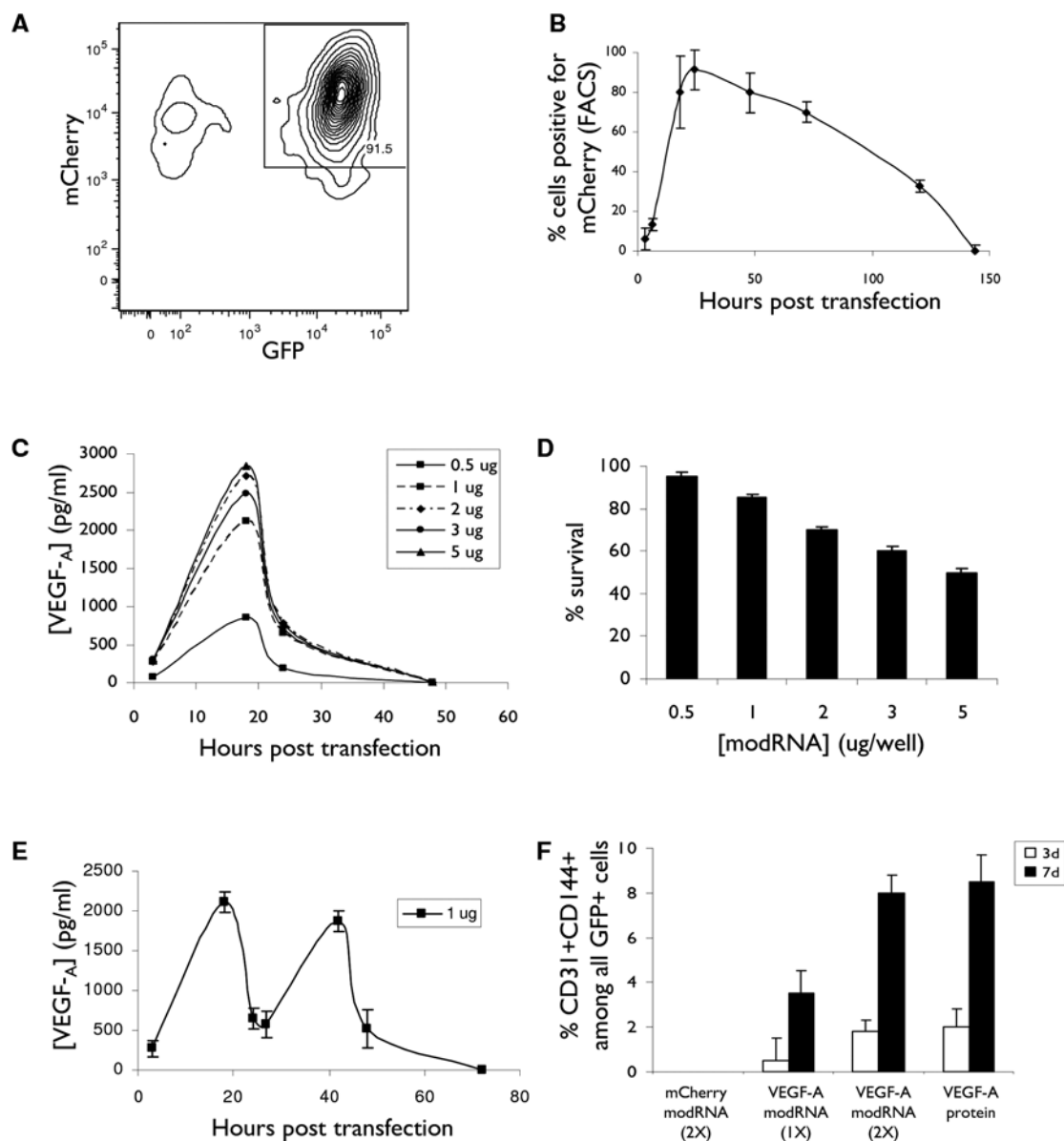
Figure 4 Endothelial differentiation of the human Isl1^{+} progenitors can be achieved by repeated transfections of the VEGF-A modRNA. **(A)** FACS result showing expression of mCherry in the day-7 purified human Isl1-cre eGFP^{+} cells 24 h post transfection of the mCherry modRNA (1 μg/10[6] cells). **(B)** Quantification of mCherry expression by FACS showing percentage of mCherry[+] Isl1-cre eGFP^{+} cells following 0-150 h post transfection. **(C)** ELISA analyses using VEGF-A-containing supernatant (refreshed 6 h before collection) cultured with the day-7 purified human Isl1-cre eGFP^{+} cells following 0-72 h post transfection of different concentrations of the VEGF-A modRNA. **(D)** Trypan blue staining showing the survival percentage of the day-7 purified human Isl1-cre eGFP^{+} cells following 24 h post transfection of different concentrations of the VEGF-A modRNA. **(E)** ELISA analyses showing the rate of VEGF-A secreted by the day-7 purified human Isl1-cre eGFP^{+} cells (supernatant refreshed 6 h before collection) following two rounds of daily transfections of 1 μg/10[6] cells with VEGF-A modRNA. **(F)** FACS analyses showing the efficiency of CD144^{+}CD31^{+} EC differentiation by one transfection or repeated transfections (following medium change) of the VEGF-A modRNA compared to the use of VEGF-A protein 3 or 7 days post treatment.

tion with the VEGF-A modRNA (1 μg/10[6] cells, medium was refreshed 6 h before collection). Our results show that the highest expression level of VEGF-A can be maintained through daily transfections (Figure 4E); however, the accumulated VEGF-A protein is stable for at least 3 days in culture as assayed by ELISA (data not
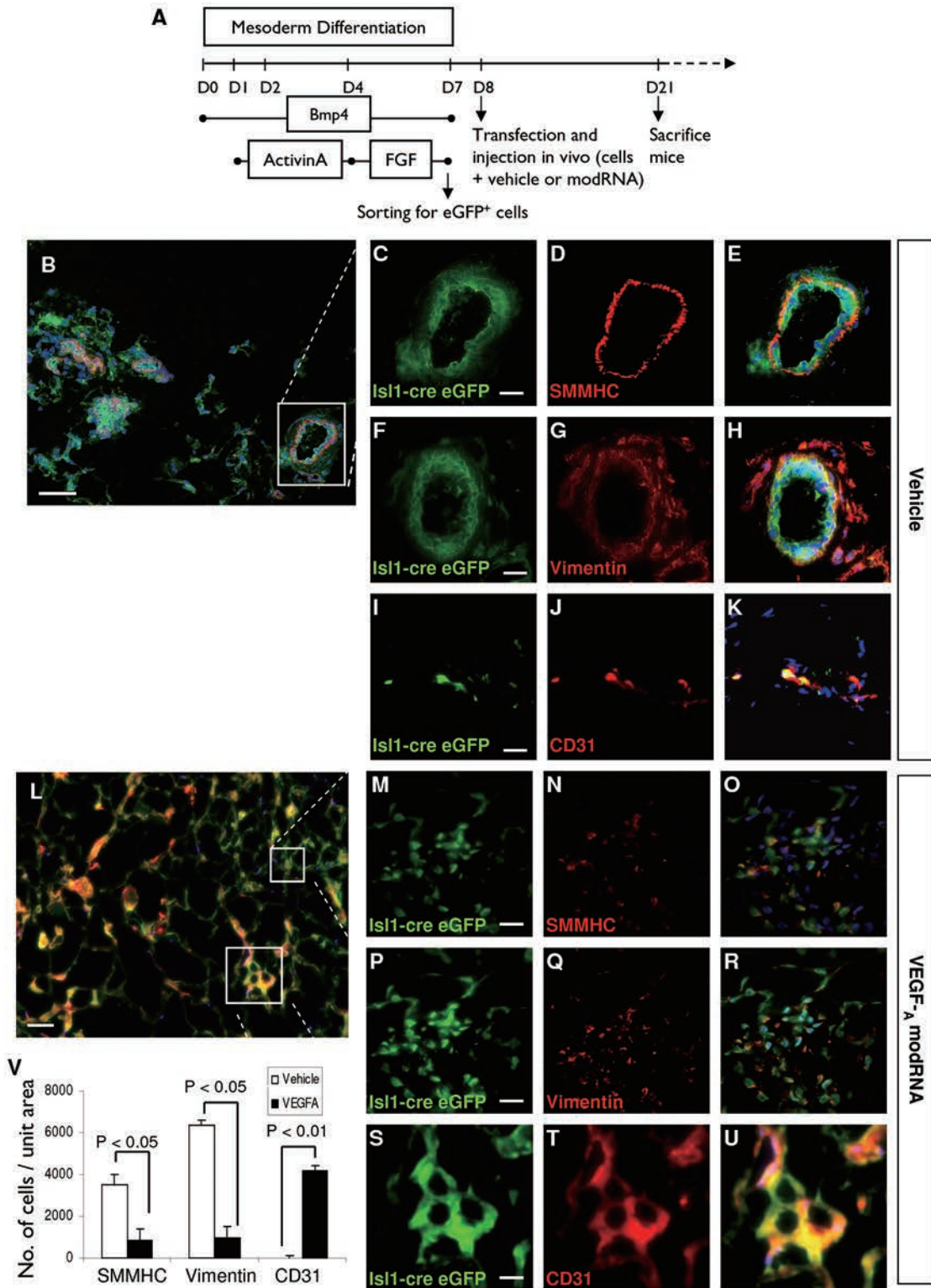
Figure 5 VEGF-A drives differentiation of the human Isl1^{+} progenitors toward an EC lineage, but away from the SMC lineage *in vivo*. **(A)** Schematic diagram of the experimental design. **(B-U)** Frozen sections from matrigel plugs incubated with the day-7 purified human Isl1-cre eGFP^{+} cells in the presence of **(B-K)** vehicle or **(L-U)** VEGF-A modRNA were stained for eGFP with smooth muscle- or endothelial cell-specific markers (scale bar in **C** = 100 μm, scale bar in **M** = 50 μm, scale bars in all the remaining panels = 25 μm). **(V)** Quantification analyzed by ImageJ showing number of double positive cells (eGFP^{+}SMMHC^{+}, eGFP^{+}Vimentin^{+} or eGFP^{+}CD31^{+}) per unit area of the matrigel plug following treatment with vehicle or VEGF-A modRNA.

shown), thus repeated transfections may be needed only when the culture medium was refreshed. Approximately 8-10% eGFP+CD31+CD144+ cells were differentiated from the day-7 purified eGFP+ progenitors following repeated transfections, which was comparable to the use of recombinant VEGF-A protein (n = 5, Figure 4F).

VEGF-A modRNA promotes cell-fate shift, proliferation and survival (reduced apoptosis) of the human Isl1+ progenitors in vivo

To examine the role of VEGF-A in driving endothelial differentiation of the human Isl1+ progenitors *in vivo*, day-7 purified eGFP+ progenitors were implanted subcutaneously (s.c.) in the vehicle- or VEGF-A modRNA-containing matrigel in NOD/SCID mice for 2 weeks (Figure 5A). Frozen sections of the vehicle- or VEGF-A modRNA-treated matrigel plugs were stained for the SMC (SMMHC, Vimentin), EC (CD31), or CM (cTNT) markers. Our immunostaining results reveal that there were more eGFP+SMMHC+ (Figure 5C-5E vs 5M-5O) or eGFP+Vimentin+ (Figure 5F-5H vs 5P-5R) smooth muscle-like structures (Figure 5B) in the vehicle-treated than the VEGF-A modRNA-treated group (Figure 5V), whereas there were more eGFP+CD31+ (Figure 5S-5U vs 5I-5K) microvessel-like structures (Figure 5L) in the VEGF-A modRNA-treated than the vehicle-treated group (Figure 5V). No eGFP+cTNT+ cells were observed in both groups (data not shown). Furthermore, we employed matrigel cytometry to quantify angiogenesis as previously described [25]. Matrigel provides an immediate source of growth factors and nutrients for EC differentiation and, therefore, a subset of eGFP+CD31+CD144+ population can be found in the vehicle-treated group. Importantly, our data reveals that there was an increase in the eGFP+CD31+CD144+ population from 6.46% in the vehicle-treated group to 16.4% in the VEGF-A modRNA-treated group (Figure 6A), indicating that VEGF-A can indeed drive differentiation of the human Isl1+ progenitors into Isl1-ECs *in vivo*. By calculating the number of eGFP+ cells isolated from the matrigel plugs, our data show that approximately 20 and 5% of the original implanted eGFP+ cells could be recovered in the modRNA-treated and vehicle-treated matrigel plugs, respectively (Figure 6C). To evaluate why a greater number of eGFP+ cells could be harvested from the modRNA-treated group, frozen sections of the vehicle- or VEGF-A modRNA-treated matrigel plugs were stained for Ki67 and Tunel (Figure 6B). There were more proliferating eGFP+Ki67+ (P < 0.05, n = 5) and less apoptotic eGFP+Tunel+ (P < 0.05, n = 5) cells in the VEGF-A modRNA-treated group than the vehicle control group. Taken together, our results reveal that VEGF-A not only promotes endothelial specification of the Isl1+ progenitors but also contributes to increased proliferation and reduced apoptosis of their daughter cells *in vivo*.

Discussion

Isl1+ endothelial progenitors and their intermediates in human cardiogenesis

The generation of diverse cardiac, smooth muscle and EC lineages in distinct compartments of the human heart is based on the expansion of multipotent cardiovascular progenitors and the formation of a diverse subset of cellular intermediates (reviewed in [18]). In contrast to the murine hearts, in many cases, these human cardiovascular intermediates coexpress early progenitor markers (e.g., Isl1), as well as differentiated markers (e.g., cTnT or SMMHC), suggesting that they are already committed towards a defined cardiac or SMC lineage [16]. In the current study, we have identified subsets of the Isl1-expressing endothelial intermediates (Isl1+CD144+ or Isl1+vWF+) in OFT region of the early human fetal hearts (gestation week 9), in addition to the previously reported human Isl1-expressing smooth muscle and cardiac myocyte intermediates [16]. Moreover, we have employed a well-characterized human ESC line that harbors a knock-in of Cre recombinase in the Isl1 locus, allowing lineage tracing of the downstream cellular intermediates. By sorting for the GFP reporter driven by the Cre recombinase expression and the differentiated EC surface markers such as CD144 and CD31, we were able to purify human cardiac-specific endothelial progenitors and to characterize them at transcriptional, translational and functional levels *in vitro* and *in vivo*.

In this study, we defined a comprehensive cardiovasculogenic signature of Isl1-ECs using FACS, immunostaining and transcriptional approaches. Isl1-ECs expressed protein markers previously defined as putative EPCs (CD34, CD105, CD117 and CD133) and ECs (CD31, CD144, eNOS, VEGFR1/2/3 and vWF). There is no known transcription factor expressed exclusively in EPCs or ECs [26]. Previous studies have shown that Id1 and Id3 double knockout [27], Foxc1−/− [28], Etv2−/− [29, 30] and Hoxa9-deficient [31] mice have severe vascular defects. Consistent with previous findings, Isl1-ECs expressed *Id1*, *Foxc1*, *Etv2* and *Hoxa9*, which are indicators of EPC development and/or EC lineage commitment. Moreover, Isl-ECs upregulated cardiac-specific genes, including *Gata4*, *Isl1*, *Mef2c*, *Nkx2.5*, *Tbx1*, *Tbx5* and *Tbx20*, compared with the noncardiac OECs. Intriguingly, Isl1-ECs also expressed Wt1, which is a cardiac-specific endothelial progenitor marker [20, 32]. Nevertheless, more studies are needed to elucidate
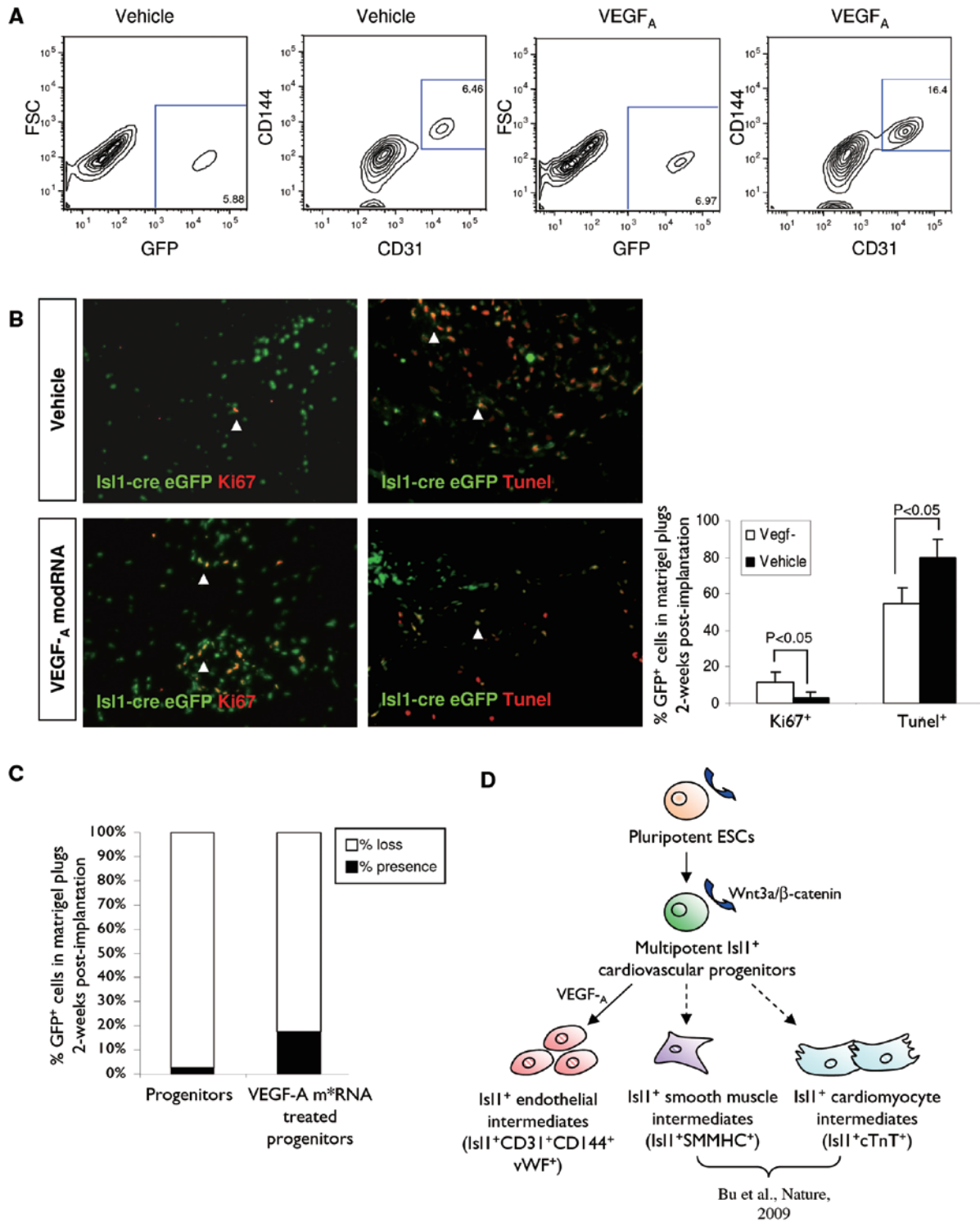
Figure 6 VEGF-A drives endothelial differentiation and promotes survival via increased proliferation and reduced apoptosis of the human Isl1^{+} progenitors *in vivo*. **(A)** FACS analyses to determine the percentage of CD144^{+}CD31^{+} ECs differentiated from the human Isl1-cre eGFP^{+} cells isolated from matrigel plugs following incubation in the presence of vehicle or VEGF-A modRNA 2 weeks post s.c. implantation. **(B**, **C)** Cell counting to determine the percentage of proliferation (eGFP[+]Ki67^{+} by immunostaining), apoptosis (eGFP[+]Tunel[+] by immunostaining) or survival (total eGFP^{+} cells by FACS) of the human Isl1-cre eGFP^{+} cells in the vehicle- or VEGF-A modRNA-treated matrigel plugs two weeks post s.c. implantation. **(D)** Model for the proposed cell fate switch of the human Isl1^{+} progenitors following VEGF-A treatment.

the molecular, cellular phenotypes and functional properties of cardiac-specific ECs compared with ECs in other organ systems.

VEGF-A is an endothelial cell-fate switch for multipotent heart progenitors

Although *in vivo* lineage tracing during murine cardiogenesis was able to indicate that a substantial portion of ECs in the mammalian heart are derived from the Isl1[11] heart progenitors [11], a direct demonstration in human hearts via clonal assays was found difficult due to inefficient differentiation of the Isl1^{+} progenitors toward the endothelial lineage. In our experience, Isl1^{+} progenitors isolated from murine embryos and ESCs, or human ESCs appeared to differentiate spontaneously into SMCs and, to some extent, CMs. Indeed, by transplanting the Isl1-expressing EBs under the kidney capsule of NOD/SCID mice, Isl1^{+} progenitors differentiated spontaneously into smooth muscle- or cardiac muscle-like cells. Although the Isl1-derived SMCs, CMs and ECs can be found in the Isl1-cre;R26R murine hearts [11], exogenous signals are needed to drive endothelial differentiation of the progenitors away from the more spontaneously differentiated smooth muscle and cardiac muscle lineages.

A recent study has documented that ventricular endocardial cells are capable of forming the coronary arteries by angiogenesis through myocardial-endothelial VEGF signaling [8]. In fact, CMs but not ECs provide the major source of VEGF [33], indicating the importance of paracrine signaling in facilitating blood vessel development in the heart. In this study, we report that a single paracrine factor VEGF-A can drive endothelial differentiation of the multipotent Isl1^{+} progenitors both *in vitro* and *in vivo*. By ablating Flk1 in the Isl1-cre mice, a reduction in the number of Isl1-ECs was observed in the endocardium [6], suggesting that Flk1 is required for the formation of the endocardium. Our result is consistent with the previous murine study that KDR was required for the VEGF-A-induced endothelial differentiation of the human Isl1^{+} progenitors. Although the loss of Isl1-ECs in the endothelium of Isl-cre;Flk1 null mice is replaced by vascular ECs [6], we are unsure whether such a compensational growth is sufficient to compensate for functions of the organ-specific ECs. We found that cardiac ECs expressed more angiocrine factors than noncardiac vascular progenitors such as OECs. Moreover, VEGF-A was the predominant ligand expressed by OFT-ECs, whereas PLGF was the predominant ligand expressed by noncardiac OECs. PLGF binds to KDR with a higher affinity than VEGF-A, indicating that the differential expression of different members of the VEGF family may also imply a functional difference among ECs derived from various organ systems. Our results are also consistent with another study that reports that cardiac ECs may have better angiogenic properties than noncardiac ECs [34]. Retroviral gene delivery of Isl1 to ECs enhances their angiogenic properties including augmentation in proliferation of ECs, adhesion to fibronectin, secretion of IL-1β and VEGF, migratory index and vascular density in the matrigel plug *in vivo* when compared with the untreated control [34].

Chemically modified VEGF mRNA promotes heart progentior vascularization, proliferation and survival following transplantation

To directly examine the role of VEGF-A in driving endothelial specification of the cardiovascular progenitors *in vivo*, we utilized a novel modRNA delivery approach to mediate highlyefficient expression of autocrine factors by the Isl1^{+} progenitors. *In vivo* transgene delivery that utilizes naked DNA plasmids or engineered viral vectors has limited efficacy and safety with low transfection efficiency, uncontrollable dose release, antiviral immunity and some risk of genome recombination or insertional tumorigenesis, whereas delivery of recombinant proteins also has other problems including short protein half-life [35-38]. On the other hand, chemical modification with the replacement of uridine and cytidine with 2-thiouridine and 5-methylcytidine, respectively, facilitates *in vivo* delivery of mRNA by increasing stability and avoiding activation of the innate immunity commonly associated with transcribed mRNA [23, 24]. The VEGF-A modRNA has been demonstrated in a parallel study *in vivo* with proangiogenic effect when directly injected intramuscularly into infarcted hearts [17]. The VEGF-A modRNA directs expansion and differentiation of the epicardial progenitors toward an EC fate, and triggers regeneration of the heart following MI via increased angiogenesis, reduced fibrosis and post-infarct remodeling, and increased survival of the recipients for at least 1 year following a single injection of modRNA. With the success of this parallel study, we reasoned that VEGF-A modRNA could also represent an improved gene delivery platform to direct cell fate decision of the human cardiovascular progenitors *in vivo*. Human Isl1^{+} progenitors were implanted subcutaneously in the vehicle- or VEGF-A modRNA-containing matrigel in NOD/SCID mice for 2 weeks. Our results reveal that there were more SMMHC^{+}/Vimentin^{+} smooth muscle-like structures in the vehicle-treated group than the VEGF-A modRNA-treated group, whereas more CD31^{+} microvessel-like structures were found in the VEGF-A modRNA-treated group than the vehicle-treated group. Taken together, our result has demonstrated a direct effect of VEGF-A in

driving endothelial specification of the human Isl1^{+} cardiovascular progenitors both *in vitro* and *in vivo*, away from the spontaneous smooth muscle and cardiac muscle differentiation pathways (Figure 6D). Moreover, VEGF-A can also promote proliferation and reduce apoptosis of the engrafted cells. Hence, it will be of interest to determine whether driving vascularization using human pluripotent stem cell-derived endothelial progenitors will have beneficial effects in the setting of cardiac injury. In addition, future studies would be warranted to determine whether transfection of VEGF-A modRNA into other tissue-specific progenitors may enhance their capability to engraft and survive in disease models of other solid organs.

Materials and Methods

Human ESC culture, EC differentiation and flow cytometry

The human Isl1-cre eGFP lineage-tracing ESC line was engineered and maintained as described previously [16]. To generate ECs via formation of EBs, a sequential administration of cytokines was implemented. Briefly, human ESCs were treated with 0.5 mg/ml dispase (Gibco) and then suspended in six-well ultra-low cluster plates (Costar) with human ESC differentiation medium containing DMEM/F12 (Gibco) supplemented with 10% knockout serum replacement (KOSR, Invitrogen), 1× non-essential amino acids (Gibco), 1× l-glutamine (Invitrogen), 1× penicillin/streptomycin (Invitrogen) and 1× β-mercaptoethanol (Gibco). On day 0, medium was supplemented with 20 ng/ml BMP4 (R&D Systems) (removed on day 7); on day 1, medium was supplemented with 10 ng/ml activinA (R&D Systems) (removed on day 4) and on day 4, EBs were transferred to adherent, Matrigel-coated plates with human ESC differentiation medium containing 1% KOSR and 8 ng/ml FGF-2 (Peprotech) (removed on day 7). From day 4 onwards, cells were incubated in hypoxia (5% oxygen). On day 7, cultures were dissociated using 0.25% Trypsin (Gibco) and the eGFP^{+} cells were purified using FACSAriaII (BD). eGFP^{+} cells were then cultured in human ESC differentiation medium containing 1% KOSR and 100 ng/ml VEGF-A (Peprotech) until the indicated time points. ECs were stained with fluorochrome-conjugated anti-CD31, anti-CD34, anti-CD105, anti-CD133, anti-CD144, anti-Flt1, anti-KDR, anti-Flt4, and anti-CXCR4 antibodies (BD), and then purified or analyzed using FACSAriaII. To generate cells for clonal assays, cells on day 7 were dissociated into single cells using 0.25% trypsin-EDTA and stained with propidium iodide (PI, BD) to exclude dead cells for sorting on the FACSAriaII. The purified eGFP[+]PI^{−} cells were plated on 24-well plates with irradiated MEF feeders and cultured in human B27 medium supplemented with 100 ng/ml VEGF-A. After 5 days of culture, colonies were picked for RNA isolation.

Isolation of ECs from human fetal hearts and human cord blood

Human fetal hearts and umbilical cords (from authorized resources) were dissociated to achieve a single-cell suspension by collagenase II (Sigma-Aldrich), OFT-ECs were isolated by FACS [39] via costaining with anti-CD31 and anti-CD144 antibodies (BD), and the double positive cells were purified using FACSAriaII. Viability dyes including PI or DAPI was also used to exclude dead cells. OECs were isolated by differential plating as described previously [40-42]. OECs expressed EC-specific surface markers, including CD31, CD144, CD146 vWF and VEGFR-2, and could improve peripheral perfusion in a murine hindlimb ischemia model [41]. OFT-ECs, OECs and HMVECs were cultured in EGM2 medium (Lonza).

Preparation of the modRNA for in vitro and in vivo transfection

Production of *in vitro* transcription template constructs (Supplementary information, Table S1) and subsequent mRNA synthesis has been described previously [24]. Briefly, open reading frames were amplified by PCR from plasmids encoding mCherry or human VEGF-A (165) (Addgene). RNA was synthesized with the MEGAscript T7 kit (Ambion), and a custom ribonucleoside blend was used comprising 3′-O-Me-m7G(5′)ppp(5′)G cap analog (New England Biolabs), adenosine triphosphate and guanosine triphosphate (USB), 5-methylcytidine triphosphate and pseudouridine triphosphate (TriLink Biotechnologies). For transfection, modRNA and RNAiMAX transfection agent were each dissolved separately in Opti-MEM (Invitrogen), combined, and then incubated for 15 min at room temperature to generate a transfection mixture. *In vitro* transfection was performed by adding the transfection mixture (1 µg modRNA) to cells plated in DMEM supplemented with 2% FBS and 200 ng/ml B18R (eBioscience) or in Pluriton Reprogramming Medium (Stemgent). Repeated transfections could be performed after medium change in cultures. *In vivo* transfection was performed by adding the transfection mixture (5 µg modRNA) directly into the cell-containing matrigel before s.c. implantation into NOD/SCID mice (Jackson lab).

RNA isolation and gene expression profiling

Total RNA was isolated from purified cells of different groups using the RNeasy mini kit (Qiagen) and reverse transcribed using Superscript III RT (Invitrogen), according to the manufacturers' instructions. Real-time qPCR profiling was performed using the angiogenesis growth factor expression arrays (SA Biosciences) analyzed on Mastercycler realplex 4 Sequence Detector (Eppendoff) via SYBR Green (QuantitectTM SYBR Green PCR Kit, Qiagen). The relative gene expression level of each sample was expressed as a relative quantitation (RQ) value determined by the 2^{-ddCT} method that represents the fold change in gene expression normalized to the housekeeping gene, *Gapdh*. The relative gene expression level of each sample was also compared with an internal control. Complementary DNA PCR primer sequences are shown in Supplementary information, Table S2.

Immunohistochemistry

Human fetal hearts were obtained from authorized sources and the pericardium was removed. Human fetal hearts, matrigel plugs or teratoma samples were freshly fixed in 4% paraformaldehyde at 4 °C overnight. The hearts were washed four times with PBS at 4 °C and equilibrated in 30% sucrose before freezing and cryosectioning. Six micrometer sections were blocked at 2% goat serum and then stained with different primary antibody combinations at 10 µg/ml at 4 °C overnight. Primary antibodies used for staining are: mouse anti-ISL1 (Developmental Studies Hybridoma Bank),

rabbit anti-cTNT, rabbit anti-CD31, rabbit anti-CD144, chicken anti-eGFP, rabbit anti-Flt1, mouse anti-SMMHC, rabbit anti-vWF (Abcam), mouse anti-Ki67 (BD), rabbit anti-KDR/Flk1 (Upstate) and mouse anti-Vimentin (R&D systems) antibodies. Alexa-Fluor-488- or Alexa-Fluor-594-conjugated secondary antibodies (Molecular Probes) were used for 30 min at room temperature, and 500 µg/ml of DAPI (Vector Labs, US) was used at room temperature for 5 min in the dark. Slides were mounted with fluorescence mounting medium (Dako, US) and allowed to dry at 4 °C overnight in the dark. For the TUNEL assay, an *in situ* cell death detection kit, fluorescein (Roche) was used according to manufacturer's instructions. Fluorescence was detected with the laser scanning confocal microscope (Zeiss, Germany) and quantification of the immunostaining images was performed by the blind cell counts (10-20 sections or > 1 000 cells per group) using the ImageJ software.

Tube formation assay

Fifteen thousand to twenty thousand of GFP⁺CD31⁺CD144⁺ ECs differentiated from Isl1-cre eGFP human ESCs were cultured in 50 µl of matrigel (BD) and 200 µl of EGM2 medium (Lonza) in each well of a 96-well plate overnight. Three dimensional tubes were visualized and fluorescence images were taken next day under the laser scanning confocal microscope.

Gene nomenclature

Gene symbol	Gene name
Cd31 (Pecam)	Platelet endothelial cell adhesion molecule
c-TnT	Cardiac troponin T
eNos	Endothelial nitric oxide synthase
Etv2 (Er71)	Ets transcription factor family member
Flk1 (KDR)	Fetal Liver Kinase 1/Kinase insert domain receptor
Foxc1	Forkhead box C1
Gata4	GATA transcription factor family 4
Hoxa9	Homeobox protein Hox-A9
Id1	DNA-binding protein inhibitor 1
Isl1	Islet-1
Mef2c	Myocyte-specific enhancer factor 2C
Nfatc-1	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
Nkx2.5	Homeobox protein Nkx-2.5
Plgf	Placenta growth factor
Smmhc	Smooth muscle myosin heavy chain
Sm22-α	Smooth muscle protein 22-alpha
α-Sma	Alpha smooth muscle actin
Tbx1	T-box transcription factor 1
Tbx5	T-box transcription factor 5
Tbx20	T-box transcription factor 20
Vegfa	Vascular endothelial growth factor-A
Wt1	Wilm's tumor gene-1

Other genes are listed in Supplementary information, Figure S1B.

In vivo matrigel plug assay and matrigel cytometry

All animal procedures were performed in accordance with protocols approved by the Massachusetts General Hospital. FACS-purified, day-7 eGFP⁺ cells derived from the Isl1-cre eGFP human ESC line were resuspended in the vehicle- or VEGF-A modRNA (1 µg/10⁶ cells)-supplemented matrigel and were then s.c. injected into the NOD/SCID mice ($n = 5$ for each group). The matrigel plugs were harvested 1-2 weeks post implantation. Human ECs from matrigel plugs can be isolated for FACS analyses as described previously [25]. Matrigel plugs were incubated for 1 h at 37 °C in PBS supplemented with 25 µg/ml hyalurinidase (MP biomedical), 25 µg/ml DNase (Sigma), 3 unit/ml Dispase and 3 unit/ml Liberase (Roche). The isolated cells were filtered using a 40 µm nylon mesh (BD), washed three times with FACS buffer (PBS supplemented with 1% BSA) and were subsequently stained with pacific blue-conjugated anti-CD31 and APC-conjugated anti-CD144 antibodies (BD) for FACS analyses. Viability dyes including PI or DAPI were also used to exclude dead cells for sorting.

Statistical analyses

The data were expressed as arithmetic mean ± SD of triplicate determinations performed under the same conditions. Statistical analysis was performed using the Student's *t*-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)