

Induction of Endothelial Cell from Fibroblast by 5 Defined Factors



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Presenter Disclosure

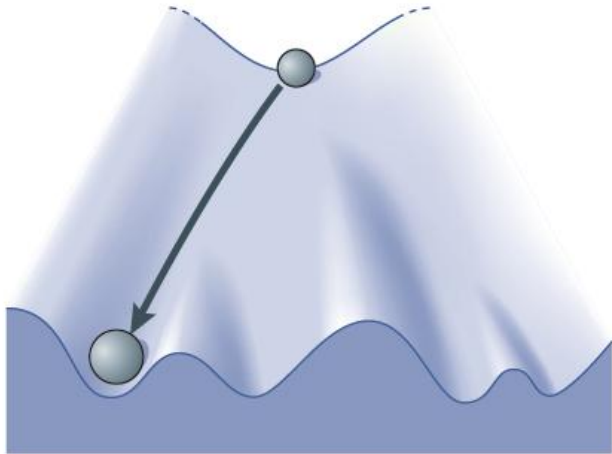
- Nothing to disclose

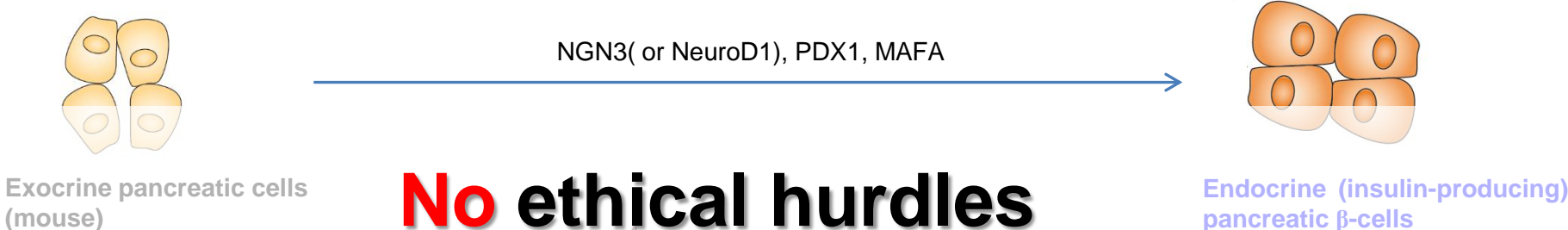
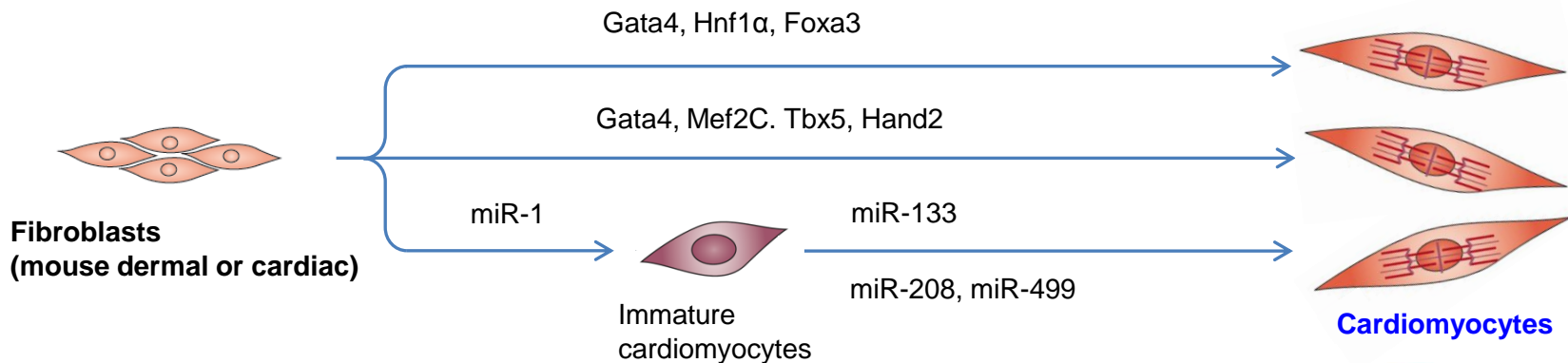
Background

- New vessel formation using endothelial cells holds great therapeutic promise.
- ESC- or iPSC-derived ECs
 - Ethical hurdles
 - No standardized protocol
 - Necessity for complex manipulation of EB
 - Low differentiation efficiency
 - Risk of contamination by feeder cells

Waddington's Epigenetic Landscape

Conventional concept

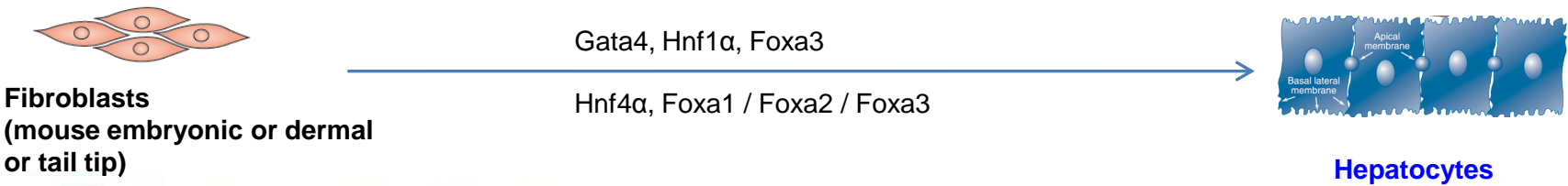
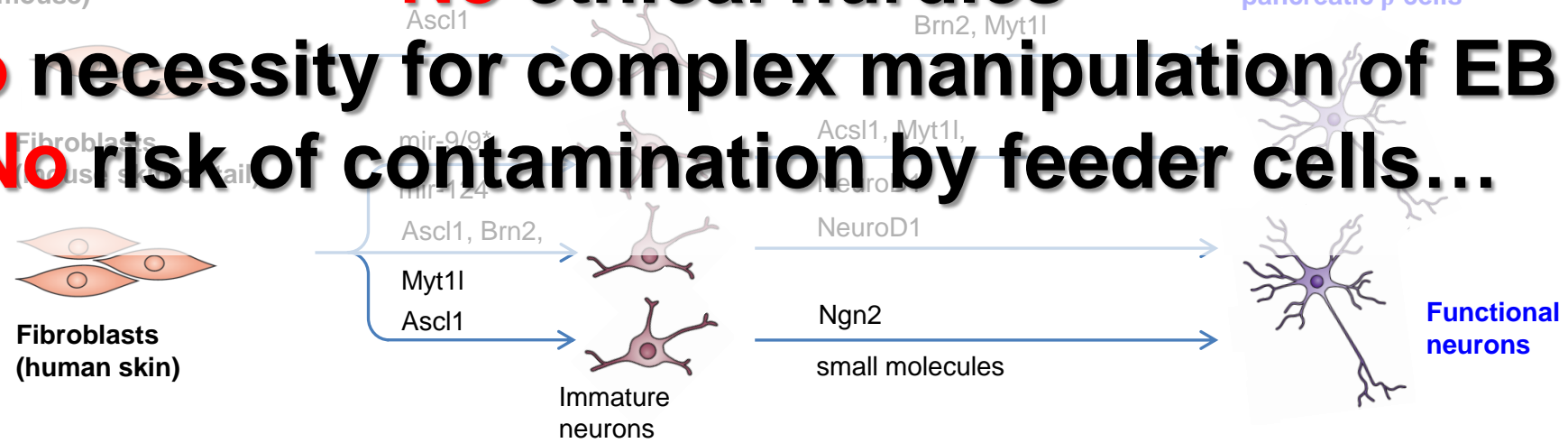




No ethical hurdles

No necessity for complex manipulation of EB

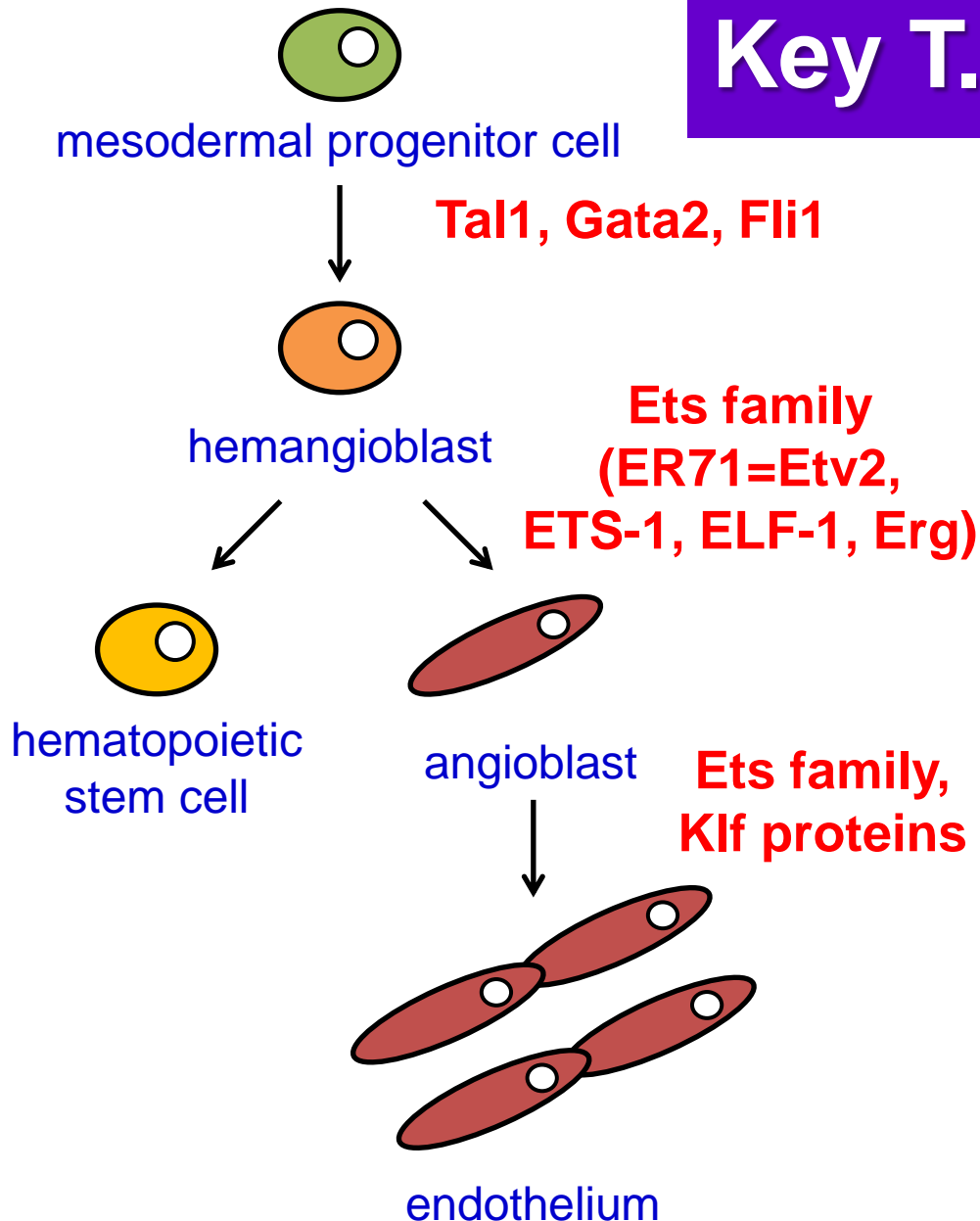
No risk of contamination by feeder cells...



Aim:

- To develop a novel methodology providing **endothelial cells from fibroblasts** via **direct conversion**.

Key T.F.s in EC development

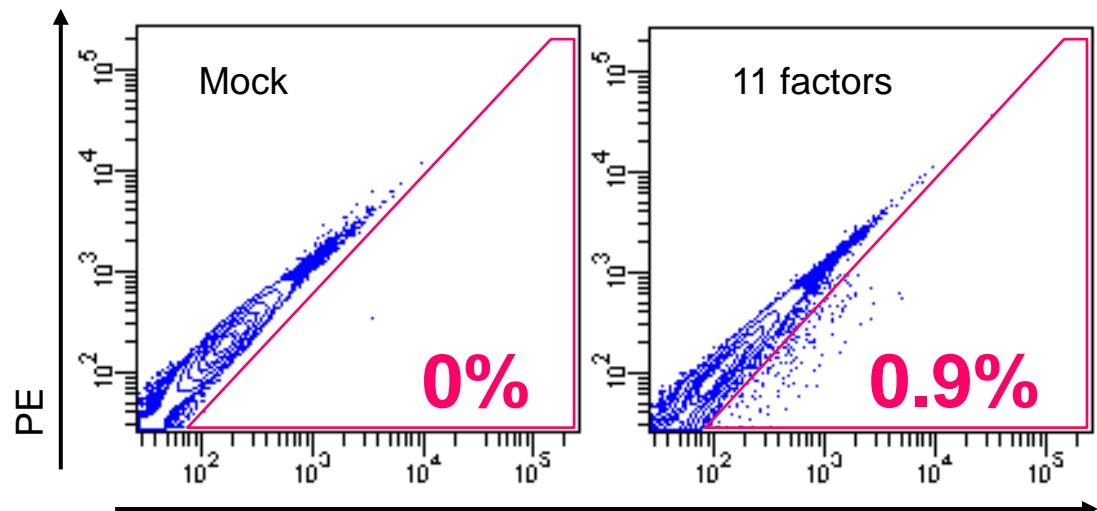
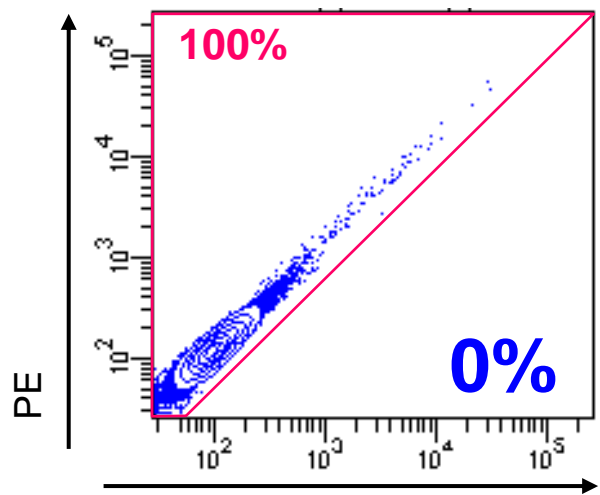
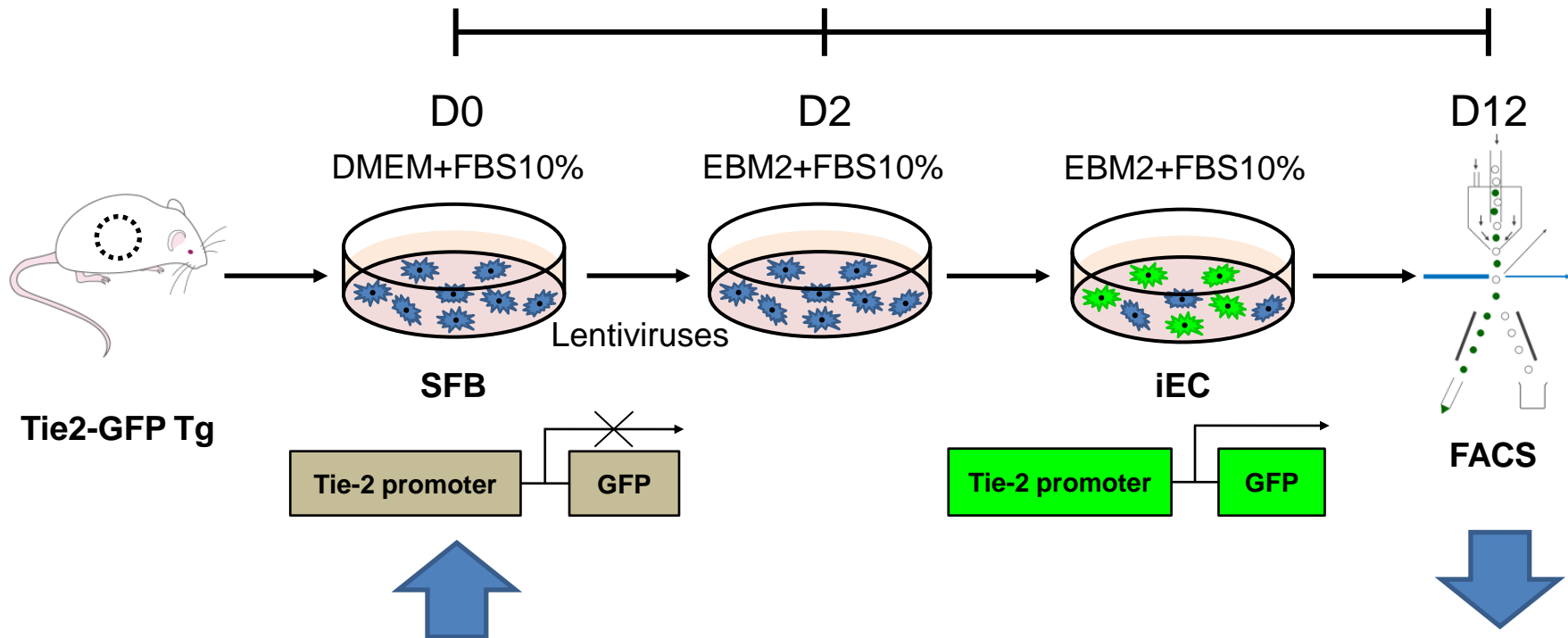


- + Co-workers of endothelial transcription
 - **FOXO1, FOXC, LMO2** (co-factor)
- + Regulator of endothelial functions
 - **Klf2**

- **Target cells: adult fibroblast**
- **Species: mouse**
- **Read-out: tie2-GFP**

11 Candidate Factors

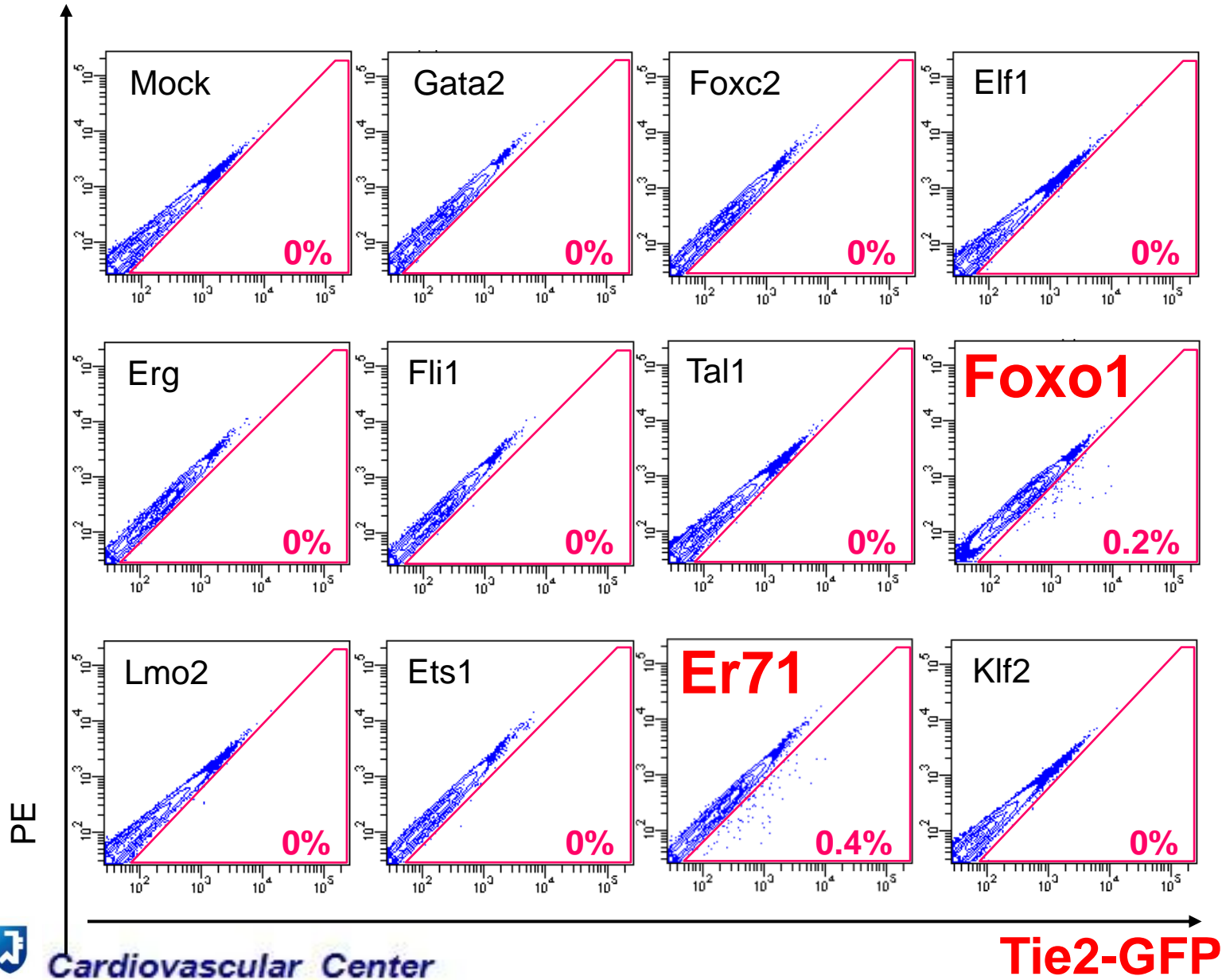
Gene symbol	Accession Number
Gata2	NM_008090
Foxc2	NM_013519
Elf1	NM_007920
Erg	NM_133659
Fli1	NM_008026
Tal1	NM_011527
Foxo1	NM_019739
Lmo2	BC057880
Ets1	BC010588
Er71(Etv2)	NM_007959
Klf2	NM_008452

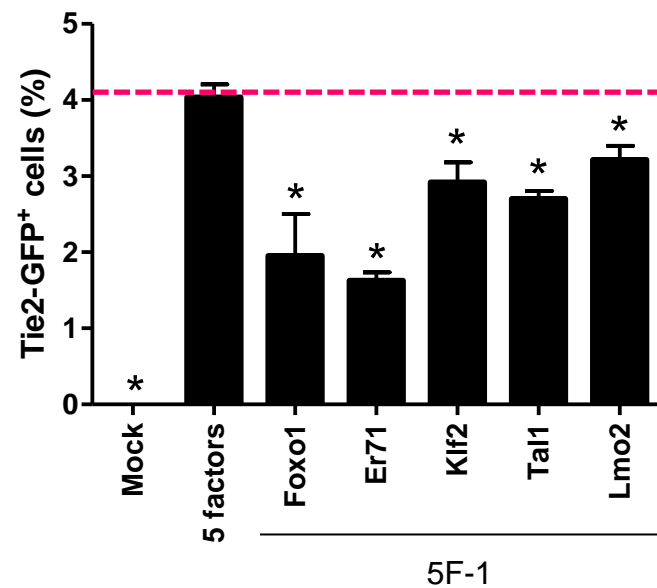
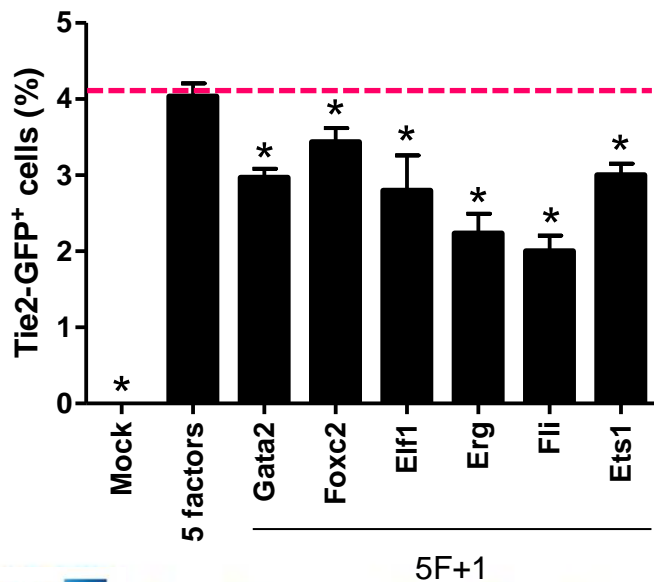
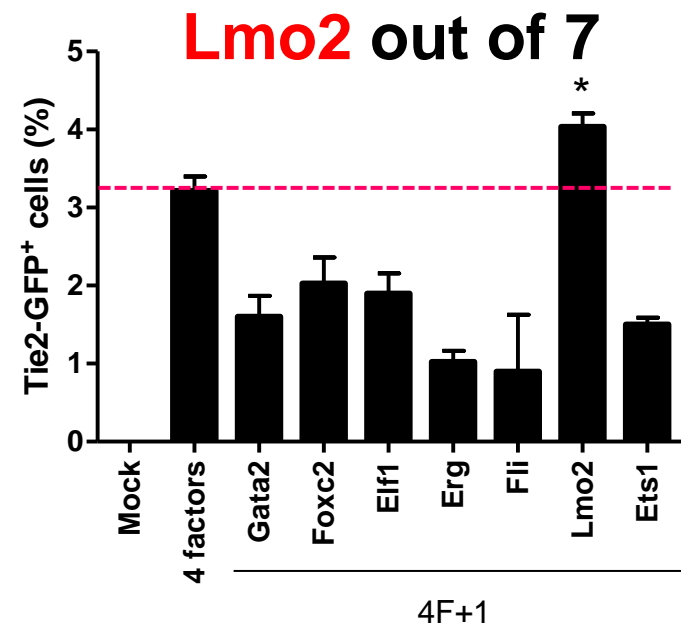
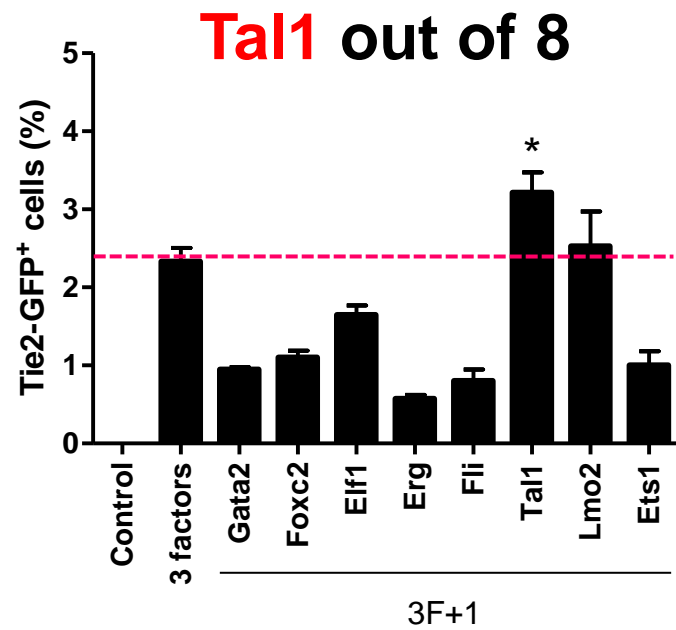
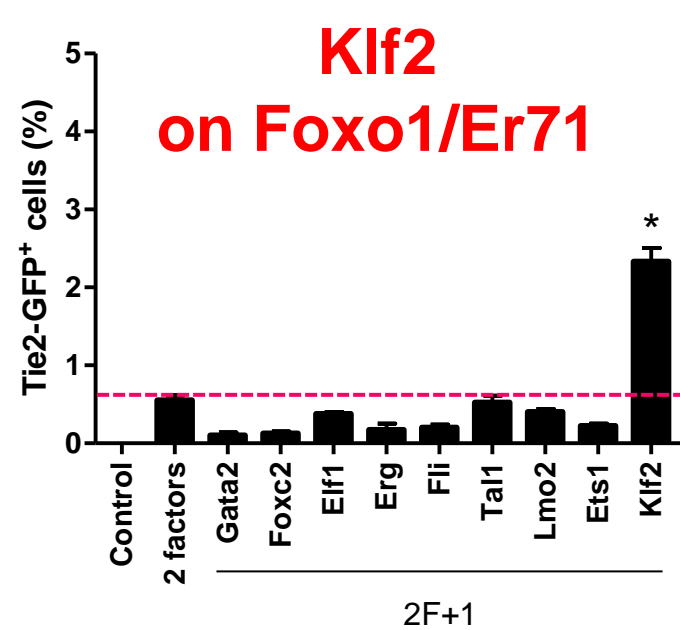


Tie2-GFP

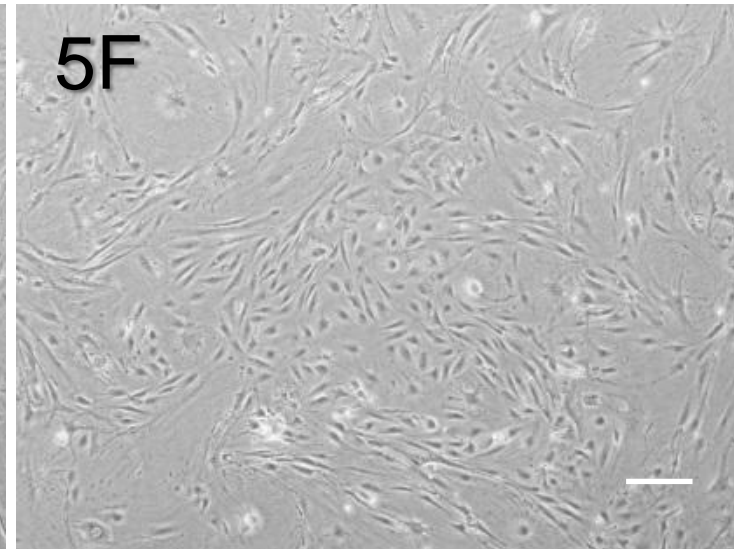
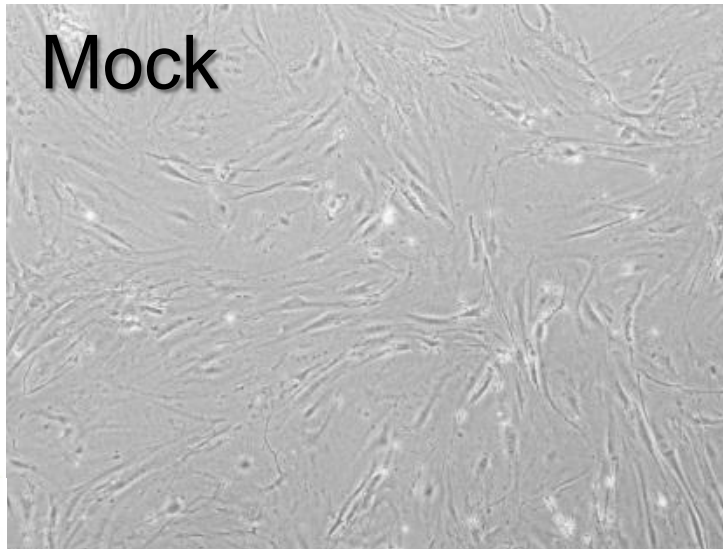
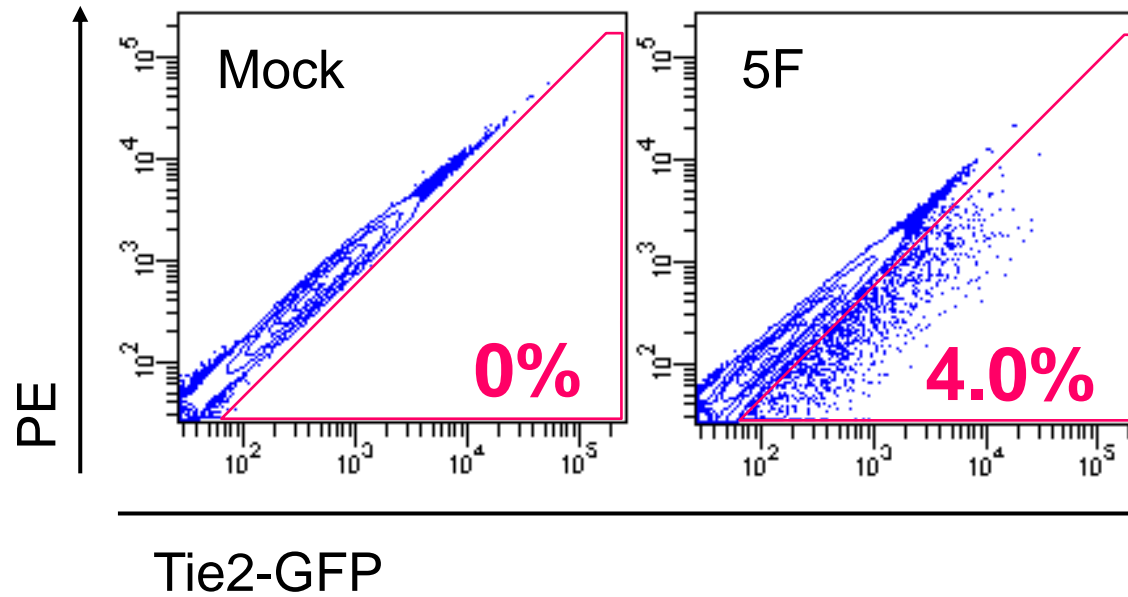
Tie2-GFP

Series of 'Single Factor' Transduction Experiments

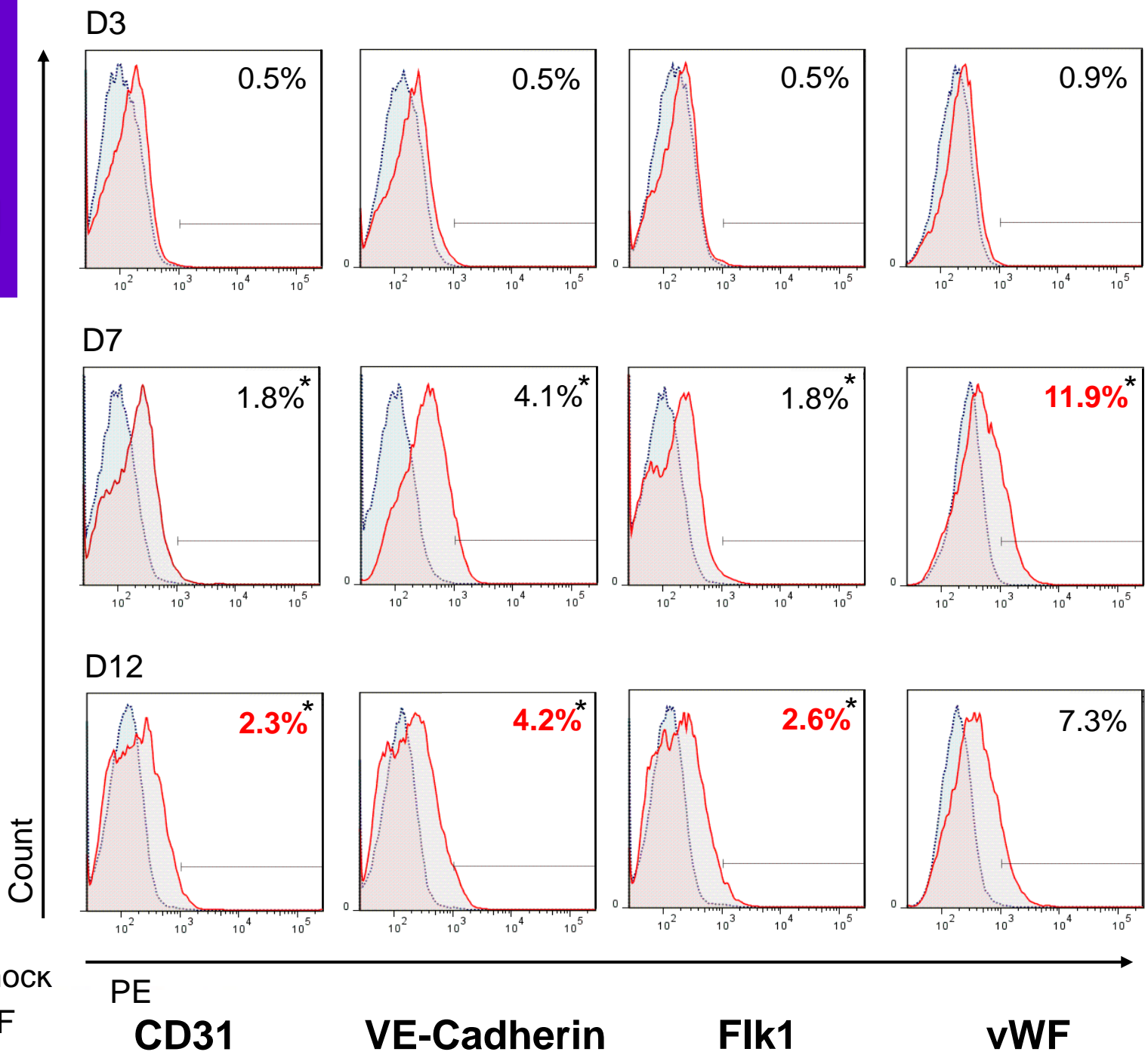




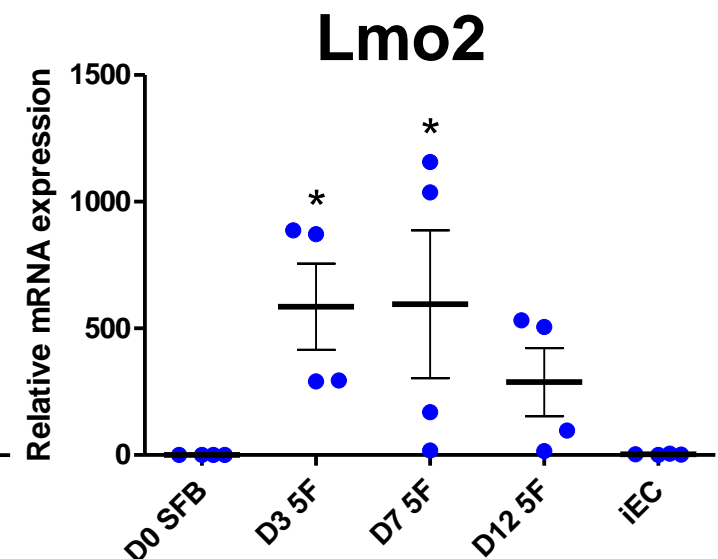
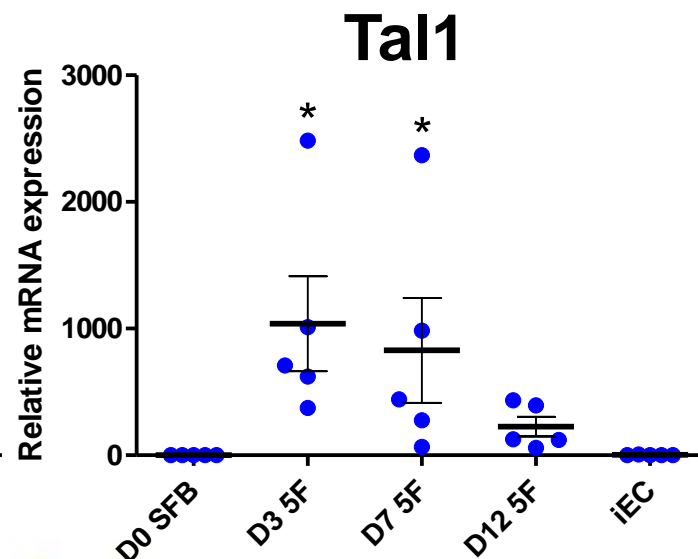
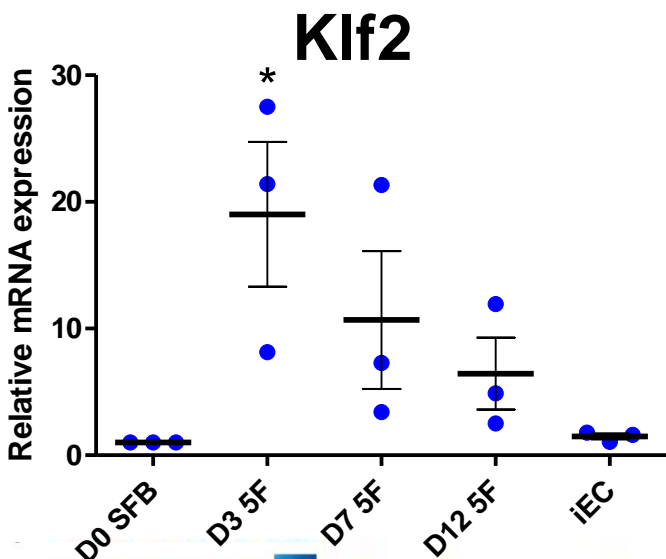
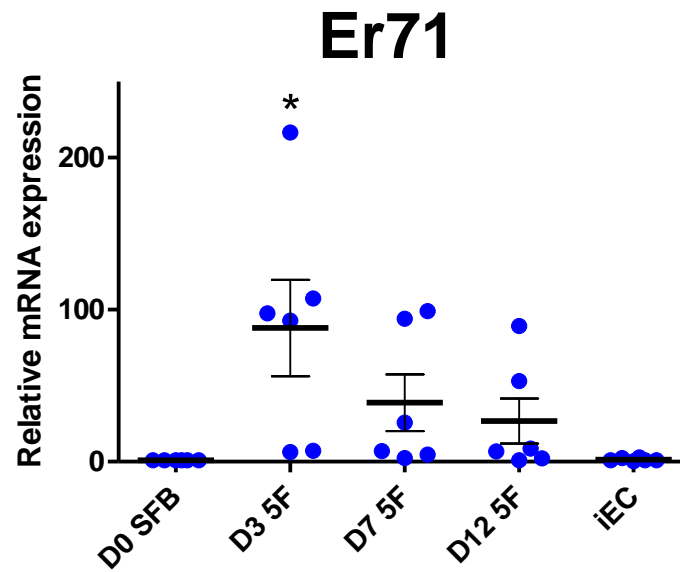
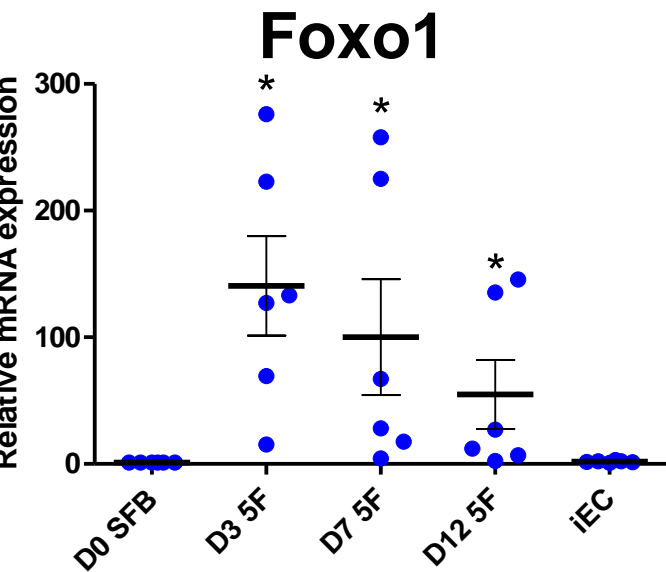
5 Key Factors for Endothelial Reprogramming



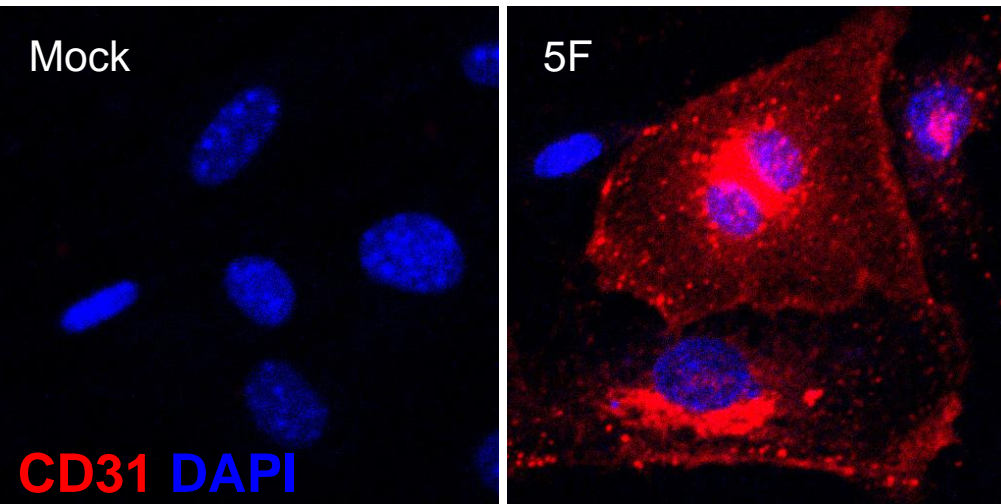
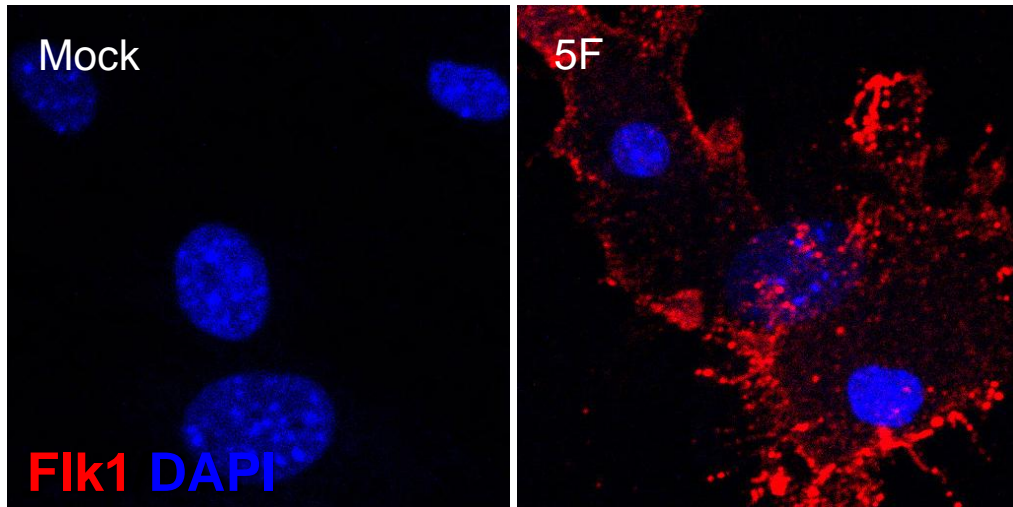
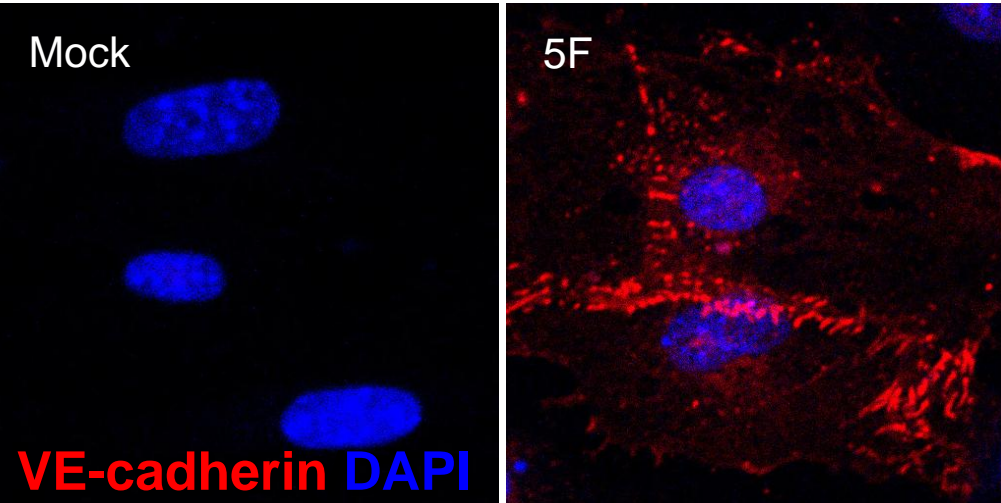
EC markers during the Programming



Lentiviral Silencing

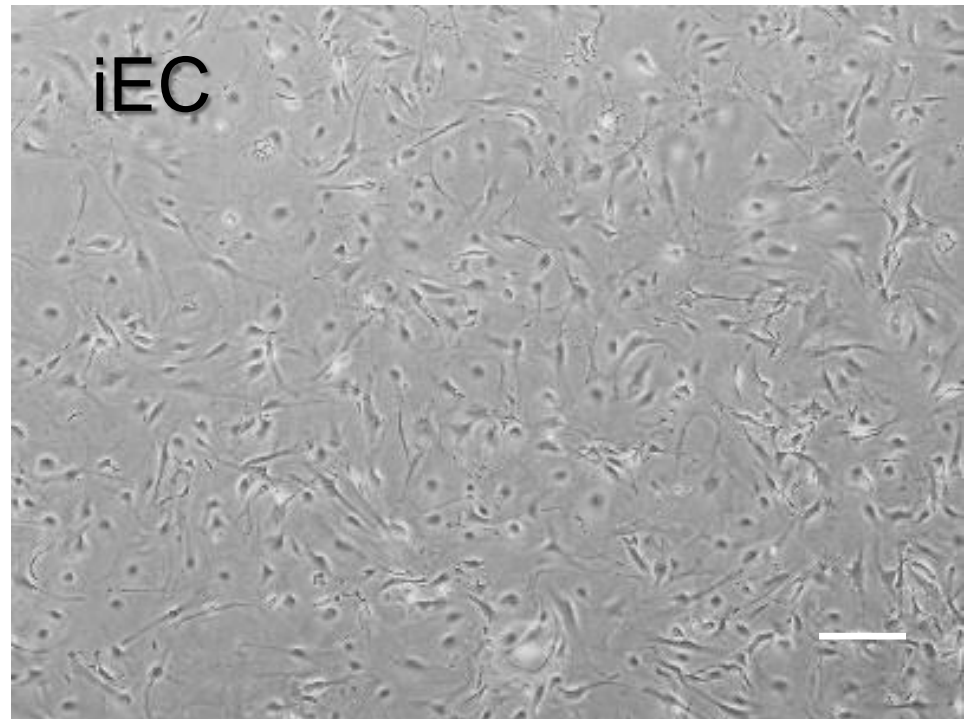
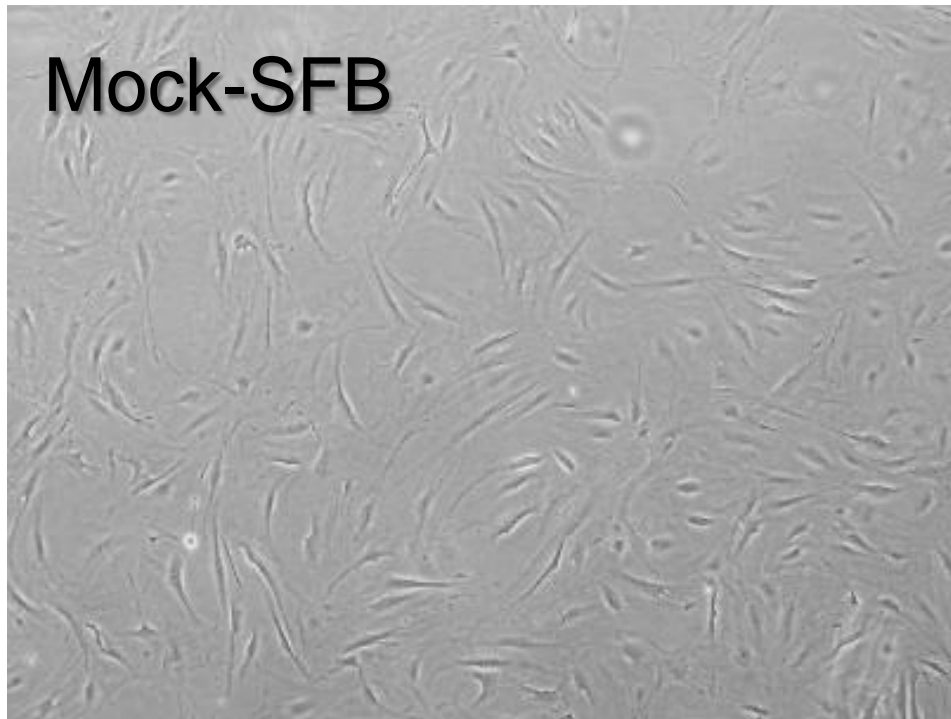


EC Markers after Endothelial Reprogramming



Before sorting by Tie2

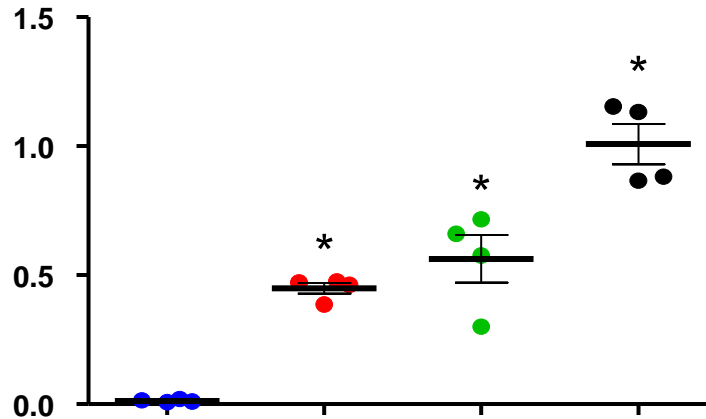
Induced Endothelial Cells: after Tie2 sorting



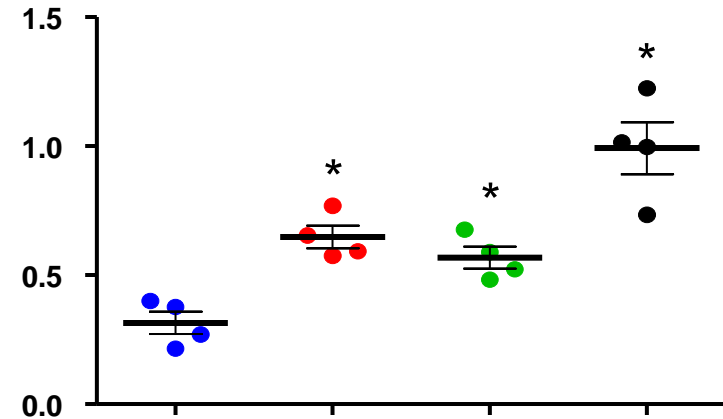
iEC Characterization: RT-PCR

Relative mRNA expression

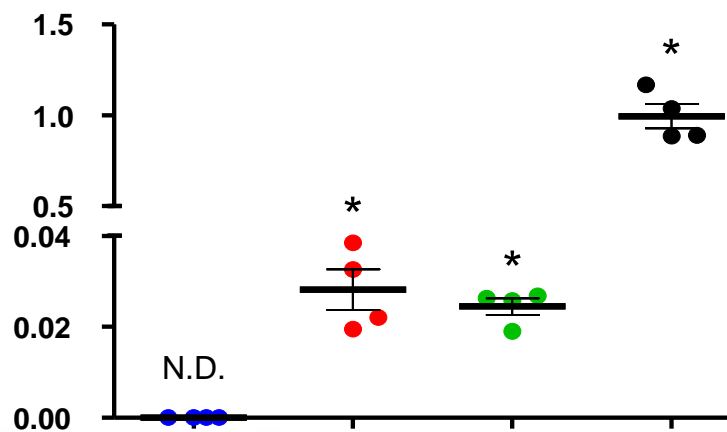
VE-cadherin



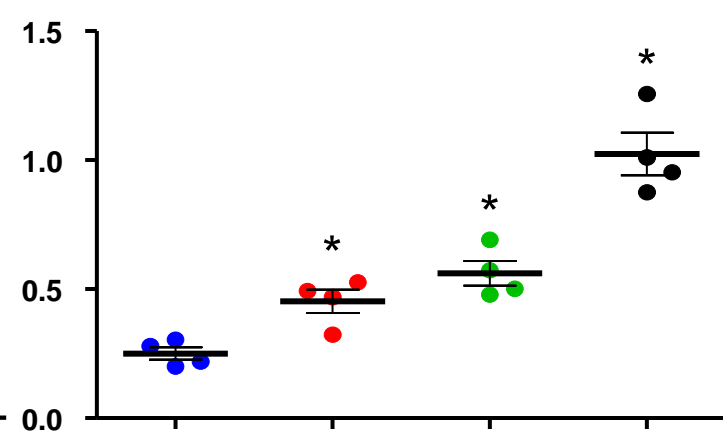
CD31



ICAM2

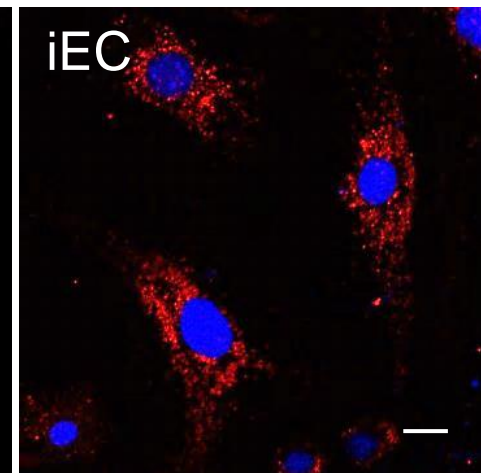
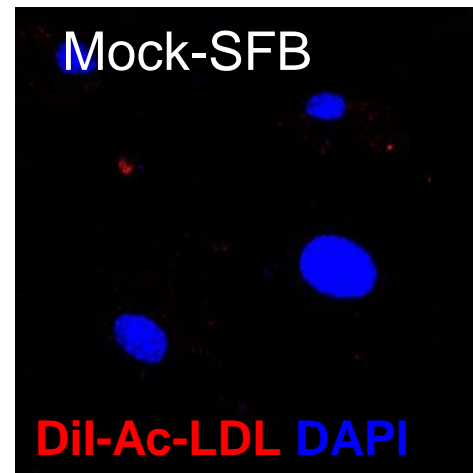
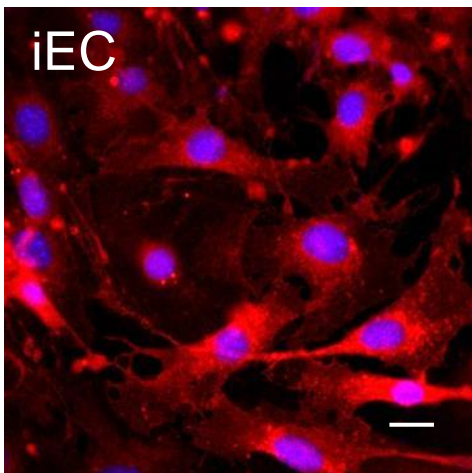
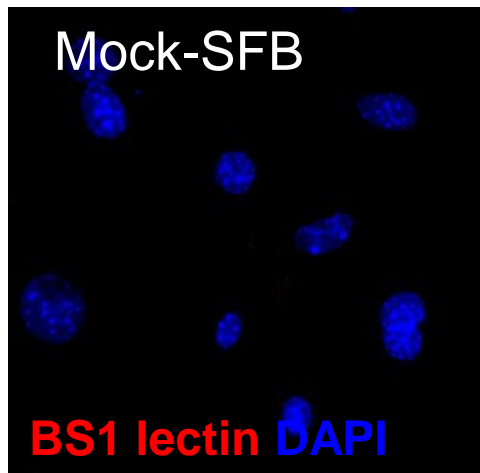
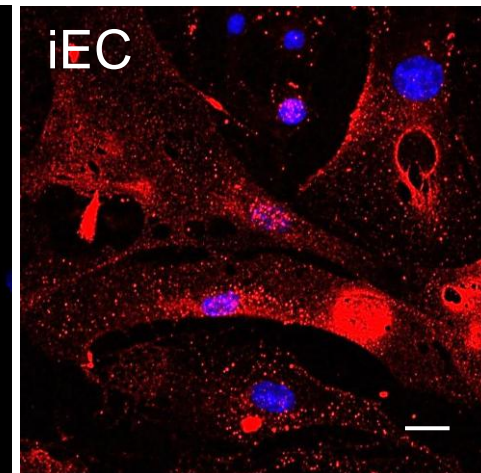
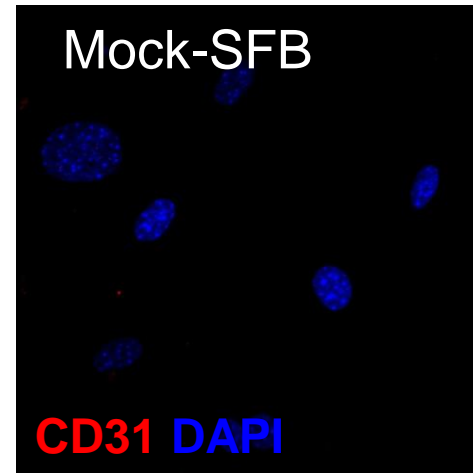
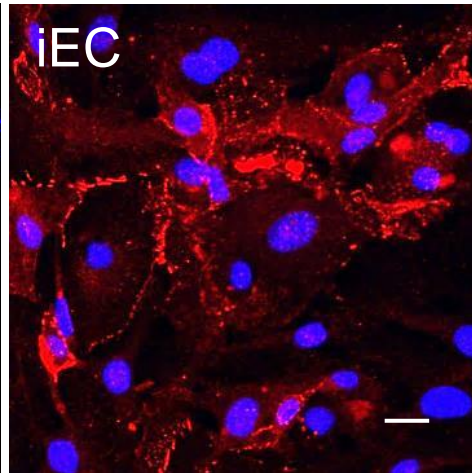
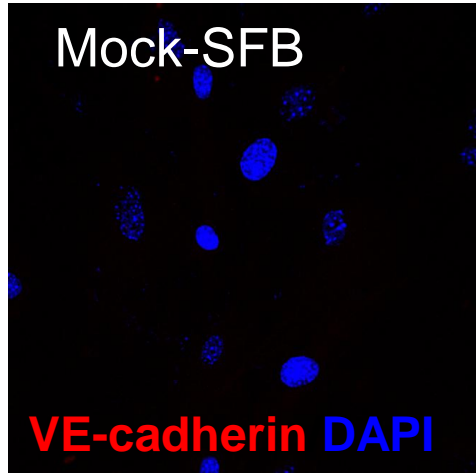


Tie2

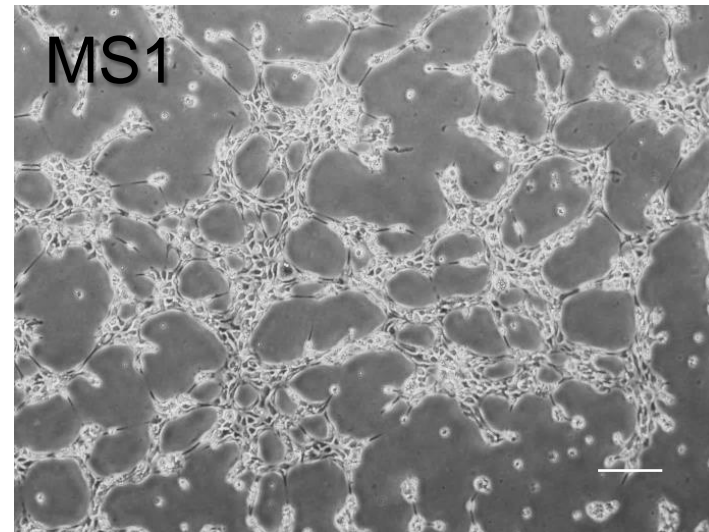
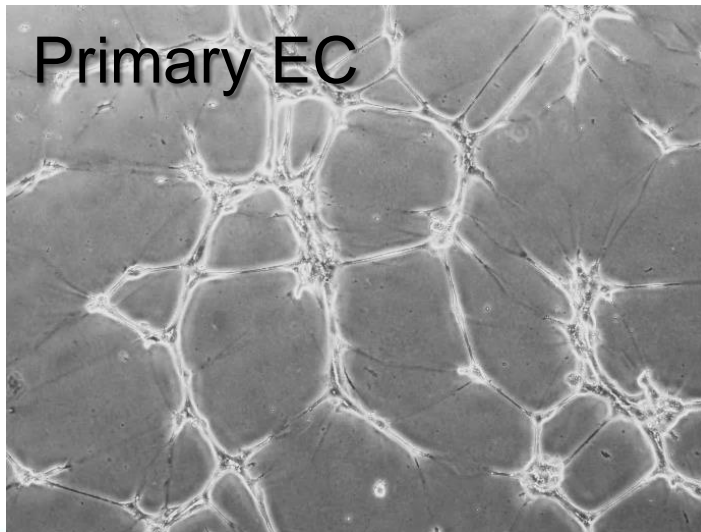
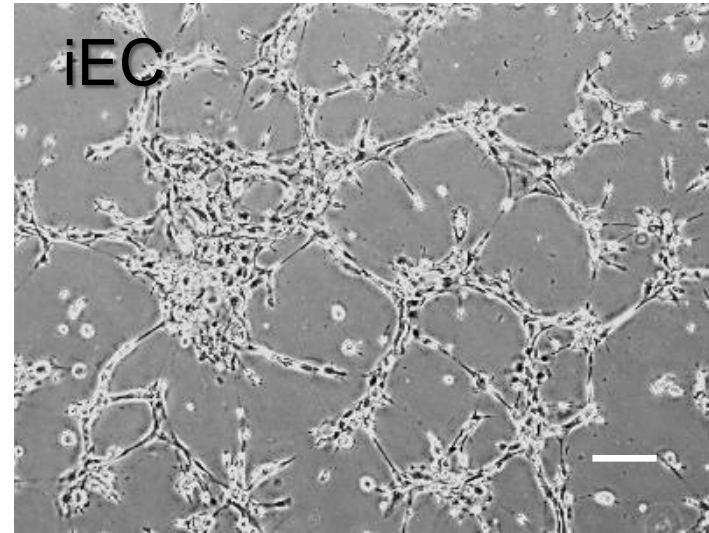
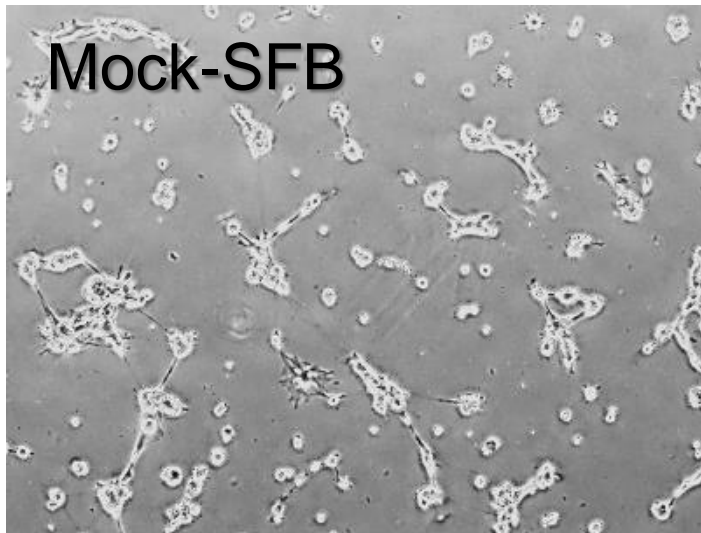


- Mock-SFB
- iEC
- Primary EC
- MS1

iEC Characterization: IF, EC Fx.

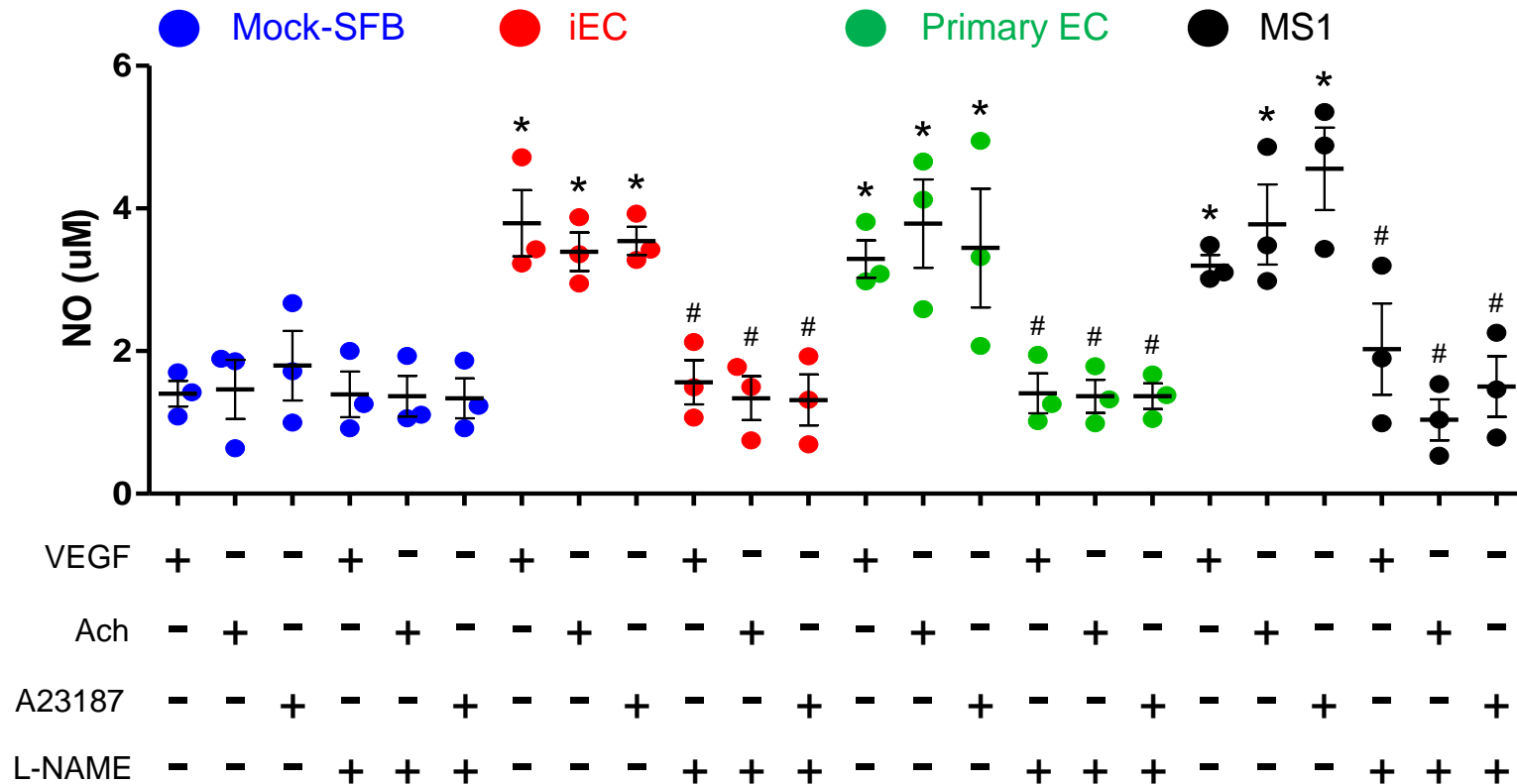


iEC Characterization: Matrigel Tube Formation



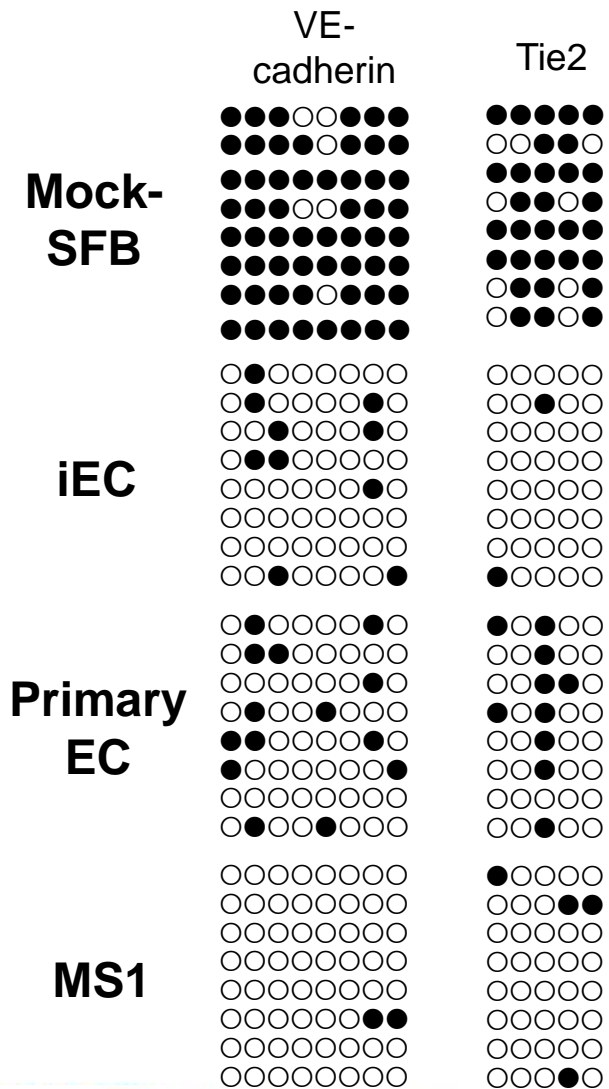
iEC Characterization: NO Production

After overnight incubation in 2 ng/ml VEGF, culture supernatants were harvested, and NO was assayed using a NO Detection Kit.

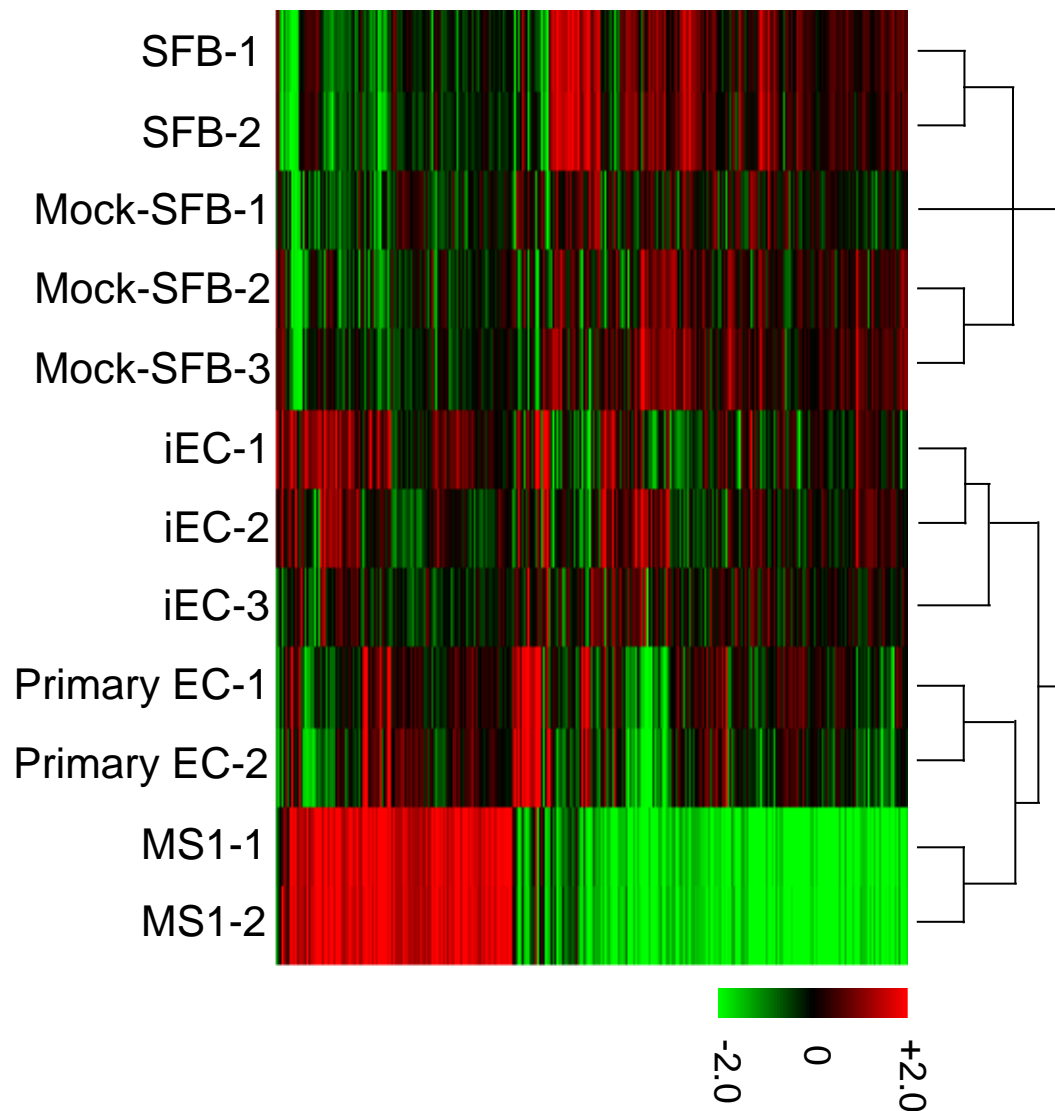


Endothelial Epigenetics / Genetics

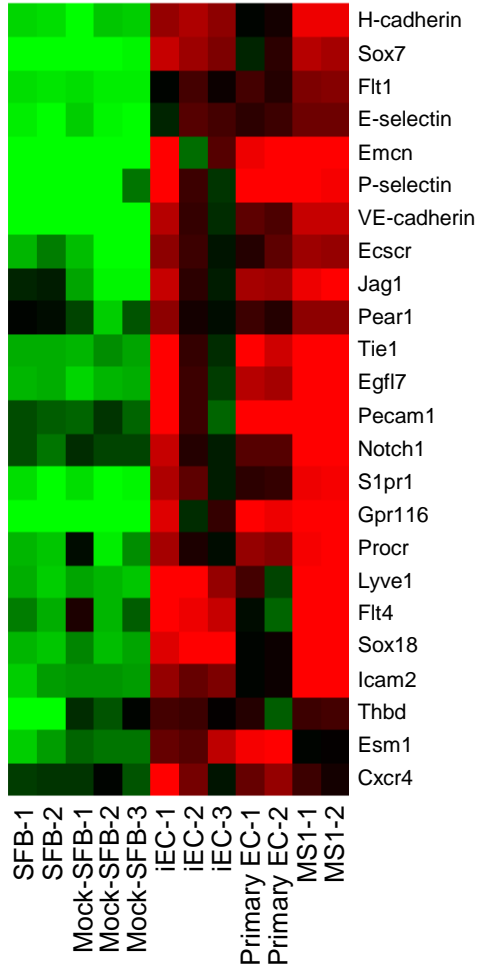
Bisulfite sequencing



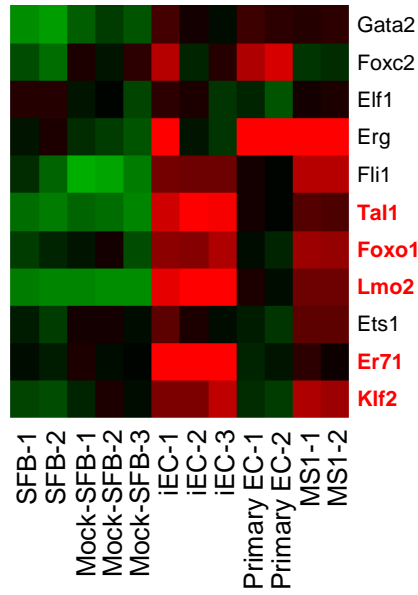
Affymetrix GeneChip Mouse Gene 1.0 ST Array



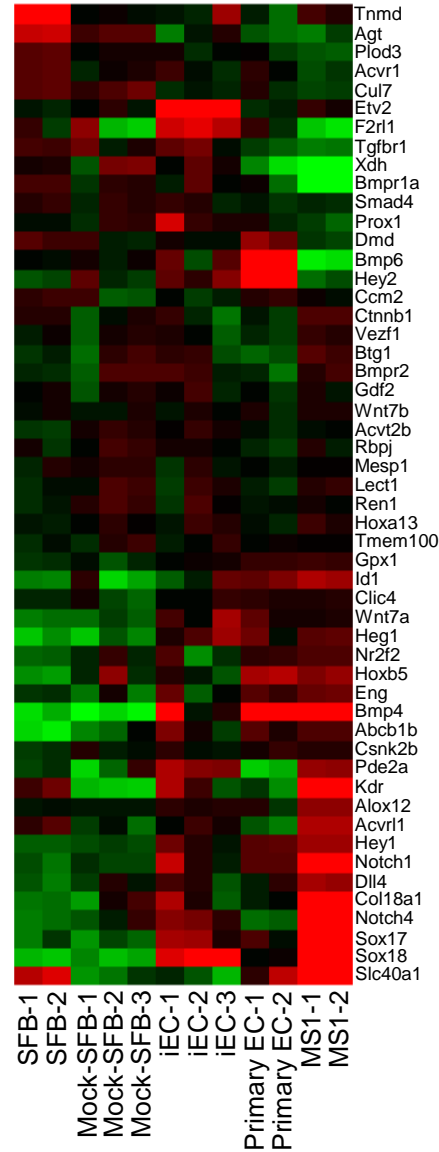
EC specific genes



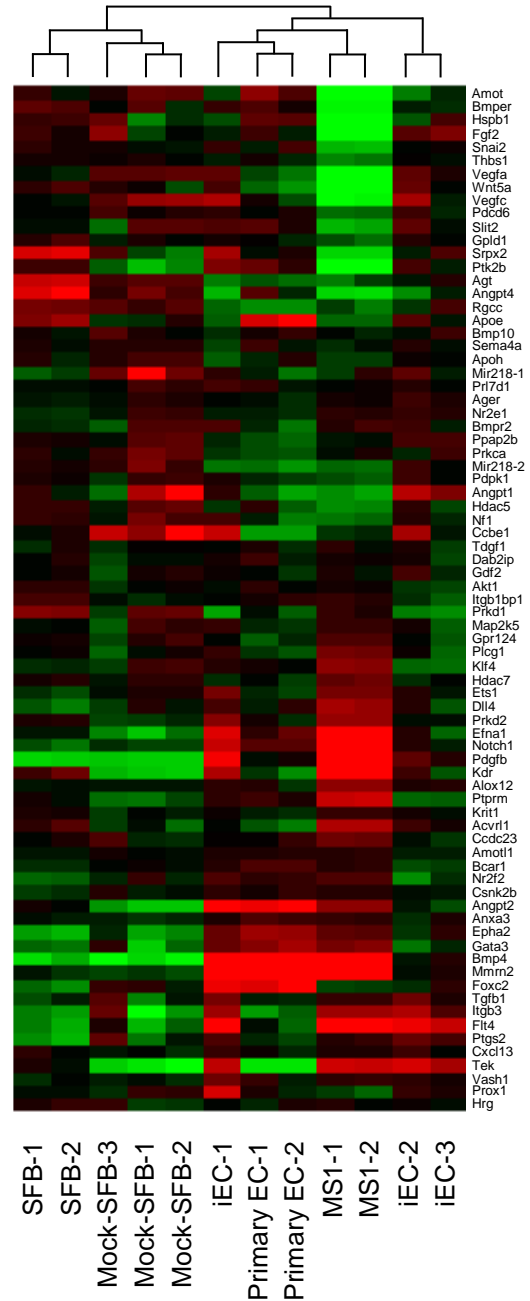
11 factors



Endothelium development (GO003158)

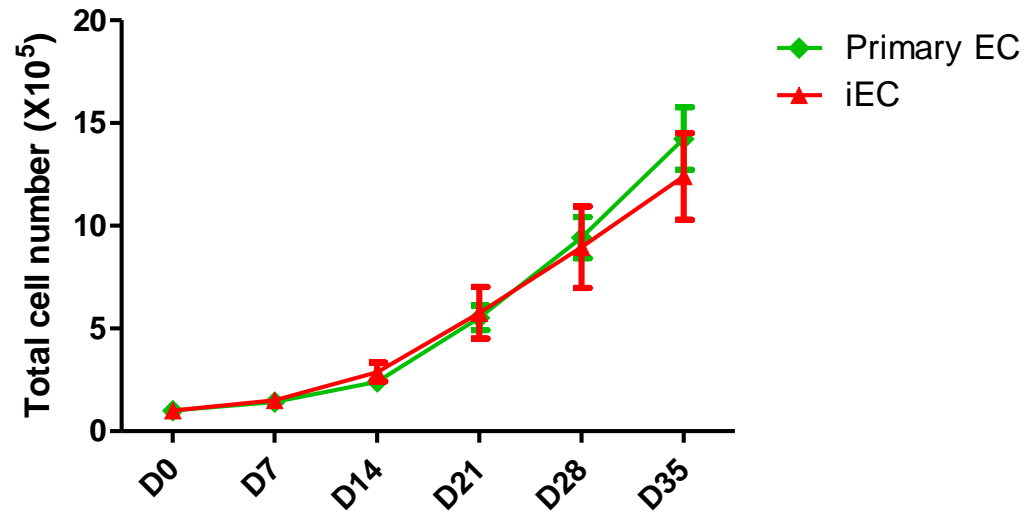


Regulation of EC migration (GO0010594)

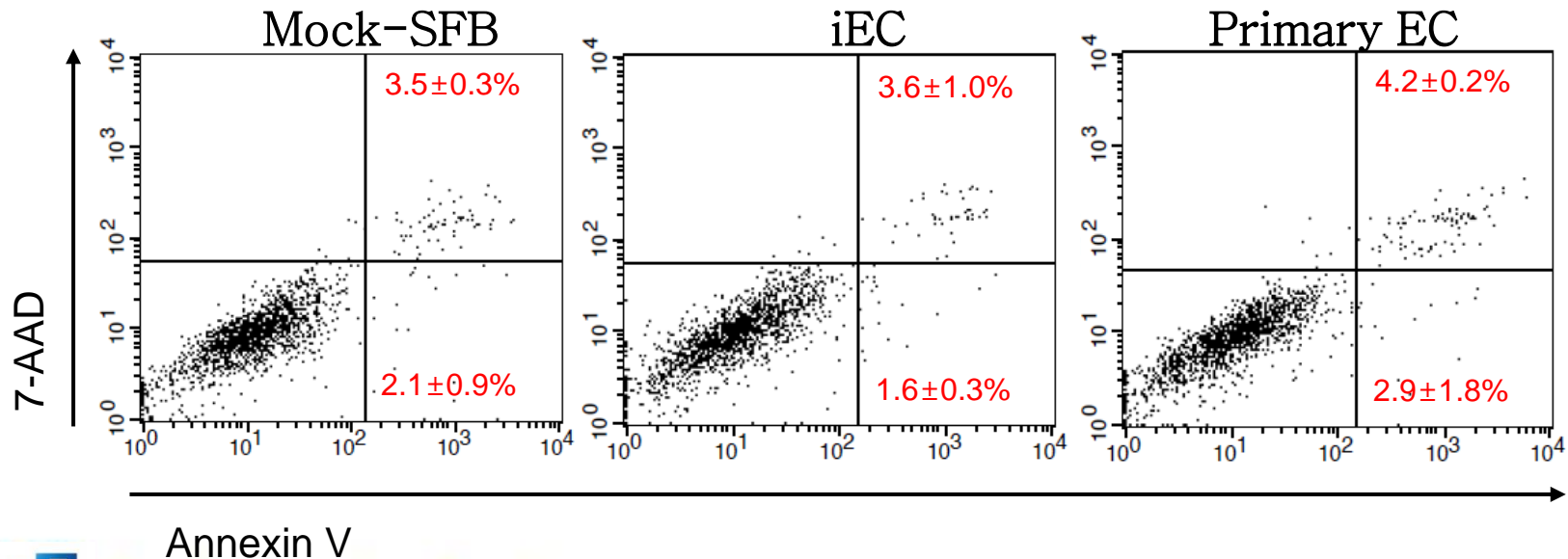


Proliferation & Apoptosis of iECs

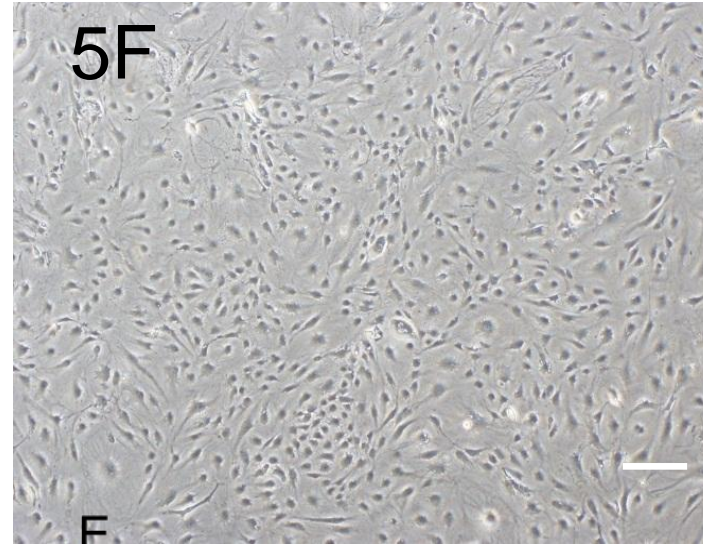
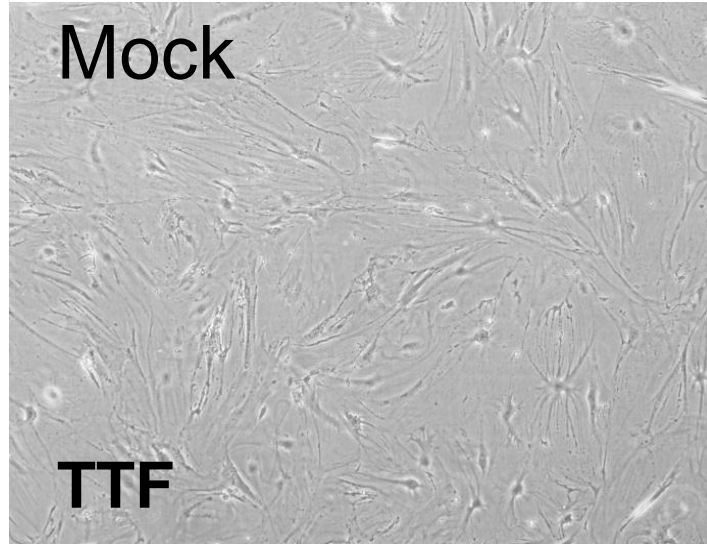
Growth Curve



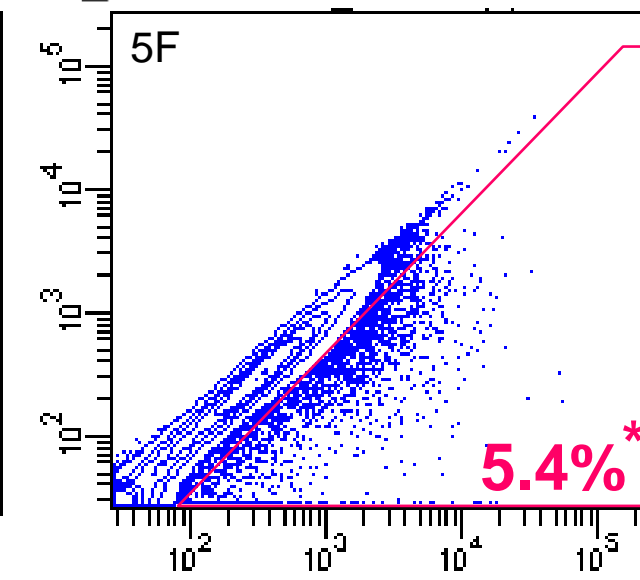
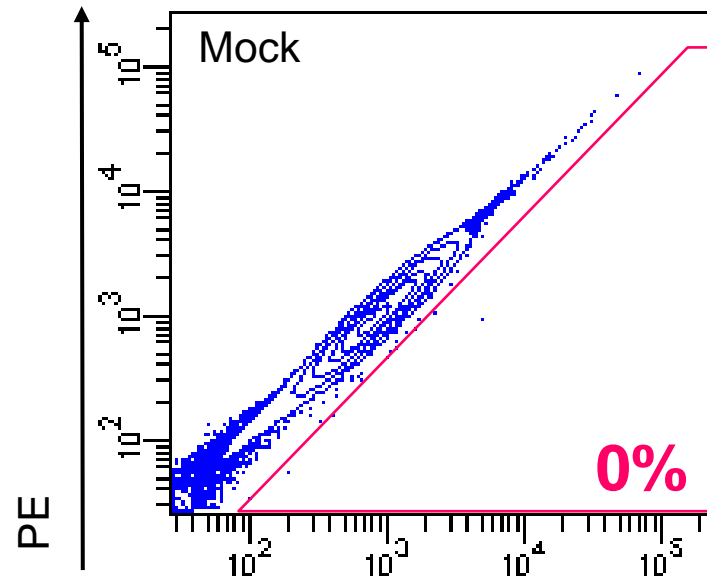
Serum Starvation



Universal Effect of iEC-5 Factors: TTF

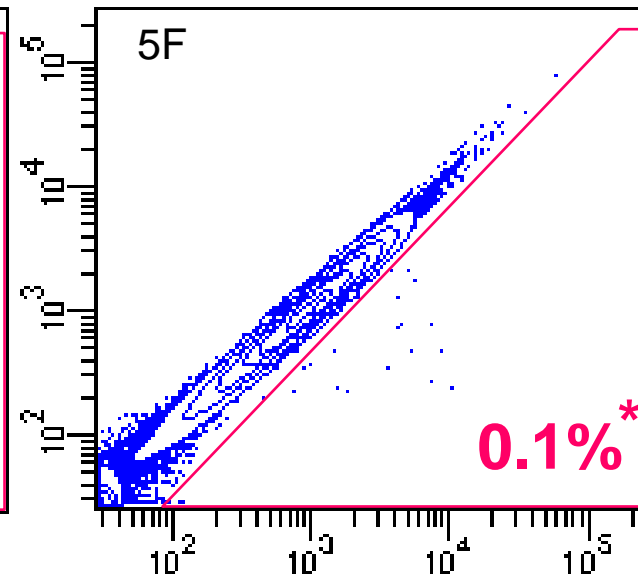
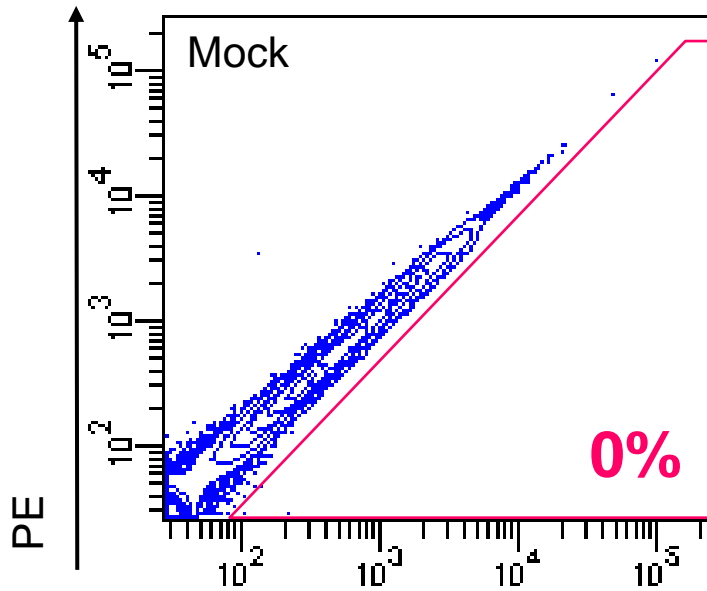
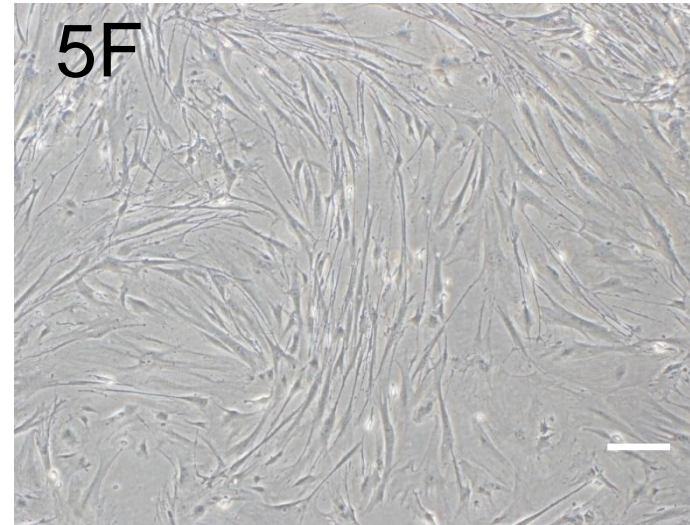
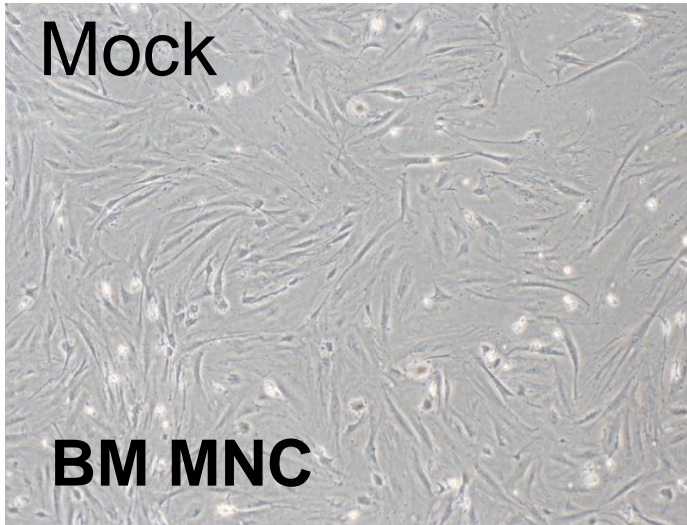


Tail-tip FB (TTF)

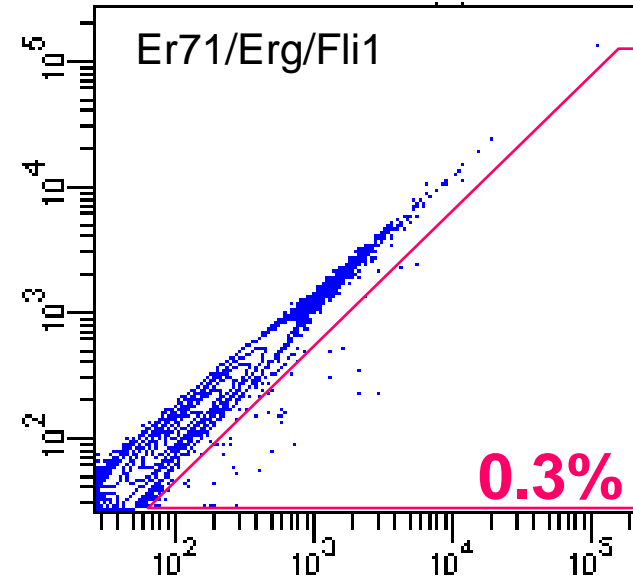
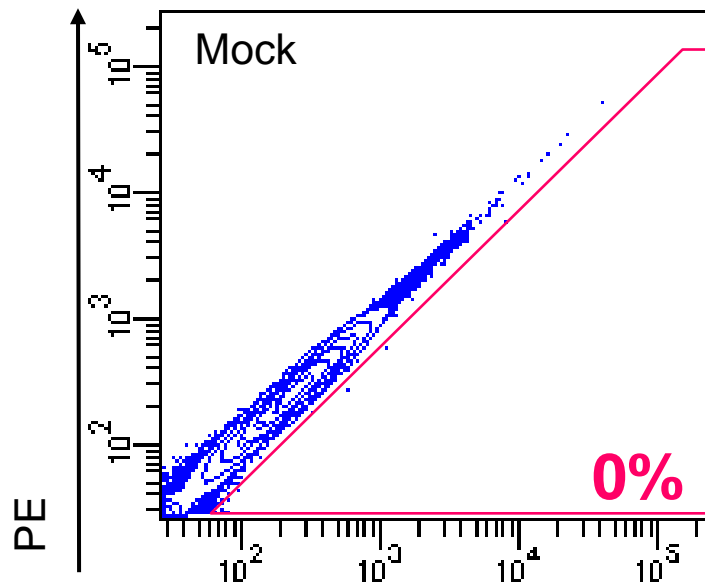
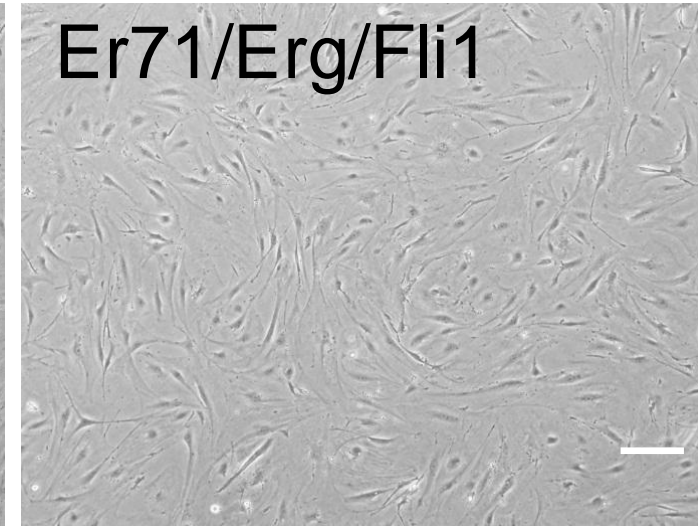
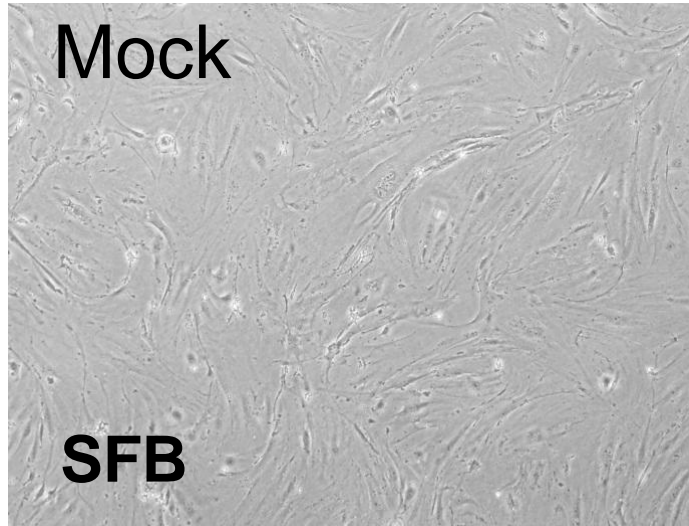


Tie2-GFP

Effect of iEC-5 Factors on **Monocytes**

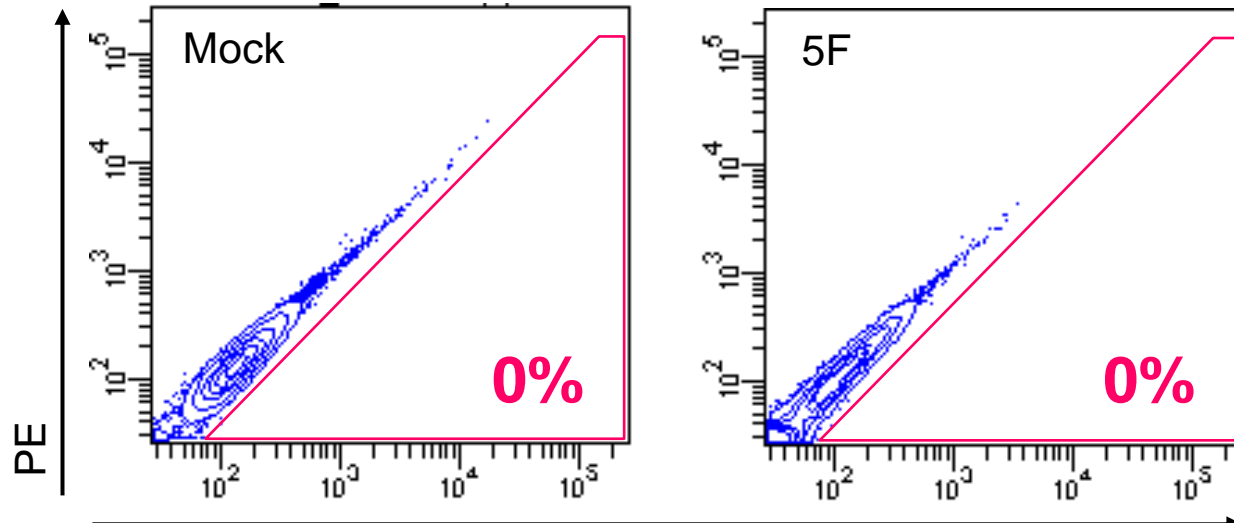


Unique Effect of iEC-5 Factors: Compared with Rafii's 3 Factors

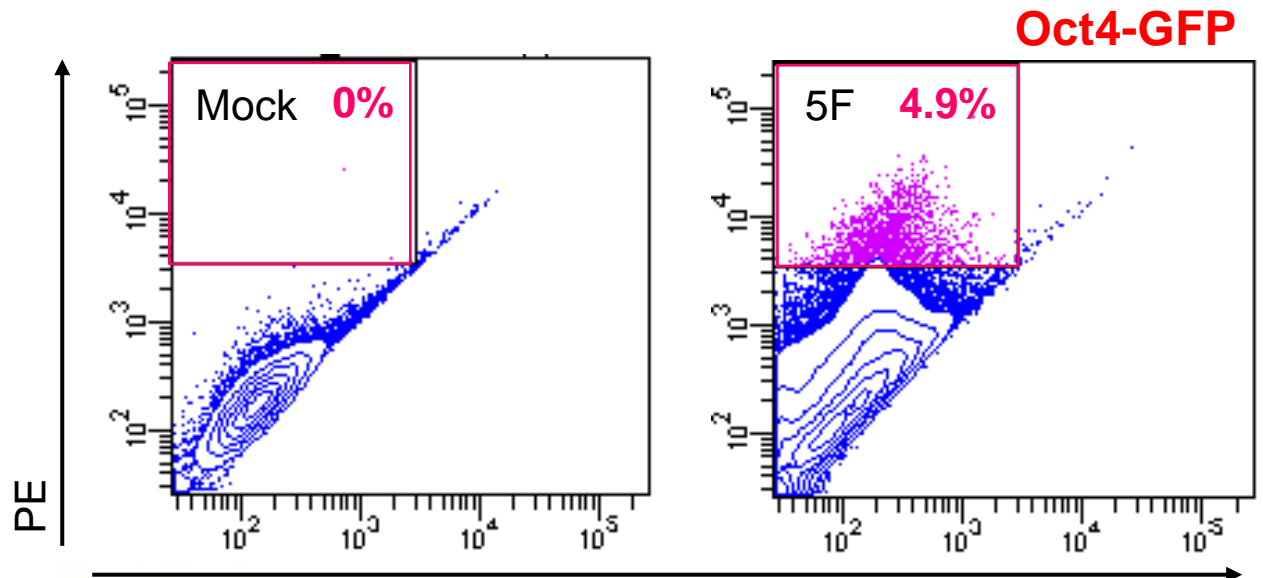


Not Through Pluripotency Induction: Oct4-GFP SFBs

Unstained

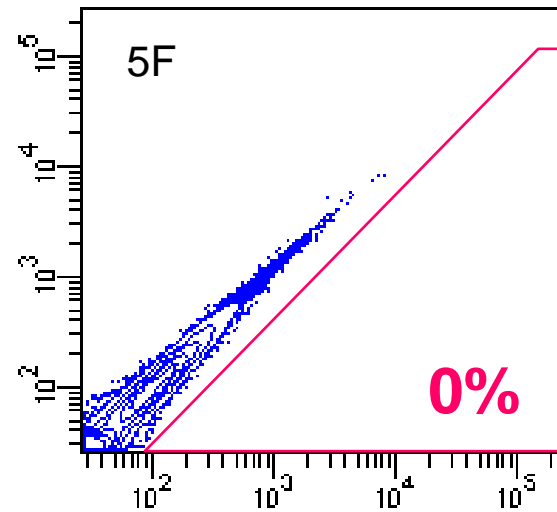
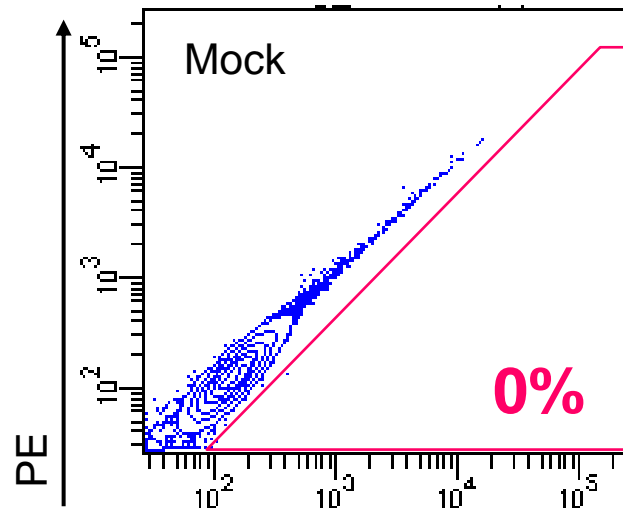


Tie2-PE ab.
stained

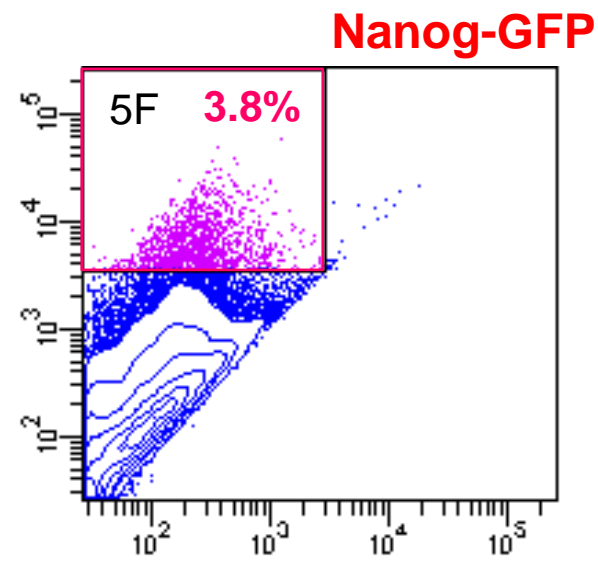
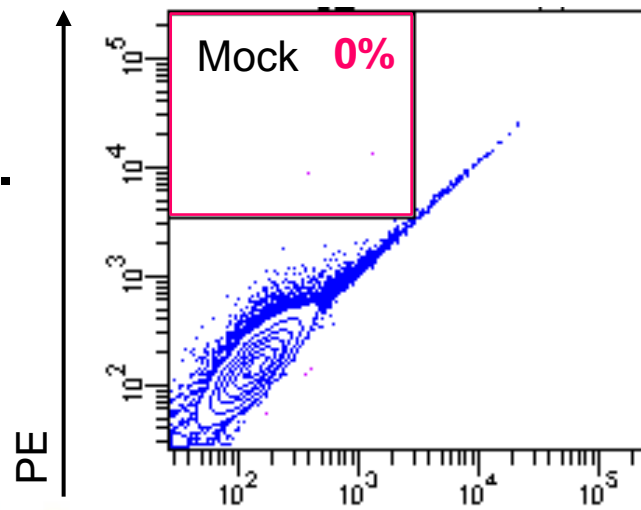


Not Through Pluripotency Induction: Nanog-GFP SFBs

Unstained



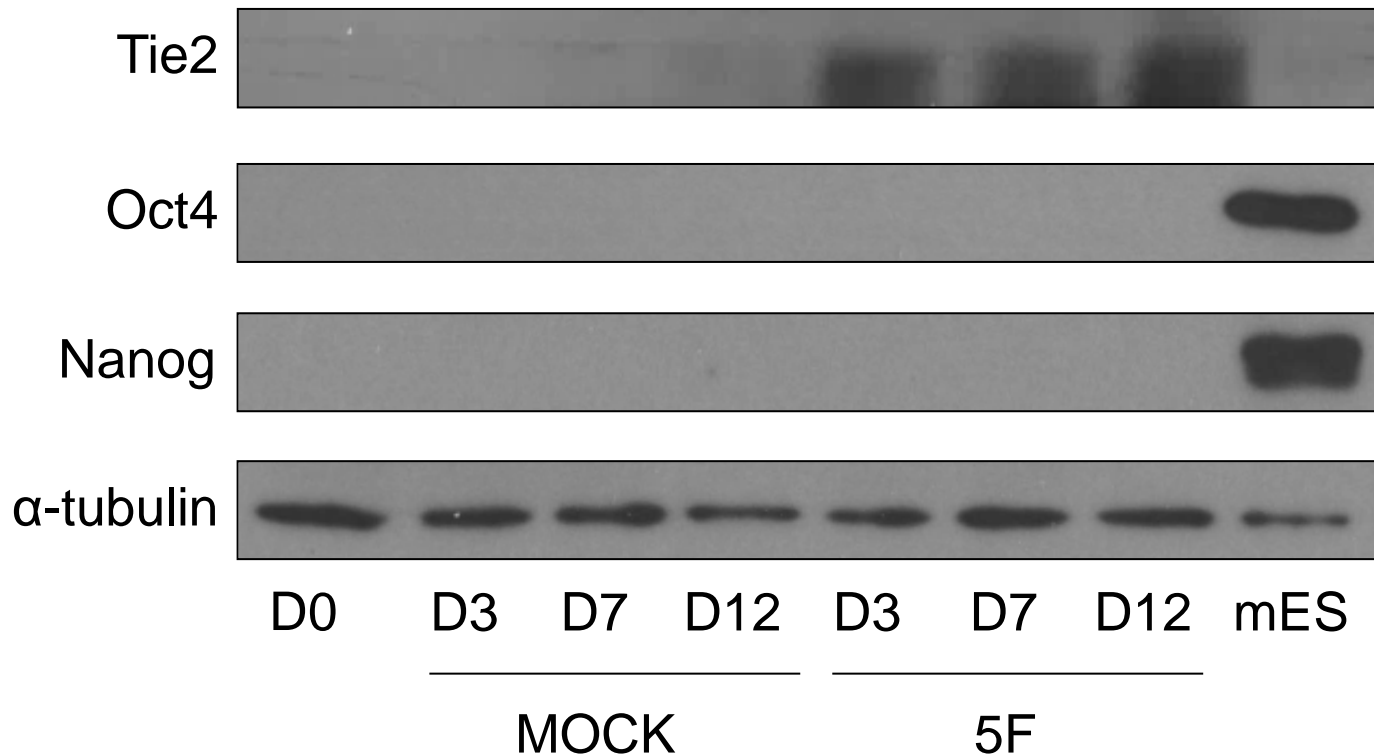
Tie2-PE ab.
stained



Nanog-GFP

Nanog-GFP

Not Through Pluripotency Induction: Oct4/Nanog stay silent during trans-differentiation

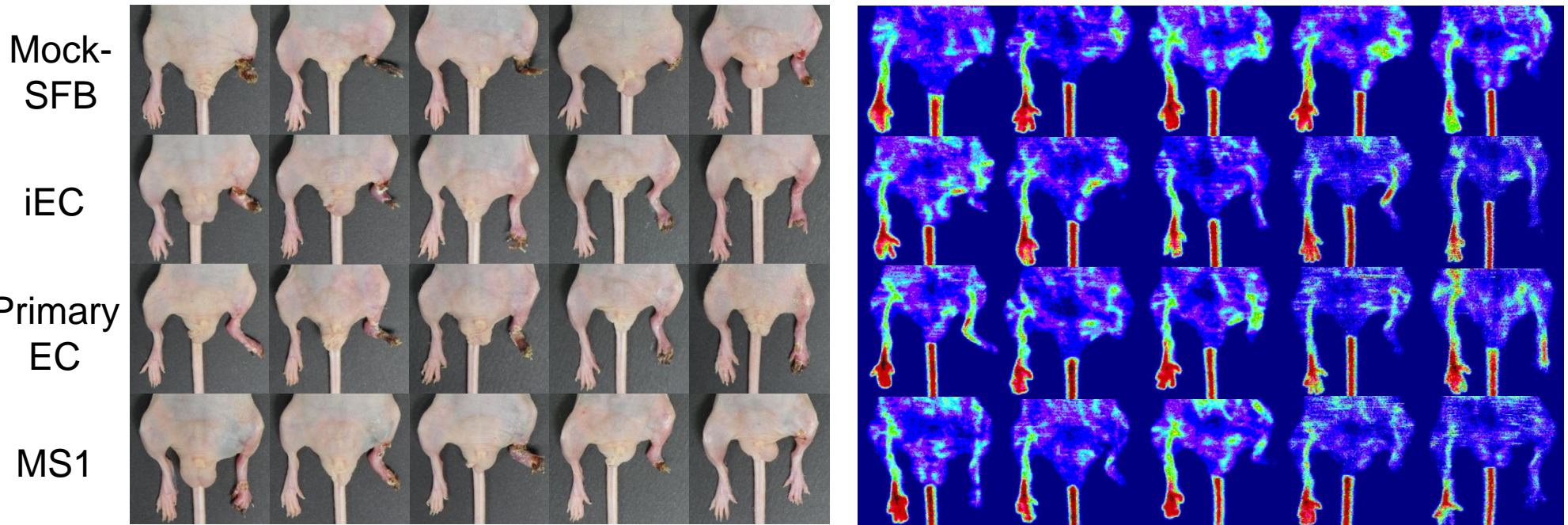


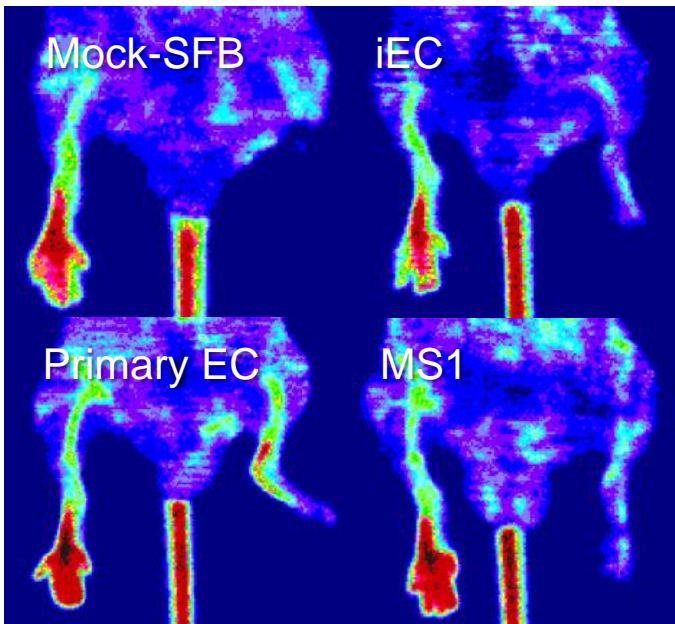
In Vivo **Functionality** of iECs

Mouse Hindlimb Ischemia Model: D14

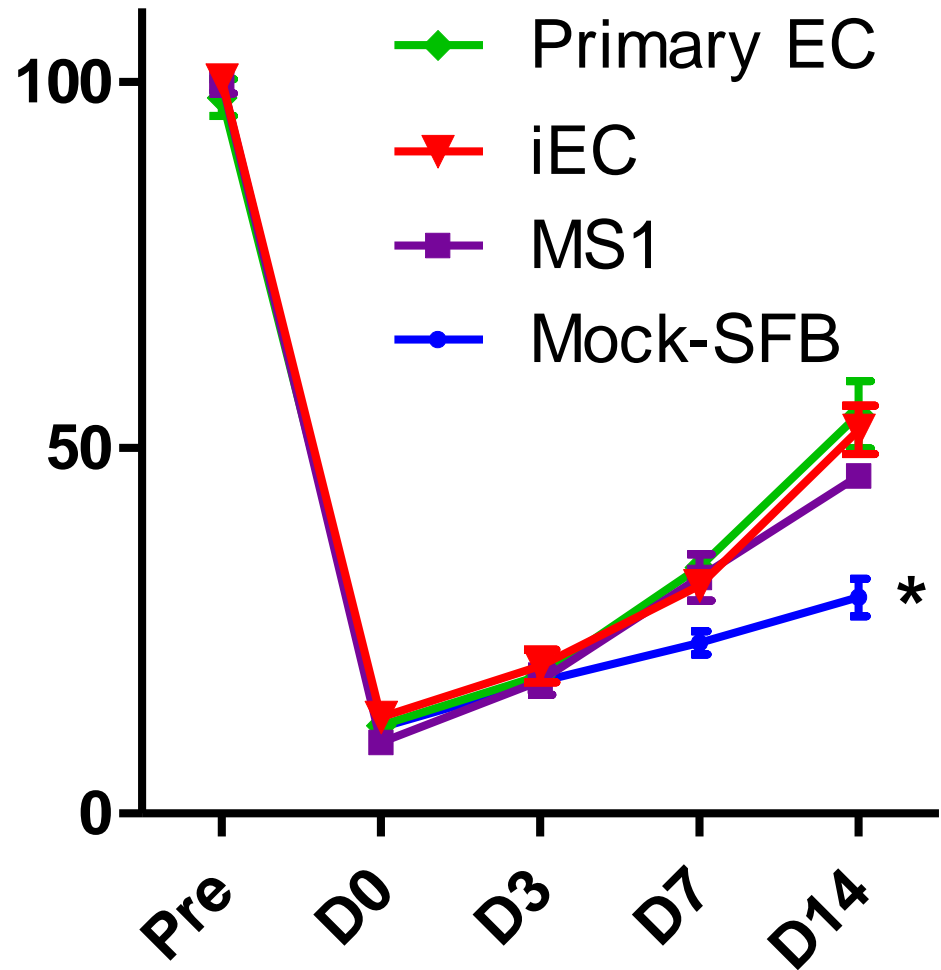
Gross Pictures

LDPI

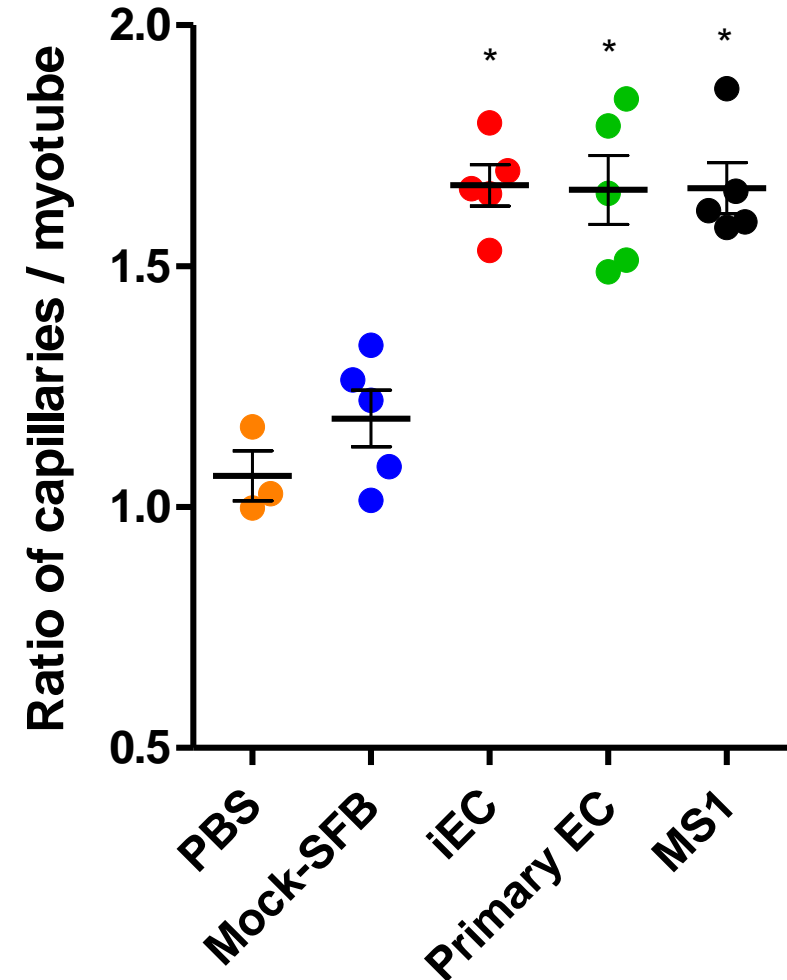
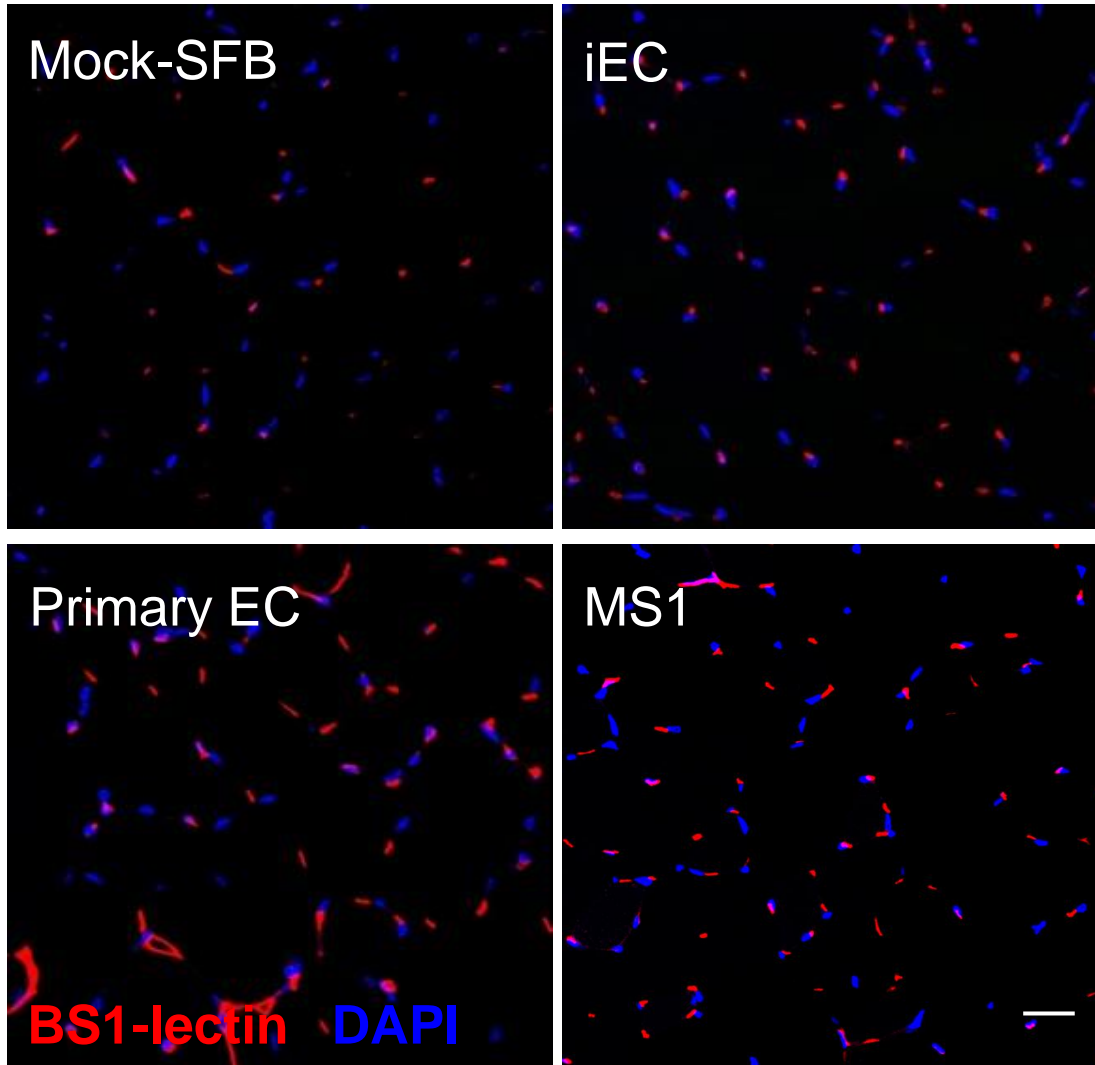




Ratio of ischemic / nonischemic limb perfusion (%)

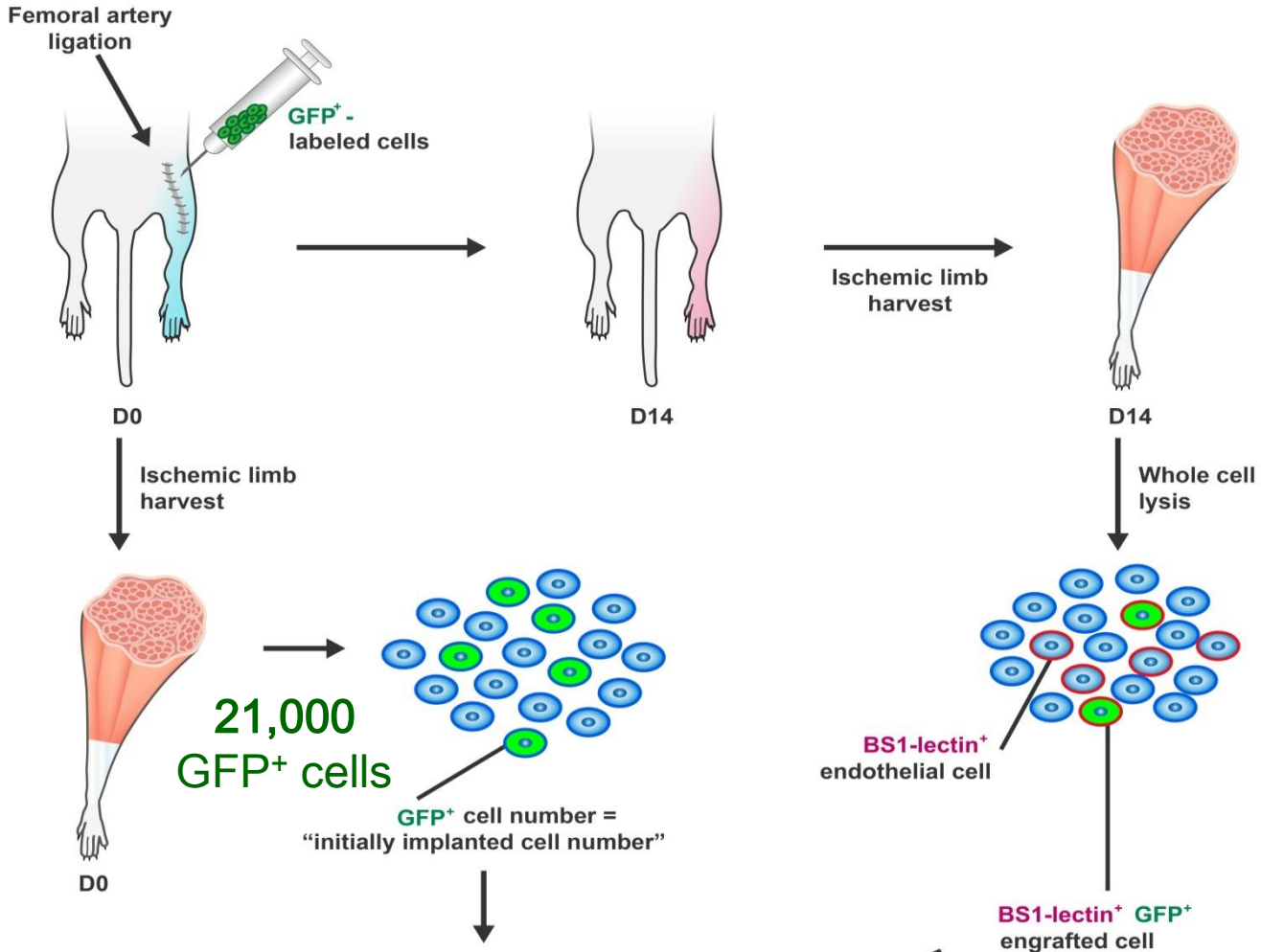


Mouse Hindlimb Ischemia Model: Capillary Density



In Vivo Engraftment Ratio = 10%

500,000
GFP+ cells



21,000
GFP+ cells

GFP+ cell number =
"initially implanted cell number"

BS1-lectin+
endothelial cell

BS1-lectin+ GFP+
engrafted cell

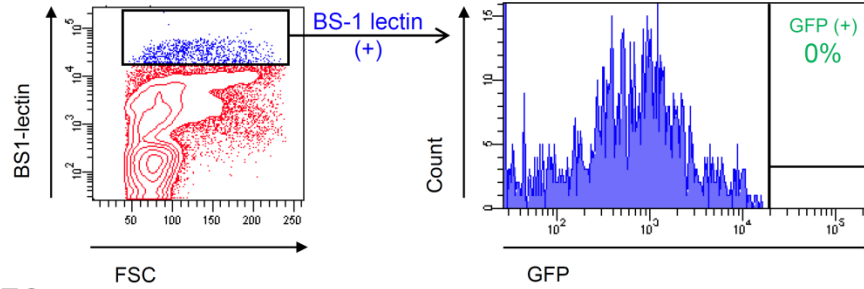
• % of GFP+ cells in capillaries =
BS-1 lectin+ GFP+ cell number /
BS1-lectin+ cell number × 100

• % of engrafted cells =
BS-1 lectin+ GFP+ cell number /
"initially implanted cell number"
× 100

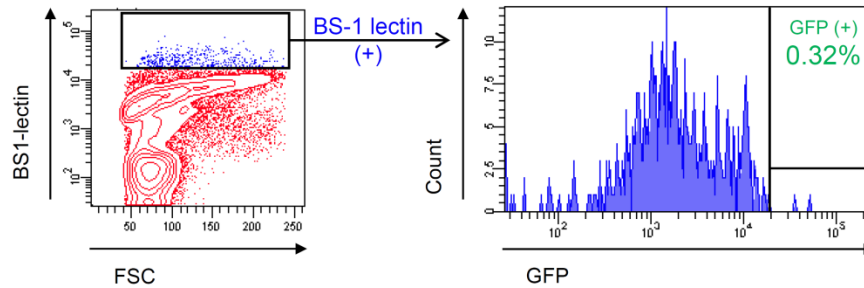
Engraftment ratio =
9.4%, 9.8%, 12.0%
in iEC, primary EC, and MS1

In Vivo Participation as EC in Capillary: 0.3%

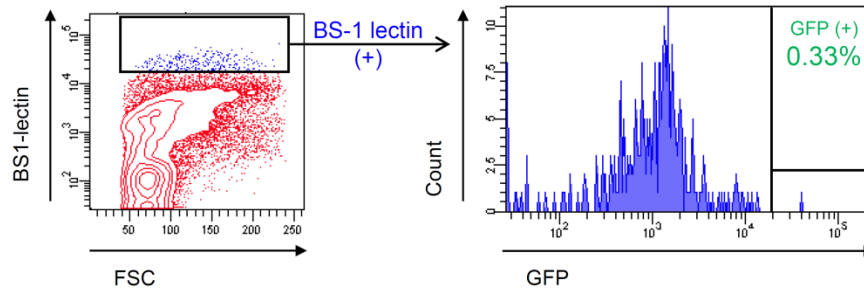
Mock-SFB



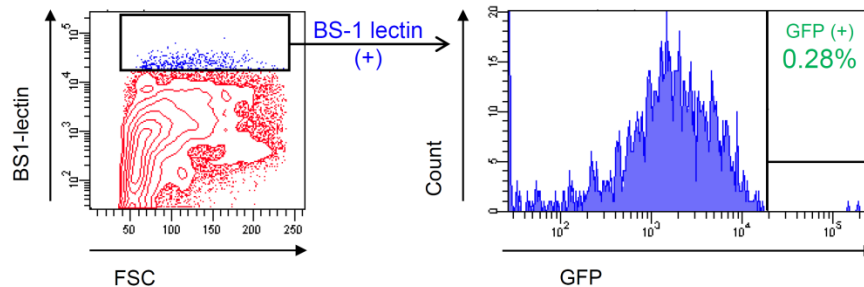
iEC



Primary EC



MS1

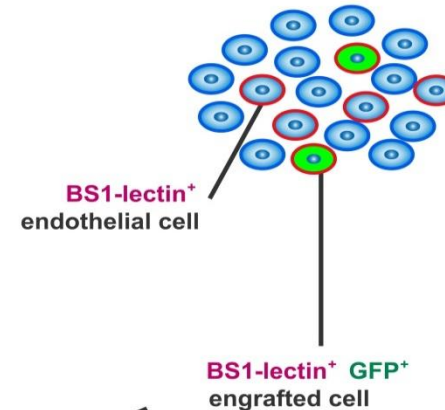


Ischemic limb harvest

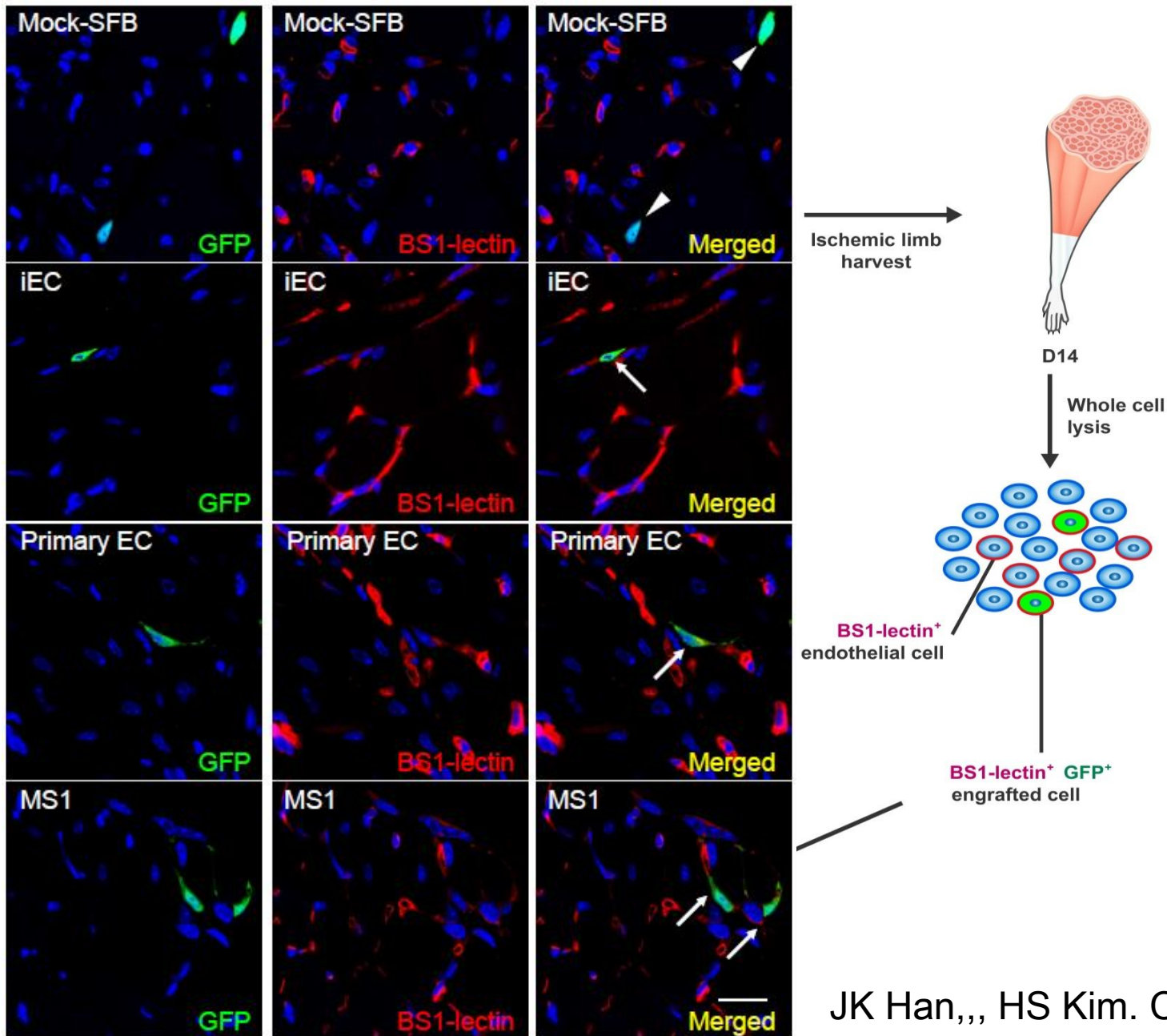


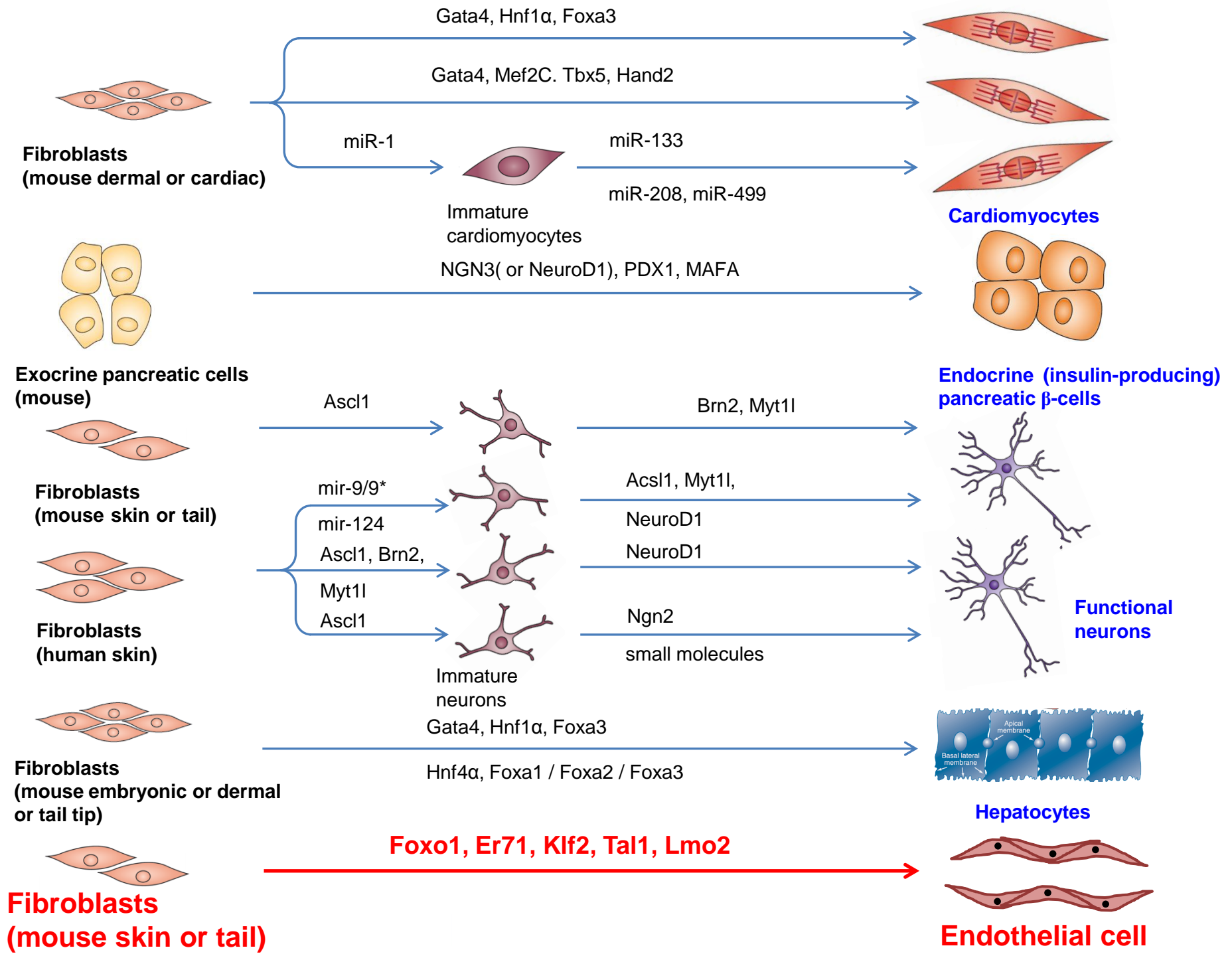
D14

Whole cell lysis

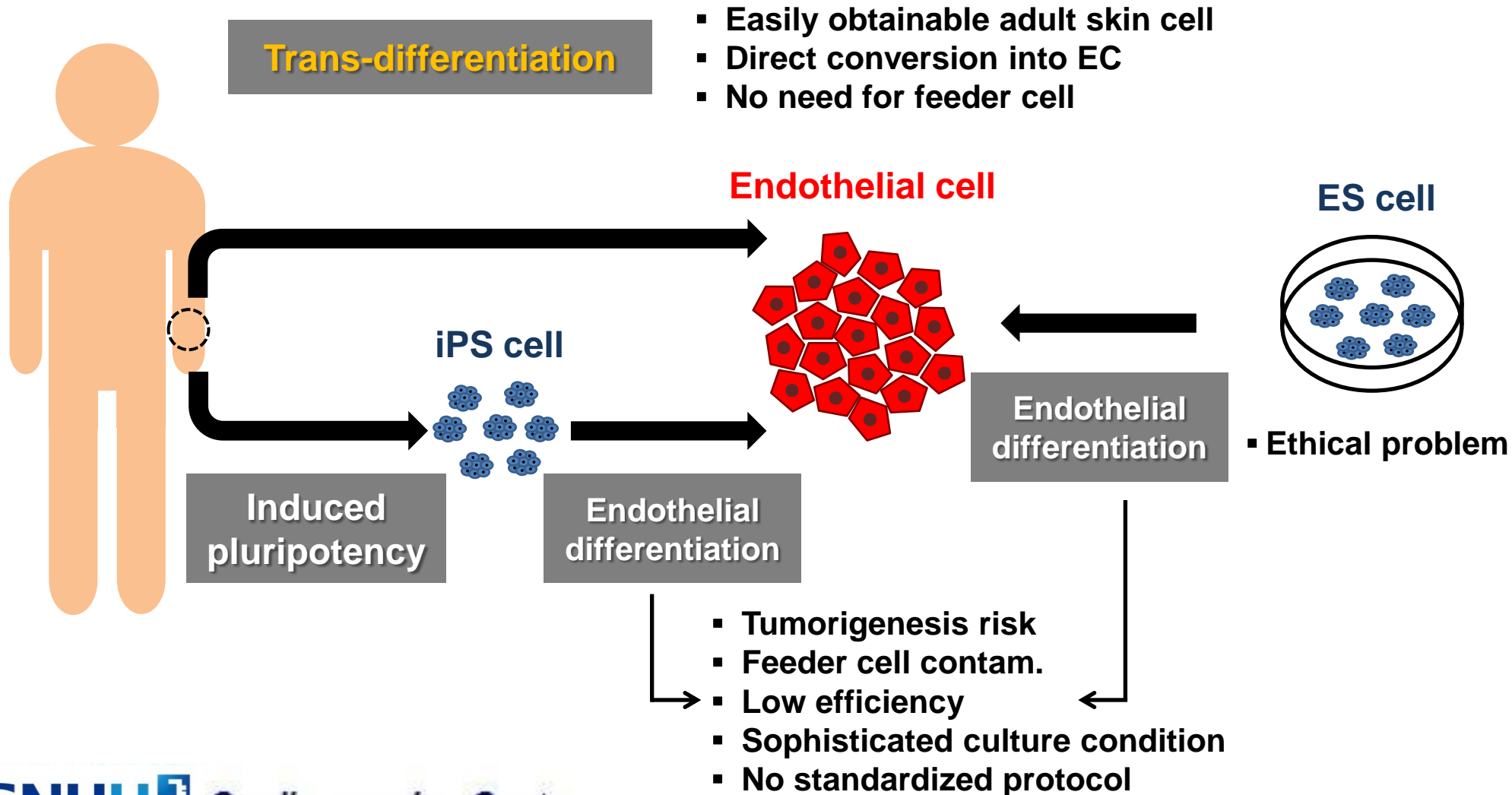


In Vivo Participation as EC : 0.3%

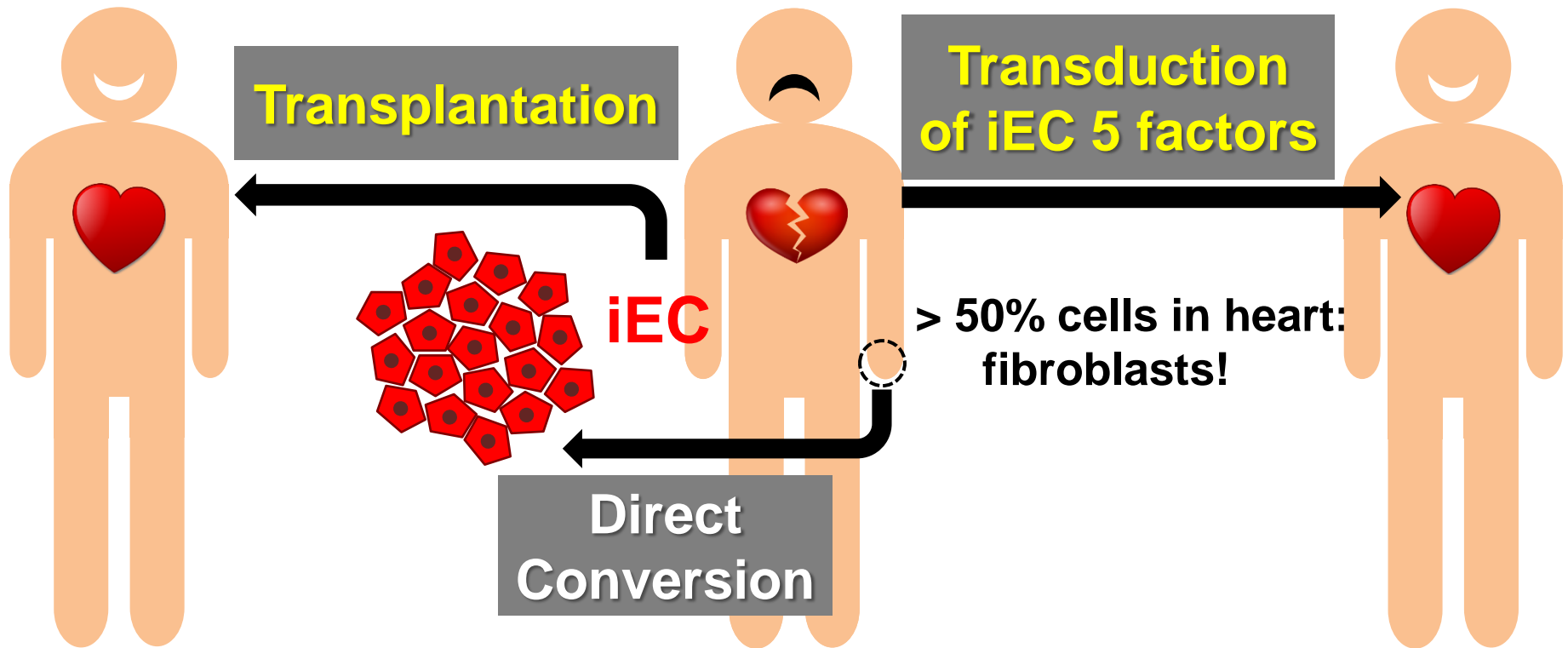




iEC vs. ESC/iPSC-derived ECs



Therapeutic Strategy



Efficient Direct Reprogramming of Mature Amniotic Cells into Endothelial Cells by ETS Factors and TGF β Suppression

- **Cell, 2012**
- **Human amniotic cells**

→ ECs

- **3 ETS factors:**

ER71/ERG1/FLI1

- **Only amniotic cells**
- Not readily available
- Immunogenicity / allograft rejection
- **Not terminally differentiated FBs**

INTRODUCTION

The generation of human endothelial cells (ECs) from nonvascular cell sources has great therapeutic potential for treatment of injured organs. Furthermore, this approach would help to

identify novel pathways that modulate the hierarchical differentiation of typical adult ECs. However, the cultivation of ECs on a clinical scale has not been achieved. Adult-derived ECs have limited expansion potential. Likewise, differentiation of pluripotent stem cells, including induced pluripotent stem cells (iPSC) or human embryonic stem cells (hESCs), into ECs (iVECs) results in the formation of ECs that proliferate poorly and drift into nonvascular lineages (James et al., 2010). Endothelial progenitor cells (EPCs) (Lyden et al., 2001; Rafii et al., 2002; Rafii and Lyden, 2005; Li et al., 2007) possess a significant expansion potential (Liu and Patient, 2007) and maintain their identity after serial passage in vitro. The shortcomings of existing strategies for generating ECs include the need to introduce exogenous transcription factors (TFs) and microenvironmental cues that establish durable tissue-specific vascular cells.

Members of the E-twenty six (ETS) family of TFs, including *ETV2* (Lee et al., 2009), *FLI1* (Lu et al., 2008), and *ERG* (McLaughlin et al., 2001), regulate vascular development and angiogenesis (De Val and Black, 2009). These TFs drive the expression of genes associated with EC development and function. In adult ECs constitutively express several ETS factors, such as *FLI1* and *ERG* (isoforms 1 and 2), whereas *ETV2* is transiently expressed during embryonic development and is absent in adult ECs (Liu and Patient, 2007). Although many of these TFs play crucial roles in EC differentiation (Liu and Patient, 2008; Pham et al., 2007), it remains unclear whether they can switch on EC genes in nonvascular cells. Here, we show that differentiation of hESCs into VECs (hESC-EC) is driven by the expression of *ETV2*, *ERG1*, and *FLI1*. However, iVECs generated by this approach are unstable and often lose their vascular identity.

In search of readily accessible and proliferative human cells for generation of authentic ECs, we identified human amniotic

Conversion of human fibroblasts to angioblast-like progenitor cells

- **Nat. Med, 2012**
- **PNAS, 2012**
- **Human FBs → ECs**
- **Y' iPSC 4 factors:**
- **OCT4/SOX2/KLF4/MYC**
- **Via partial iPSC or progenitor cells status**
- **Concerns over tumorigenic potential**

Here we present a method for the simple and efficient conversion of human fibroblasts to CD34⁺ progenitor cells with bipotent differentiation potential. We use a reprogramming strategy in which complete reprogramming to pluripotency is shortened or bypassed and the cells transition through a plastic intermediate state. This allows redefinition of CD34⁺ progenitor cells and subsequent differentiation into functional endothelial and smooth muscle cells. We thus demonstrate, to our knowledge, that a reprogramming strategy involving partial de-differentiation is feasible in human cells for the generation of multipotent progenitors.

Lineage conversion of fibroblasts to angioblast-like cells
 Prior to establishing our lineage-conversion conditions, we established a mesodermal induction medium (MIM) for efficient differentiation of human fibroblasts into endothelial progenitor cells. The MIM was analyzed in an in vivo mouse model of mesodermal development in different human PSC (hPSC) lines¹². We established a mesodermal induction medium (MIM) for efficient

Directed conversion of fibroblasts into endothelial cells capable of angiogenesis and reendothelialization in tissue-engineered vessels

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The generation of induced pluripotent stem cells is an important tool for regenerative medicine. However, the main restriction is the risk of tumor development. In this study we found that during the early stages of somatic cell reprogramming toward a pluripotent state, specific gene expression patterns are altered. Therefore, we developed a method to generate partial-iPSC (PiPS) cells by transferring four reprogramming factors (OCT4, SOX2, KLF4, and c-MYC) to human fibroblasts for 4 d. PiPS cells did not form tumors in vivo and readily displayed the potential to differentiate into endothelial cells (ECs) in response to defined media and culture conditions. To clarify the mechanism of PiPS cell differentiation into ECs, SET translocation (myeloid leukemia-associated) (SET) similar protein (SETSIIP) was identified to be induced during somatic cell reprogramming. Importantly, when PiPS cells were treated with VEGF, SETSIIP was translocated to the cell nucleus, directly bound to the VE-cadherin promoter, increasing vascular endothelial-cadherin (VE-cadherin) expression levels and EC differentiation. Functionally, PiPS-ECs improved neovascularization and blood flow recovery in a hindlimb ischemic model. Furthermore, PiPS-ECs displayed good attachment, stabilization, patency, and typical vascular structure when seeded on decellularized vessel scaffolds. These findings indicate that reprogramming of fibroblasts into ECs via

reprogramming may regulate signal pathways able to direct the differentiation of reprogrammed cells before the pluripotent state. Therefore, “skipping pluripotency” is a way to convert a somatic cell from one type to another. In this study we have established a method to generate partially induced pluripotent stem (PiPS) cells. This method includes transferring of the genes encoding the four transcription factors (OCT4, SOX2, KLF4, and c-MYC) to human fibroblasts, and culture in reprogramming media for 4 d. PiPS cells did not form tumors in vivo and had the potential to differentiate into ECs in response to defined media and culture conditions. We demonstrated that these PiPS cell-derived ECs are functional in angiogenesis in infarcted tissues in ischemic limb and in reendothelialization in tissue-engineered vessels ex vivo.

Results

Alterations of Gene Expression During Fibroblast Cell Reprogramming as Early as Day 4. Human fibroblasts were virally transduced with genes encoding the four transcription factors OCT4, SOX2, KLF4, and c-MYC, cultured in reprogramming media for 4, 7, 14, and 21 d, and subjected to microarray analysis. The results revealed that 198 genes were altered at day 4, 107 genes at day 7, 97 genes at day

Summary

- The first study demonstrating that adult fibroblasts can be directly converted to ECs by defined factors.
- These **iEC 5 factors** are Foxo1, Er71, Klf2, Tal1 and Lmo2.
- iECs exhibit endothelial features and functions *in vitro* and *in vivo*.

Conclusions

- Our study provides further evidence that **cell fate determination is not eternal, but plastic** by the formation of new transcriptional network.
- Our findings identify the **molecular background of endothelial differentiation and trans-differentiation.**
- This study makes significant progress towards **future clinical application.**

Direct Conversion of Adult Skin Fibroblasts to Endothelial Cells by Defined Factors

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Background—Cell-based therapies to augment endothelial cells (ECs) hold great therapeutic promise. Here, we report a novel approach to generate functional ECs directly from adult fibroblasts.

Methods and Results—Eleven candidate genes that are key regulators of endothelial development were selected. Green fluorescent protein (GFP)–negative skin fibroblasts were prepared from Tie2-GFP mice and infected with lentiviruses allowing simultaneous overexpression of all 11 factors. Tie2-GFP⁺ cells (0.9%), representing Tie2 gene activation, were detected by flow cytometry. Serial stepwise screening revealed 5 key factors (Foxo1, Er71, Klf2, Tal1, and Lmo2) that were required for efficient reprogramming of skin fibroblasts into Tie2-GFP⁺ cells (4%). This reprogramming strategy did not involve pluripotency induction because neither Oct4 nor Nanog was expressed after 5 key factor transduction. Tie2-GFP⁺ cells were isolated using fluorescence-activated cell sorting and designated as induced ECs (iECs). iECs exhibited endothelium-like cobblestone morphology and expressed EC molecular markers. iECs possessed endothelial functions such as *Bandeiraea simplicifolia*-1 lectin binding, acetylated low-density lipoprotein uptake, capillary formation on Matrigel, and nitric oxide production. The epigenetic profile of iECs was similar to that of authentic ECs because the promoters of VE-cadherin and Tie2 genes were demethylated. mRNA profiling showed clustering of iECs with authentic ECs and highly enriched endothelial genes in iECs. In a murine model of hind-limb ischemia, iEC implantation increased capillary density and enhanced limb perfusion, demonstrating the in vivo viability and functionality of iECs.

Conclusions—We demonstrated the first direct conversion of adult fibroblasts to functional ECs. These results suggest a novel therapeutic modality for cell therapy in ischemic vascular disease. (*Circulation*. 2014;130:1168-1178.)

Key Words: cell transdifferentiation ■ endothelial cells ■ fibroblasts

iEC Project

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