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Supplemental Information

Enhanced Hemangioblast Generation and Improved Vascular Repair and Regeneration from Embryonic Stem Cells by Defined Transcription Factors

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Inventory of Supplemental Information

Figure S1, related to Figure 1;
Figure S2, related to Figure 1;
Figure S3, S4 and Table S1, related to Figure 3;
Figure S5, related to Figure 5;
Figure S6, related to Figure 6-7;
Figure S7, related to Figure 6-7;
Table S1, Groups of affected pathway by co-expression of ER71, GATA2 and Scl, related to Figure 3;
Table S2, Primer Sets Used for qRT-PCR in This Study, related to Figure 1, 3-7;
Table S3, Primers for ChIP-PCR analysis, related to Figure S4;
Table S4, The Sequence of Morpholinos (MOs), related to Figure 2.
Figure S1. ER71, GATA2 and SCL as potent enhancers of FLK-1⁺ hemangioblast generation from ES cells, related to Figure 1. (A) Genes preferentially expressed in the FLK-1⁺SCL⁺ hemangioblast. FLK-1⁻SCL⁻, FLK-1⁺SCL⁻, FLK-1⁺SCL⁺, were sorted from day 3 Scl<sup>chrCD4</sup> EBs. Each cell population, a pool of sorted cells from multiple experiments, was subjected to gene expression analysis by qRT-PCR. qRT-PCR assays performed in triplicate, normalized to Gapdh, and analyzed according to the ∆∆CT method. (B) A2lox ES cells or R1 ES cells were differentiated and analyzed for FLK-1 and PDGFRα expression by FACS on day 3 and 4. Numbers indicate the percentage of live-gated cells within each quadrant. Representative results from three independent experiments are shown. (C) Cell cluster score of microtiter wells containing adherent and round hematopoietic cells 4 days following transfer of the blast colony. The percentage of each cell type, hematopoietic, adherent and both cell types, derived from 136 (-DOX) and 145 (+DOX) blast colonies from three independent replatings is shown. -DOX, 17/136, hematopoietic; 14/136, adherent; 105/136, both cell types. +DOX, 19/145, hematopoietic; 12/145, adherent; 114/145, both cell types. Note that ~70% of the colonies contained both adherent and round hematopoietic cell clusters regardless DOX treatment. (D) VE-CADHERIN (Red) and SMAα (green) co-staining of the single blast colony derived adherent cells. Scale bar, 50µm. (E) Statistical analysis of hematopoietic and endothelial cell outcome of the FLK-1⁺ progeny on OP9. See also Figure 1J. *p<0.05, **p<0.01, 3 independent experiments. (F) iEGS ES cells were differentiated for 3 days, with or without DOX (1 ug/ml) added on day 2. At day 3, FLK-1⁺ cells are sorted, infected with GFP retrovirus, cocultured with OP9 cells in 1:5 ratio in hanging-drops for overnight. Cells are harvested the next day, mixed in with matrigel, and subcutaneously injected into NSG mice (1 x 10<sup>6</sup> cells/mouse). After 2 weeks, matrigel plugs were harvested, fixed, cryosectioned, and subjected to immunofluorescence. CD31 and
SMAα staining (G), quantification of the GFP⁺CD45⁺ cells in matrigel plugs was determined by FACS (H). G is a representative image from 8 independent matrigel plugs (–DOX and +DOX). Error bars in H represents S.D of 2 independent matrigel plugs (–DOX) or 3 independent matrigel plugs (+DOX). Scale bar, 20 µm for CD31 staining; 50 µm for SMAα staining. Dotted lines in yellow indicate the vessel-like structure and stars indicate EGS-induced FLK-1⁺ derived cells.
Figure S2. FLK-1 and PDGFRα expression pattern is not altered by various candidate factors, related to Figure 1. Inducible ES cell lines (A2lox ES cells targeted with the indicated cDNAs) of the candidate factors were treated with DOX (1 μg/ml) between days 2 and 4 of differentiation. Cells were analyzed by FACS for FLK-1 and PDGFRα (A), hCD4 (SCL) and FLK-1 (B) expression. On day 3 and 4. Numbers indicate
the percentage of live-cell gated within each quadrant. Representative results from three experiments are shown.
Figure S3. ER71 functions upstream of Gata2 and Scl, related to Figure 3. (A) Kinetic analysis of \( Er71, Gata2 \) and \( Scl \) expression in \( Er71^{-/-} \) and wild-type EBs by qRT-PCR. Genes were normalized against \( Gapdh \), \( **p < 0.01 \), compared to \( Er71^{-/-} \) EBs, 3 independent experiments. (B) Inducible ES (\( iEr71, iGata2 \) or \( iScl \)) cells were differentiated in serum with doxycycline added on day 2. Expression levels of \( Er71, Gata2 \) and \( Scl \) were normalized to \( Gapdh \), and then the ratio of the gene quantity (+DOX) to gene quantity (-DOX) was determined to yield fold change shown on the y-axis. Error bars indicate S.D from three independent experiments.
Figure S4. ER71 binds to the Scl promoter region, related to Figure 3. (A) Screening the sequence of consensus Ets transcription factor binding sites in evolutionary conserved region. (B) ChIP assays of ER71 recruitment in Scl gene. iEr71-V5 ES cells
were differentiated in serum with doxycycline addition on day 1. EBs were collected on day 3 and performed ChIP-PCR analysis. Numbers on the x-axis indicate the locations of amplicons of each qPCR primer set on Scl. The value normalized against IgG IP values is shown on the y-axis. Error bars indicate S.D from three independent experiments. (C) Ets binding-sites in evolutionarily conserved area H4 is shown.
Figure S5. Enhanced cell viability, expression of hypoxia-induced survival factor, angiogenic paracrine factor, and preservation of ECM structures in FLK-1\(^{+}\)(+DOX)+hMSC spheroid, related to Figure 5. Quantification of (A) Bcl-2 and (B) p53 expression determined by qRT-PCR (*p < 0.01, compared to the FLK-1\(^{+}\)(+DOX) spheroid group, 3 independent experiments). The spheroids generated between EGS-induced FLK-1\(^{+}\) and hMSCs in different ratios (1:1, 1:5, and 1:10) are shown in x-axis.
(C) Mitochondrial metabolic activity determined by MTT assay (*p < 0.01, compared to the FLK-1*(+DOX) spheroid group, 3 independent experiments). (D) Immunofluorescent staining of HIF-1α and angiogenic paracrine factors (VEGF, FGF2, and HGF). Scale bar = 300 µm. (E) Quantification of HIF-1α expression determined by qRT-PCR (*p < 0.01, compared to the FLK-1*(+DOX) spheroid group, 3 independent experiments). (F) Profiles for the cumulative release of angiogenic paracrine factor, HGF from single cells and spheroids as determined by ELISA (*p < 0.01, compared to the FLK-1*(+DOX) spheroid group, 3 independent experiments). (G), (H) Quantification of relative ECM amount determined by densitometer (*p < 0.01, compared to the FLK-1*(+DOX)+hMSC, 1:5, 3 independent experiments).
Figure S6. Improvement of ischemic hindlimb salvage and angiogenesis in the lesion by transplanting reduced amount of FLK-1\(^{+}\)(+DOX)+hMSC spheroids, related to Figure 6-7. (A) Representative photographs and physiological status of ischemic hindlimbs 28 days after cell transplantation. All photographs were taken at the
same magnification. Blood perfusion ratio of ischemic limbs was also measured by laser Doppler imaging at day 0, 7, 14, 21, and 28 after treatments. *$p < 0.01$ compared to the no treatment group, 8 mice/group. (B) H&E staining and Masson’s trichrome staining of histological ischemic limb sections 28 days after treatments (Blue indicates fibrosis, scale bar = 200 µm). The percentage of fibrotic area is shown, *$p < 0.01$ compared to the no treatment group, 8 mice/group. Total amount of microvessels were determined by CD31 and SMA$\alpha$ immunoblot and qRT-PCR analysis. (C) Immunofluorescent staining of CD31 and SMA$\alpha$, and quantification of microvessels in ischemic hindlimb tissues at 28 days after reduced amount of FLK-1$^+$ (+DOX)+hMSC spheroids transplantation. Arrows points the typical vessel-like structure. Scale bar indicates 200µm in low magnification and 50µm in high magnification (*$p < 0.01$ compared to the no treatment group, 8 mice/group).
Figure S7. Endothelial and smooth muscle cell restricted potential of EGS induced FLK-1\(^+\)-hMSC spheroids, related to Figure 6-7. (A) The contribution of FLK-1 cells to the vessels as shown by Y chromosome-positive (Green) cells. Scale bar, 100 µm. (B) Series of single fluorescent channel with merged images for DAPI, Y-chromosome and CD31 of ischemic hindlimb tissues at 28 days after FLK-1\(^+\)(+DOX)+hMSC spheroids transplantation. Scale bar indicates 100 µm. (C) H&E staining results showing teratoma
formation after injecting ES cells subcutaneously. Scale bar indicates 50 µm. (D) H&E staining results showing no teratoma formation after injecting FLK-1*(+DOX)+hMSC spheroids subcutaneously. Scale bar indicates 200 µm. (E) Immunoblot analysis for microvessels (SMA-α, CD31), cartilage (COLLAGEN Type II), and bone (OSTEOCALCINE, OSTEONECTIN).
### Table S1. Groups of affected pathway by co-expression of ER71, GATA2 and Scl, Related to Figure 3.

| Pathway map title                                                                 | Significance score [-log10 (P value)] |
|-----------------------------------------------------------------------------------|--------------------------------------|
|                                                                                  | Day 2.5    | Day 3   |
| **Epithelial-to-mesenchymal transition (EMT)**                                    |            |         |
| Development_Regulation of epithelial-to-mesenchymal transition                     | 9.41       | 9.56    |
| Development_TGF-beta-dependent induction of EMT via SMADs                          | 6.08       | 8.86    |
| Development_TGF-beta-dependent induction of EMT via RhoA, PI3K                     | 5.78       | 2.59    |
| Development_TGF-beta-dependent induction of EMT via MAPK                           | 4.82       | 3.76    |
| Development_NOTCH-induced EMT                                                     | 4.12       | 5.56    |
| **Cytoskeleton remodeling, cell adhesion**                                        |            |         |
| Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodeling                       | 3.80       | 6.22    |
| Cell adhesion_Cadherin-mediated cell adhesion                                      | 4.22       | 5.56    |
| Cell adhesion_ECM remodeling                                                       | 5.22       | 3.99    |
| Cell adhesion_Endothelial cell contacts by junctional mechanisms                   | 4.23       | 4.62    |
| **Development**                                                                   |            |         |
| Development_WNT signaling pathway. Part 2                                          | 5.99       | 5.30    |
| Development_WNT5A signaling                                                        | 3.32       | 2.03    |
| Development_BMP signaling                                                          | 2.72       | 4.50    |
| Development_VEGF-family signaling                                                  | 3.68       | 1.80    |
| Development_VEGF signaling via VEGFR2 - generic cascades                           | 2.02       | 4.06    |
| Development_Flt3 signaling                                                         | 2.08       | 4.04    |
| Development_FGF-family signaling                                                   | 3.66       | 1.72    |

### Table S1. Groups of affected pathway by co-expression of ER71, GATA2 and Scl.

Sixteen pathway maps, statistically significantly changed in day 2.5 and day 3 iEGS EBs using MetaCore enrichment analysis, belong to 4 general biological processes: epithelial EMT development, cytoskeleton remodeling and cell adhesion. These maps were used as source for composition of process-specific lists of genes with expression changes.
Table S2. Primer Sets Used for qRT-PCR in This Study, related to Figure 1, 3-7

| Primer  | Forward Sequence | Reverse Sequence |
|---------|------------------|------------------|
| Gapdh   | aggtcggtgtgaacggatttg | tgtagaccatgtagtgaggtca |
| Hand1   | ggcagctacgcacatcatac | cttggcatcgggaccatag |
| Hhex    | agtgacgaccagacagccgagc | cttagccacacccatgaa |
| Sox17   | acctcacccctcagctccaa | tgtgctgtccagaggtcag |
| Ets1    | aca gag tac ttt cgg atc aag ca | acg ctc tca aaa gag tcc tgg |
| Tie1    | ttt tct tgg cct ctc atg ttg | cgc acg atg cga tca tcc tt |
| Mixl1   | acg cag tgc ttt cca aac c | ccc gca agt gga tgt ctg g |
| Bmp4    | ttk ctc gta acc gaa tgc tga | cct gaa tct cgg cga ctt ttt |
| Bmp6    | aga agc ggg aga tgc aaa agg | gac agg ggc tgt tag aga tcc |
| Cer1    | ctc tgg gga agg cag acc tat | cca caa aca gat ccg gct t |
| Fst     | tgc tgc tac tct gcc agt tc | gtt ctg caa cac tct tcc tgg |
| Fzd7    | cgg ggc ctc aag gag aga a | gtc ccc taa acc gag cca g |
| Wnt5a   | caacgtcagagacctctca | catcctggtgccggaact |
| Wnt5b   | cac cag ttt cga cag agg c | gca tct ctc cgg cta cct atc t |
| Lef1    | tggattcccacagcgggt | cgtaggagctgcgccgacag |
| Tcf4    | atg ggc ggc aac tct tgg at | cgt agc cgc gct gat cta t |
| Itga8   | tgg ctc gga ttc cca aag gag | gtt ccc cga cca ata tgc ca |
| Esam    | tgg ctc ggg gtt tgt tgt tgt ct | tct acc gct tcc aat tgt tgt ag |
| Kdr     | ctc ttt gag cca gca atg tct | tct tgt gac cca gca atg tc |
| Flt4/Vegfr3 | ctggcaaatgtgtaactcatga | acaaccggtgtctctactg |
| Twist1  | gga caa gct gag cca gat tca | cgg aga agg cgt agc tga g |
| Snail1  | cag acg ctc cct tgt gtcc t | ggt cag caa aag cac ggt t |
| Snail3  | ggt ccc cca cta cgg gaa ac | ctc tag ggg gct act ggg att |
| Gsc     | cag atg ctc ccc tac atg aac | tct ggg tac tgt tcc tgg |
| Cdh2    | agcgcagcttacgcaaggg | tcgctgtctctactgagacttt |
| Cdh11   | tgt tgt ctc gga aca att ctt t | gcc tga ggc atc aat gtt ta |
| Elk3    | ttcttcagcagcgatgatcagc | gttgaggactgctcgccg |
| Er71    | cag agt cca gca ttc acc ac | agg aat tgt cac agc tga at |
| Gata2   | ccaccaaatccaagaagac | agactgagggaagggggt |
| Scl     | cac tag gga gta tgt ctcttg | ggt gta ggg acc atc aag aat ct |
| Lmo2    | atg tcc tgc gcc atc gaa ag | cgg tcc cct atg ttc tgc tg |
| Gata1   | atg gaa tcc aga cga gga ac | ctc ccc aca att ccc act ac |
| Runx1   | ctt cct ctc ctc cgt tgt a | ctc cgg agt aat tgt cat cg |
| Cdh5    | cac tgt tgt ggg agc acc ctt c | ggg gca gcc att cat tgt tct |
| Tie2/Tek| gacgtattaaacacagcgtctatcgg | ggggaatgtcactaaggggtccag |
| Cd31    | ctc cca gtc cga aaa tgg aac | ctt cat cca ccc ggg cta tc |
| Fli1    | atggagcggcttagatgg | gaagcgtatctctagcgtt |
| Sox7    | agatgctgggaaagctatgg | agagggagctgaggaagag |
| Sox18   | gacccgagctctgctcac | ggtaacagagttggaaa |
| Foxc2   | aacaccaacagcagctccttc | gctgtctgtgctagggcag |
| NfatC1  | tga cca ccc atg cca ctc tg | tct ccc tcc cga tgt ctg tc |
| Pdgfra  | aga aac gat cgt tgt gag atct | ttg gat gga tgg gag ttt ga |
| Pdgfrβ  | ttc cag gag tga tac cag ctt | agg ggg cgt gat gag tac g |
| Gene   | Primer Sequence  |
|--------|-----------------|
| Isl1   | atg atg gtg gtt tac agg cta ac | tcg atg cta ctt cac tgc cag |
| Mesp1  | gtc act cgg tcc tgg ttt aag | acg atg ggt ccc acg att ct |
| Mesp2  | cca cgg aac ctt cag agc tga cta aag | ata ttc agt ggg ggt cct tcc agg |
| β-major Globin | gac cca ggc cta ctt cga tag c | tga ggc tgt cca agt gat tca |
| b-H1 Globin | gaa acc ccc gga tta gag cc | gag cca agg tct cct tga ggt |
| Lyve1  | cttacctcaggatgtcctga | gtccaacacgggttttaaatg |
| Myl4   | aag aaa ccc gag cct aag cag g | tgg gtc aaa ggc aga gtc ct |
| Myl7   | ggc cca aag tgt ctc ttc taa | tgc aga tga tga cct ccc tgt |
| cTnT   | cag agg agg cca acg tag aag | ctc cag cgg gga lct tgt gt |
| SMAα   | gtccacacatcagggagtaa | tcggatacttccagcctagga |
| Tagln  | ccaacaaaggtcactctactga | atctgaggggtctacatca |
| Cnn1   | gcc cag aaa tac gac cat cag | ttc gca aag aat gat ccc gc |
| Myh11  | gtt tgt tgg tca acc cct ac | gat gtt gcc cag cat ctc at |
| HIF-1α | tcaagtctgactcagcactgtaag | tattcgaggctgtgctagctg |
| hVEGF  | gcaagaagagagggcagaatcc | acactccaggttctgctatt |
| hFGF2  | gaagagcgacacctcactcagtaag | ctcggcagttcggttcaggt |
| hHGF   | gttacttgctgatggagccta | atgatcctcagcagatag |
| mCd115 | tggccctcatgtgaggacaaagaa | tggcagagagatccgaatatac |
| Bcl-2  | ctgtctgctacgtcagctagtc | cagatcgggttcctcagtc |
| p53    | gatgactgcctggaggagct | ctgggtgcttatagggg |
| hCD31  | gctgtgtggtggaaggaggtgc | gaagtgctggaggtgtgc |
| mCD31  | gagcccaatcacgttgctttgtt | tctctctgctctctcagtt |
| hSMα   | ccagctatgtgtaagaagaggg | gtgaatctcactctgcatcaggt |
| mSMα   | tgtgtgctgactcaggtgagatg | cttctgcatctgtcagcagaa |
| β-actin | atcctgaagacactctatgc | aacagcagctcagtaacagtc |
| Gapdh  | aacatgtggcattggtgaagg | acacatgggtggtaggaaca |
Table S3. Primers for ChIP-PCR analysis, Related to Figure S4

| Primer description | Forward sequence 5’ to 3’ | Reverse sequence 5’ to 3’ |
|--------------------|---------------------------|--------------------------|
| H2                 | gaggagggcgctgccatttc      | cgccctagaattgcttctgtg    |
| H3                 | cggggagactctctctcttcc     | ctcacggcaacccactctcaac   |
| H4-1               | cagatccgtagaggggtcgc      | cccacacacatccggaattta   |
| H4-2               | ccacctccctctctctccac      | acttagctggcttgtaggtc     |
| H6                 | ctggaggttttcaggttctc      | cctctagttgctgctactcg    |
| H7                 | cttcccatagttgctcttttgg    | ctttcacccggttggtggt     |
| H8-1               | aatgagatctccgcccttg       | tccctctctggtcattgag     |
| H8-2               | ctgtggcagctctctggat       | aagtttgctggtggttgg      |
| H8-3               | gggatgagaagcaggtcaat      | gcctaggggtcgattggac     |

Table S4. The Sequence of Morpholinos (MOs), Related to Figure 2

| Morpholino          | Sequence 5’ to 3’                               |
|---------------------|--------------------------------------------------|
| gata2a (splice site| 5’-CATCTACTCACCAGTCTGCGCTTTG-3’                 |
| MO-1                |                                                  |
| etsrp MO-2          | 5’-TTGGTACATTTCCATATCTTAAAGT-3’                  |
| scl MO-1            | 5’-AAAGCGCGCGTTACCTGTAA TAGTG-3’                 |
| scl MO-2            | 5’-TAAAATGCTCTTACCATCGTTGATT-3’                  |
Supplemental Experimental Procedures

ES cell differentiation, in vitro analyses, and Blast colony assay

ES cell culture, in vitro differentiation, FACS and qRT-PCR analyses were performed as described previously (Lugus et al., 2007; Park et al., 2004; Park et al., 2006). Scl<sup>hCD4</sup> ES cells, carrying a non-functional human CD4 in the Scl locus, were generated, differentiated, sorted and replated as described (Chung et al. 2002). Inducible ES cell lines (ild1, ild2, iEts1, iEts2, iGadd45-gamma, iKlf5, iTbx3, iSox7, iSox18, iPitx2C, iFoxh1) were generated as described (Lee et al. 2008). Briefly, the cDNAs of candidate factors were targeted into the Hprt locus of A2Lox ES cells, which constitutively express the reverse tetracycline transactivator (rtTA) from the Rosa26 locus, and contain a targeting site upstream of the Hprt locus, such that a transgene is under the regulation of the tetracycline operator after site-specific recombination. In these inducible ES cell lines, nonfunctional human CD4 (hCD4) was knocked in into the Scl locus as a surrogate marker for Scl.

Blast colony assay and further differentiation to hematopoietic, endothelial, and smooth muscle cells were performed as described previously (Choi et al., 1998; Faloon et al, 2000; Chung et al. 2002; Ema et al, 2003). Briefly, Blast colonies were generated by replating EBs on day 3-3.5 of differentiation, or sorted FLK-1+ cells from day 2.75-3 EBs in the presence of VEGF (5 ng/ml), kit ligand (KL, 1% conditioned medium or 100ng/ml purified) and D4T endothelial cell conditioned medium (CM) at 25%, and counted 4 days later. Individual blast colonies were picked from methylcellulose media after 4 days culture into type IV collagen-coated 96-well plates in the presence of cytokines (VEGF, 5ng/ml; IGF-1, 10ng/ml; bFGF, 10ng/ml; IL-11, 50ng/ml; Epo, 2U/ml; KL, 100ng/ml) and cultured further for 2-4 days.
**OP9 culture system**

*iEGS* ES cells were differentiated in serum and treated with doxycycline (1 µg/ml) on day 2. Day 3 EBs were collected, trypsinized, sorted FLK-1+ cells by staining with biotin-α-FLK-1 (1:200, BioLegend) and SA-PE (1:500, eBiosciences). Sorted FLK-1+ mesoderm populations were cultured on OP9 cells in α-MEM with 20% selected FBS, L-glutamine (2mM) and Ascorbic acid (50µg/ml) (Nakano et al., 1994). After 5-6 days culture, there was about 20-30 fold expansions in cell number (from 4x10⁴ FLK-1+ mesoderm input to >1x10⁶ differentiated progeny). The resulting differentiated progeny cells of FLK-1+ mesoderm were analyzed by FACS and gene expression analyses.

**Flow cytometry**

EBs were harvested and dissociated into single cells using accutase (eBioscience). Primary antibodies include PE-α-FLK-1 (1µg/ml, AVAS12 α1), PE-α-CD31 (1µg/ml, MEC 13.3), FITC-α-CD41 (2.5µg/ml, MWReg30) (BD PharMingen), APC-α-PDGFRα (1µg/ml, APA5, eBioscience), Biotin-α-PDGFRα (1µg/ml, APA5, eBioscience), APC-α-CD45 (1µg/ml, 30-F11, Biolegend), biotin-α-VE-CADHERIN (2.5µg/ml, eBioBV13, eBioscience) and biotin-α- LYVE-1 (1µg/ml, ALY7, eBioscience). Secondary detection reagents include streptavidin (SA)/APC (0.5µg/ml, eBioscience), and SA/PE (0.5µg/ml, eBioscience). Species-specific IgG isotype controls were used as negative controls. Data were acquired on a Facscalibur flow cytometer (Becton Dickinson) and analyzed using FlowJo (Treestar) software. Cells were sorted from day 3 differentiated ES cells (Embryoid bodies, EBs) using FACS AriaII cell sorters (BD Biosciences).

**Human mesenchymal stem cell (hMSC) culture**
hMSCs were purchased (female origin, Lonza Walkersville Inc, Walkersville, MD) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Hyclone, Logan, UT) supplemented with 100 units/mL of penicillin (Gibco BRL, Gaithersburg, MD), 10% (v/v) fetal bovine serum (FBS, Gibco BRL), and 100 µg/mL of streptomycin (Gibco BRL) at 37 °C in a humidified atmosphere containing 5% CO₂. All experiments were performed using hMSCs within five passages. The method used to generate hMSC, control FLK-1⁺+hMSC, and FLK-1⁺(+DOX)+hMSC spheroids were based on hanging drop method. Briefly, 30 µL drops of the culture medium containing cells were applied onto the inside of the lid of a petri dish that contained phosphate-buffered saline (PBS) to prevent drying out (Bhang, et al. 2011). After 24 hours of incubation in an incubator, the spheroids formed in the drops were retrieved using a Pasteur pipette.

**Hypoxic cell Culture**

To generate a hypoxic culture condition, cell culture dishes were placed in hypoxia chamber (MCO-18M, Sanyo, Japan) containing 1% oxygen and 5% CO₂ at 37°C. hMSC and FLK-1⁺(+DOX)+hMSC spheroids were cultured in cell culture medium without FBS supplement.

**Cell death evaluation**

The viability of hMSC, FLK-1⁺(+DOX), and FLK-1⁺(+DOX)+hMSC spheroids or single cells under hypoxic condition was determined by double staining with fluorescein diacetate (FDA, Sigma) and ethidium bromide (EB, Sigma). In this staining, FDA stains the cytoplasm of viable cells green, whereas EB stains the nuclei of nonviable cells red. The staining solution was freshly prepared by mixing 10 mL of FDA stock solution (1.5 mg/ml of FDA in dimethyl sulfoxide), 5 mL of EB stock solution (1 mg/mL of EB in PBS), and 3 ml of PBS (pH = 8.0). Cells were incubated in the staining solution for 3-5 min at
37°C. After staining, the samples were examined using a fluorescence microscope (Olympus, Tokyo, Japan). Cell viability was also evaluated by colorimetric quantification of a neutral red dye (i.e., 3-amino-7-dimethylamino-2-methylphenazine hydrochloride) taken up by viable cells.

**Enzyme-linked immunosorbent assay (ELISA)**

Concentrations of angiogenic growth factors in conditioned media obtained from single cells and spheroids, hMSC and FLK-1*(+DOX)+hMSC, cultures were determined using ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s protocol. Concentrations were expressed as the amount of growth factor per 10⁴ cells at the time of harvest. The number of cells was determined by a total DNA quantification assay. Total amount of DNA in the cells was determined by quantifying the DNA-binding Hoechst 33258 dye (Invitrogen). A solution of the same volume of cell lysate and Hoechst 33258 dye solution (20 µg/mL) was prepared, and its fluorescence was measured by a microplate reader (Tecan, Durham, NC) at an excitation wavelength (λ_{ex}) of 380 nm and an emission wavelength (λ_{em}) of 420 nm. The amount of total DNA was calculated by extrapolation from a calibration curve.

**Epithelial to mesenchymal transition (EMT) signaling array**

The mouse EMT RT² Profiler PCR Array that profiles the expression of 84 key genes in EMT pathway was purchased from SA-Bioscience (PAMM-090A, Invitrogen). Total RNAs were isolated from day 4 iEGS EBs (±DOX on day 2-4), converted to cDNAs, and used for screening by real-time PCR following the manufacturer’s instructions. QRT-PCR assays performed in triplicate, normalized to 4 housekeeping genes (Gusb, Hsp90ab1, Gapdh and Actb), and analyzed according to the ΔΔCT method with the SA-Biosciences proprietary software.
Matrigel plug assay

FLK-1<sup>+</sup> cells sorted from day 3 iEGS EBs were infected with GFP retrovirus by centrifugation at 3000 rpm for 1.5 hours. GFP-infected FLK-1<sup>+</sup> cells were mixed with OP9 cells in 1:5 ratio and cultured overnight in hanging-drops. For each injection, total 1 x 10<sup>6</sup> cells in spheroids were mixed with 800 µl unpolymerized Matrigel (BD) at 4°C. The mixed solution was injected subcutaneously (one plug per animal) into mildly sedated (ketamine 100 mg/kg, IP) NOD/SCID/IL2Rg<sup>-/-</sup> (NSG) mice (The Jackson Laboratory) using a 20 gauge-needle. Matrigel plugs were excised, treated with methanol/10% DMSO for 1 hour on ice and further fixed in 4% paraformaldehyde for 1 hour on ice. Plugs were immersed in 30% sucrose solution for overnight at 4°C and embedded in O.C.T. frozen section medium (Sakura Finetek).

Zebrafish husbandry and strains

Adult Zebrafish were raised and maintained according to established methods (Westerfield, 1993). Embryos were obtained from natural mating and staged according to morphology as described. All studies on wild-type fish were carried out in the Oregon AB background. etsrp<sup>v11</sup> mutant line was obtained from Brant Weinstein (Pham et al., 2007).

Microinjection of morpholinos

All morpholinos (MOs) used in our studies were previously characterized and shown to be reasonably effective and specific (Galloway et al., 2005; Juarez et al., 2005; Sumanas and Lin, 2006). To knockdown etsrp, we injected 3ng of 1:1 mix of anti-etsrp MO1 and MO2 (Sumanas and Lin, 2006). To knockdown scl, we injected 3ng or 12.5ng of 2:3 mix of anti-scl MO1 and MO2 (Juarez et al., 2005). gata2-specific MO (Gene
Tools LLC) has been described previously (Galloway et al., 2005) and was injected at a dose of 33.4ng. For anti-scl+gata2, anti-etsrp+scl and anti-etsrp+scl+gata2 MOs, we injected a 1:1 combination of the mixes described above. All MOs were injected at the 1-cell stage using sequences in Table S4.

**Whole-mount in situ hybridization**

For single-color in situ hybridization, antisense riboprobes were labeled with digoxigenin (DIG; Roche). The hand2 and fli1a probes were used. Whole-mount in situ hybridization was performed as described (Thisse and Thisse, 2008).

**Teratoma formation**

(FLK-1⁺(+DOX)+hMSC) spheroids transplantation was compared with ES cell transplantation by transplanting (FLK-1⁺(+DOX)+hMSC) spheroids subcutaneously (n = 6 each). The amount of transplanted ES cells was identical with the number of FLK-1⁺(+DOX) (5 x 10⁶ cells/mouse).

**Immunostaining for ES/EBs, Matrigel plugs and tissue sections**

*iEGS* EBs were plated onto Collagen I-coated slides (BD Biosciences) on day 2 w/o DOX and further differentiated till day 4, fixed in 2% formaldehyde in PBS, and blocked with 1% BSA/0.5% saponin in PBS. Primary antibodies (Abs): biotinylated E-CADHERIN (2.5 µg/ml, R and D Systems), N-CADHERIN (2.5 µg/ml, BD Transduction Laboratories). Secondary Abs detection: AlexaFluor594 goat-α-mouse IgG and SA-AlexaFluar488 (2 µg/ml, Molecular Probes). Nuclei were stained with DAPI (1 µg/ml, Molecular Probes). Single blast colonies were cultured in type IV collagen-coated 96-well plates (BD Biosciences) for 4 days, fixed in 4% PFA in PBS, permeabilized with 0.2% Triton-X100 in PBS and blocked with 5% BSA. Primary Abs: CD31 (rat clone MEC13.3; BD),
biotinylated VE-CADHERIN (2.5 µg/ml, Biolegend), FITC-SMAα (1A4, Sigma). Secondary Abs detection: AlexaFluor594 goat-α-rat IgG and SA-AlexaFluor488 (2 µg/ml, Molecular Probes). Nuclei were stained with DAPI (1 µg/ml, Molecular Probes). FLK-1+(DOX)+hMSC spheroids were fixed with 4% (v/v) paraformaldehyde for 10 min at room temperature (RT) and washed with PBS for 3 times. Immunofluorescent staining with HIF-1α (Abcam, Cambridge, UK) was used to observe hypoxic environment in spheroids. For detection of angiogenic factor secretion in spheroids, samples were immunofluorescently stained with HGF (Abcam), VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), FGF2 (BD Transduction Laboratories, Lexington, UK), respectively, followed by examination using a fluorescent microscope (Nikon TE2000, Tokyo, Japan).

Excised Matrigel plugs embedded in O.C.T. medium (TISSUE-TEK® 4583, Sakura Finetek USA Inc., Torrance, CA) were used to prepare cryosections of 10µm thickness. Non-specific binding of antibodies was blocked by incubation with 5% normal goat serum for 1 hour at 4°C. The following primary antibodies were used for double staining: α-mouse CD31 (rat clone MEC13.3; BD) and anti-GFP (chicken ab13970; Abcam). Secondary detection: AlexaFluor594 Goat α-rat IgG and AlexaFluor488 Goat α–chicken IgG (Invitrogen).

Ischemic limb muscles harvested 3 and 28 days post treatment were embedded in O.C.T. compound followed by freezing and slicing into 10 µm-thick sections at -22 °C. Ten slides were selected out of the beginning, middle, and end part of each sample. Immunofluorescent staining for Y-chromosome using CEP Y DNA probe kit (Vysis, Downers Grove, IL) was conducted to detect transplanted control FLK -1+ cells and FLK -1+(DOX) cells from the sections as described previously (Jiang, et al., 2004). Immunofluorescent staining with caspase-3 (Abcam) was used to detect apoptotic cells.
For the detection of microvessels in ischemic regions, sections were immunofluorescently stained with CD31 (PECAM, Abcam) and anti-smooth muscle actin (SMA)-α (Abcam), respectively. Twenty different images per slide were randomly acquired from three different samples and analyzed at ×100 and ×400 magnification. CD31-positive vessels with single-layered round morphology and SMA-α positive vessels with multiple-layered round morphology were counted, respectively. Fluorescent vessels with round morphology were counted and calculated as vessel number per mm². Rhodamine (red) and FITC (green) conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were used to visualize the stained vessels. Cellular nuclei were counter-stained with DAPI (Vector Laboratories, Burlingame, CA).

**Immunoblot analysis**

Immunoblot analysis was performed as previously described (Park et al., 2004) Briefly, iEGS ES cells were differentiated in serum free conditions, treated w/o DOX (1 µg/ml) on day 2, collected EBs on day 4 and lysed for immunoblotting. Antibodies were used as follows: Phospho-SMAD1/5 (Ser463/465, 41D10), SMAD1 (D59D7), Phospho-AKT (Ser473, D9E), AKT (#9272), Phospho-ERK1/2 (Thr202/Tyr204, D13.14.4E), ERK1/2 (3A7), Phospho-GSK3β (Ser9, 5B3), GSK3β (27C10), LEF1 (C12A5) (Cell Signaling), N-CADHERIN (32/N-Cadherin, BD Transduction Laboratories), GAPDH (Santa cruz).

Ischemic hindlimb muscles (5 samples per group) were homogenized using a Dounce homogenizer (50 strokes, 4°C) in an ice-cold lysis buffer (15 mM Tris-HCl (pH 8.0), 0.25 M sucrose, 15 mM NaCl, 1.5 mM MgCl₂, 2.5 mM EDTA, 1 mM EGTA, 1 mM DTT, 2 mM NaPPi, 1 µg/mL pepstatin A, 2.5 µg/mL aprotinin, 5 µg/mL leupeptin, 0.5 mM phenylmethylsulfonl fluoride, 0.125 mM Na₃VO₄, 25 mM NaF, and 10 µM lactacystin). After quantification and separation, the proteins were probed with antibodies against
CASPASE-3, VITRONECTIN, LAMININ, BCL-2, P53, SMA-α, CD31, COLLAGEN type II, and OSTEONECTIN from Abcam; VEGF, HGF, OSTEOCALCIN from Santa Cruz Biotechnology; and FGF2 from BD Transduction Laboratories.

**Histology**

Ischemic limb muscles retrieved 3 and 28 days post treatment were fixed with a formaldehyde solution, dehydrated with a graded ethanol series, and embedded in paraffin. 4 µm sections obtained from the specimens were stained with hematoxylin and eosin (H&E) to examine muscle degeneration and tissue inflammation. Masson’s trichrome staining was also conducted to assess tissue fibrosis in ischemic regions.
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