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Cochrane, A., Kelaini, S., Bojdo, J., Vilà-González, M., Drehmer, D., Caines, R., ... Margariti, A. (2017). Quaking is a Key Regulator of Endothelial Cell Differentiation, Neovascularization and Angiogenesis. Stem Cells. DOI: 10.1002/stem.2594

Published in:
Stem Cells

Document Version:
Publisher's PDF, also known as Version of record

Queen's University Belfast - Research Portal:
Link to publication record in Queen's University Belfast Research Portal

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Download date:24. Feb. 2017

STEM CELLS

REGENERATIVE MEDICINE

Quaking is a Key Regulator of Endothelial Cell Differentiation, Neovascularization and Angiogenesis

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Key words. Differentiation • Endothelial cell • STAT • Angiogenesis • RNA Binding Proteins • mRNA Splicing

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Received August 25, 2016; accepted for publication January 24, 2017; available online without subscription through the open access option.

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1066-5099/2017/$30.00/0

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/stem.2594

ABSTRACT

The capability to derive endothelial cell (ECs) from induced Pluripotent Stem (iPS) cells holds huge therapeutic potential for cardiovascular disease. Objective- This study elucidates the precise role of the RNA-binding protein Quaking isoform 5 (QKI-5) during EC differentiation from both mouse and human iPS cells and dissects how RNA-binding proteins can improve differentiation efficiency towards cell therapy for important vascular diseases. iPS cells represent an attractive cellular approach for regenerative medicine today since they can be used to generate patient-specific therapeutic cells towards autologous cell therapy. In this study, using the model of iPS cells differentiation towards ECs, the QKI-5 was found to be an important regulator of STAT3 stabilisation and VEGFR2 activation during the EC differentiation process. QKI-5 was induced during EC differentiation, resulting in stabilisation of STAT3 expression and modulation of VEGFR2 transcriptional activation as well as VEGF secretion through direct binding to the 3' UTR of STAT3. Importantly, mouse iPS-ECs overexpressing QKI-5 significantly improved angiogenesis and neovascularization and blood flow recovery in experimental hind limb ischemia. Notably, human iPS cells overexpressing QKI-5, induced angiogenesis on Matrigel plug assays in vivo only seven days after subcutaneous injection in SCID mice. These results highlight a clear functional benefit of QKI-5 in neovascularization, blood flow recovery and angiogenesis. They, thus, provide support to the growing consensus that elucidation of the molecular mechanisms underlying EC differentiation will ultimately advance stem cell regenerative therapy and eventually make the treatment of cardiovascular disease a reality. STEM CELLS *2017; 00:000–000*

SIGNIFICANCE STATEMENT

Cardiovascular disease is a leading cause of mortality worldwide with pathology being driven by the dysfunction of endothelial cells (ECs). Induced pluripotent stem (iPS) cell technology is offering new avenues to generate

ECs. Although there has been significant progression in the field, currently, there are not any fully defined protocols in existence aimed at generating high-fidelity, stable ECs. We provide robust evidence that the RNA-binding protein QKI has a unique role in inducing EC differentiation from iPS cells and remarkably improving angiogenesis, neovascularization and blood flow recovery (almost 100%) in an in vivo model of vascular regeneration. These findings can advance stem cell regenerative therapy towards the treatment of cardiovascular disease.

Subject Codes: iPS Cells; endothelial cell differentiation, vascular disease, angiogenesis, QKI-5.

Introduction

Cardiovascular disease is a leading cause of mortality worldwide with pathology being significantly driven by progressive vascular endothelial cell (EC) dysfunction which regulates key pathogenic events such as infiltration of inflammatory cells, vascular smooth muscle proliferation and platelet aggregation [1, 2]. As cardiovascular disease progresses, ECs become depleted from the vascular lumen, and, without adequate replacement, non-perfusion and tissue ischaemia ensues. Repair and regeneration of ECs has been an important research focus for many years. As an important therapeutic avenue, the delivery of adult progenitor or stem cells to repair damaged vasculature has faced many limitations, such as identification and availability of appropriate, efficacious cell-types for therapy [3]. However, recently, the ability to derive ECs from induced pluripotent stem (iPS) cells has extended the scientific scope for regenerative medicine [4, 5].

Cell reprogramming is offering new avenues for regenerative medicine with iPS cells proving able to differentiate into nearly all types of cells within the body [6-8]. This is a unique characteristic of iPS cell technology, which offers a significant potential for cell-based therapies towards repairing tissues or organs destroyed by injury, degenerative diseases, aging or cancer [4, 5, 8, 9]. Generation of iPS cells also offers a promising strategy to create patient-specific cells [10-12] [7, 13] with building evidence that these cells have therapeutic efficacy in animal models of disease [5, 14] [15] [16] [3]. The concept of cell reprogramming is powerful [17-20] and has, so far, allowed the development of dynamic approaches to generate functional cells of interest and to switch cell fate [20]. We and others have recently demonstrated novel strategies of direct reprogramming towards functional ECs [21, 22]. Both iPS cells and directly reprogrammed cells (partial iPS –PiPS cells) can serve as useful tools not only for derivation of functional cells but also for establishing efficient protocols of differentiation and developing platforms to investigate the underlying mechanisms.

Vascular cell differentiation is achieved through the coordination of diverse molecular pathways [21, 23-28]. Due to this, there is a need to better understand the complex dynamics behind RNA regulation and how this, in turn, influences transcriptional control and protein expression. Elucidation of these complex molecular signals, which are evoked during iPS and PiPS cell differentiation towards ECs, may allow specific targeting of their activities to enhance cell differentiation and promote tissue regeneration. In an effort to elucidate the imperative mechanisms of RNA regulation during EC differentiation from iPS cells, the present study focused on the RNA-binding protein Quaking (QKI). QKI is a member of the "STAR" (signal transduction and activation of RNA) family of proteins. These proteins are characterized by the presence of at least one RNA-binding motif (QKI containing a KH domain), SH2 and SH3 domains and potential phosphorylation sites. This suggests their involvement in signal transduction pathways [29] [30], pre-mRNA splicing [31], exportation of mRNAs from the nucleus [32], protein translation and mRNA stability. Functional studies of the mouse QKI gene have revealed that it has a key function in embryonic blood vessel formation and remodeling [33] [34, 35]. During vascular cell differentiation and reprogramming processes, QKI could play an important regulatory role in gene expression at the post-transcriptional level in either a miRNA-dependent or independent manner. Alternative splicing of genes, a process that RNA binding proteins are highly involved in, generates many forms of mature mRNA from the same gene is an essential process in development and a potent way to regulate gene expression at a post-transcriptional level. QKI has a number of alternative splicing isoforms, three of which have been associated with vascular development; QKI-5, QKI-6 and QKI-7. This further reinforces the importance of QKI in protein regulation and development. Each isoform contains identical RNA binding domains and differ only by their carboxy-terminal ends [36, 37]. In this study we focus on the QKI splicing isoform 5, (QKI-5). QKI-5 is most highly expressed during EC differentiation from iPS cells, it is an essential player during embryogenesis and notably, QKI-5 null mice are embryonic lethal [38, 39].

We provide strong evidence that QKI-5 plays a critical role in the differentiation of ECs derived from iPS cells and acts as a key regulator of CD144 stabilization and VEGFR2 activation through STAT3 signaling. The RNA binding protein QKI-5 directly binds to 3' UTR of STAT3 and induced STAT3 mRNA stabilisation. Remarkably, QKI-5 improved neovascularization and blood flow recovery in a hind limb ischemic model. Notably, human iPS cells overexpressing QKI-5 induced angiogenesis in Matrigel plug assays in vivo only seven days after subcutaneous injection in SCID mice, highlighting, thus, a pivotal role of QKI-5 in neovascularization, blood flow recovery and angiogenesis.

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Materials and Methods

Cell culture media, serum, and cell culture supplements were purchased from ATCC, Millipore, LONZA, and Thermo Fisher Scientific. Antibodies against VE-cadherin (CD144) (ab33168), PECAM (CD31) (ab28364), Flk-1 (ab9530), STAT3 (ab119352), pSTAT3 (ab76315), JAK-1 (ab47435), GAPDH (ab8245), OCT4 (ab19857), eNOS (ab66127), QKI (ab126742) were purchased from Abcam. Antibodies against vWF (SC-8068), QKI (SC-103851) were purchased from Santa Cruz. Antibodies against QKI-5 (AB 9904) were purchased from Millipore.

Mouse iPS and ES cell Culture and Differentiation

Mouse iPS cells were generated in our laboratory using a similar approach as previously described [40, 41]. Mouse iPS cells were cultured in gelatin-coated flasks (PBS containing 0.02% gelatin from bovine skin; Sigma-Aldrich) in DMEM (ATCC) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Thermo Fisher Scientific), 10 ng/ml recombinant human leukemia inhibitory factor (Millipore); and 0.1 mM 2-mercaptoethanol (Invitrogen) in a humidified incubator supplemented with 5% CO_2. The cells were passaged every 2 days at a ratio of 1:6. Differentiation of iPS cells was induced by seeding the cells on type IV mouse collagen (5 μg/ml)-coated dishes in differentiation medium (DM) containing α-MEM supplemented with 10% FBS (Invitrogen), 0.05 mM 2-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin in the presence of 25 ng/ml VEGF for the time points indicated.

Human iPS cells differentiation and HUVECs

Human iPS cells were pre-differentiated in low attachment plates using StemPro serum free media supplemented with BMP4, Activin A, FGF and VEGF for 5 days. The pre-differentiated cells were seeded on fibronectin (Sigma-Aldrich), whilst KDR endothelial precursor cells were magnetically sorted on day 6 using MicroBeads Kit (Miltenyi BIotec) and culturing in EGM-2 media (LONZA) for 3 to 9 days. QKI-5 was overexpressed or knockdown by lentiviral gene transfer on day 3 after KDR selection and the cells were harvested on day 3 and subjected to further analysis or labeled for the in vivo Matrigel plug assays. HUVECs were seeded on collagen I coated plates and cultured in EGM-2 media. QKI-5 was overexpressed by lentiviral gene transfer and subjected to further analysis.

Experimental hind limb ischemia

The mouse hind limb ischemia model was performed as previously described [21, 42]. iPS-ECs were infected with FUW-QKI-5 or control empty vectors on day 4 of EC differentiation. 48 h later the cells were trypsinised and labelled with Molecular Probes Vybrant Cell Labelling (MP22885) before being injected intramuscularly into the adductors of ***C57BL/6*** wild type mice.

Statistical Analysis

Data are expressed as the mean±SEM and were analyzed using GraphPad Prism 5 software with a two-tailed Student's *t* test for two groups or pairwise comparisons or ANOVA. A value of *: p<0.05. * p<0.05, ** p<0.01, *** p<0.001 was considered significant.

Detailed Methods and Materials are in *SI Appendix,* Experimental Procedures

Results

Pluripotent stem cells differentiation towards endothelial cells

Mouse iPS cells were differentiated towards ECs by seeding on collagen IV and differentiation media (DM) supplemented with 25 ng/ml Vascular Endothelial Growth Factor (VEGF) (DM+V) for 2 to 10 days. The differentiated ECs adopted a typical EC morphology (Fig. 1A-B). The efficiency of EC differentiation from mouse iPS cells was very high as FACs analysis is shown in Fig. 1C (86.7% positive cells for CD144 (VE-Cadherin), and 87.2% positive cells for CD31 were obtained). These results were confirmed in both mRNA (Fig. 1D) and protein levels (Fig. 1E, F). During EC differentiation the pluripotent marker OCT4 was suppressed allowing the expression of the EC specific markers CD144 and CD31 to occur in a time point dependent manner (Fig. 1F). Indeed, immunofluorescence confocal microscope images revealed that ECs derived from iPS cells expressed EC markers (CD144, FLK-1, eNOS and vWF) which are characteristic of ECs (Fig. 1G). We also observed that the expression of the transcription factor STAT3 which is a known essential player in VEGF signaling during vascular development. We are reporting that STAT3 was progressively induced during EC differentiation (Fig. 1H). Taken together, this data clearly demonstrates that mouse iPS cells have been differentiated towards ECs based on a highly efficient approach.

The RNA-binding protein QKI is induced during EC differentiation from iPS cells

QKI-5 was significantly induced during EC differentiation from iPS cells and this occurred in parallel with the EC markers CD144 and CD31 (Fig. 2A). It is important to note that only QKI-5 mRNA splicing isoform was induced during EC differentiation. QKI-6, and QKI-7 expression levels were also tested showing no change or decrease, respectively (Figure 2A). Time-course experiments also demonstrated that QKI-5 was progressively induced after 4 days post-differentiation from iPS cells (Fig. 2B). There was a comparable induction of CD144

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and STAT3 (Fig. 2B). Immunofluorescence images demonstrated that the alternative splicing isoform QKI-5 was localized to the EC nucleus in both mouse iPS-derived ECs and in HUVECs (Fig. 2C-E). Also in both EC-types, CD144 and CD31 were expressed in the plasma membrane (Fig. 2D and E). These results demonstrated that QKI-5 expression is induced during EC differentiation from mouse iPS cells, and its expression is maintained in mature ECs.

A number of additional experiments were performed to determine whether QKI-5 plays a regulatory role in iPS cell differentiation towards ECs. Initially, a pure population (100%) of differentiated ECs was obtained based on CD144 selection, as it is a robust EC-specific marker. Based on this pure population of ECs derived from iPS cells, a high expression of QKI-5 was observed and this was comparable to the EC markers CD144, VEGFA, and the transcription factor STAT3 (Supplementary Fig. S1 A-F).

Further experiments performed on mouse embryonic stem cells (ES cells) differentiated to ECs, using a similar approach as the iPS cells, verified that QKI-5 expression was induced in parallel with CD144 expression (Supplementary Fig. S2 A-D). These results indicated that QKI-5 may be implicated in differentiation of ECs derived from mouse iPS and ES cells. In order to elucidate the underlying mechanisms regulated by QKI-5 in EC differentiation, QKI-5 was overexpressed by lentiviral gene transfer at day 4 of EC differentiation using ECs derived from iPS cells. The cells were harvested on day 6 and subjected to further analysis. QKI-5 overexpression significantly enhanced expression of the endothelial markers CD144, CD31, eNOS, VEGFA and the transcription factor STAT3 (Fig. 2F and G). Surprisingly, QKI-5 overexpression also induced the in activation of the CD144 (VE-Cadherin) promoter as revealed by luciferase assays (Fig. 2H).

Importantly, QKI-5 also induced the expression of the arterial specific EC marker Ephrin B2, but it did not affect the expression of venous (CoupTFII), and lymphatic (Lyve1) markers. Moreover, QKI-5 induced EC differentiation in an EC-specific manner, while markers of other cells lineages such as neuronal (Nestin), cardiac (Mef2c) or smooth muscle cell (SMA) were not altered by QKI-5 overexpression (Fig. 2I). This data reveal that QKI-5 induced EC differentiation towards an arterial lineage.

QKI-5 is capable of initiating EC differentiation from iPS cells

Further experiments have revealed that QKI-5 leads to the stabilization of VE-Cadherin adhesion molecule during the EC differentiation process. When differentiated ECs were treated with Actinomycin D for 6 hours or cyclohexamide for 24 hours, CD144 (VE-Cadherin) expression was stabilized in the presence of QKI-5 (Supplementary Fig. S3A-B and S3C-D), respectively). Moreover, when differentiated ECs were treated with Actinomycin D in a time point experiment from 0 to 24 hours CD144 expression was stabilised as a decay curve is shown in Supplementary Figure S3E**.** These results demonstrate that QKI-5 induced the expression and stabilisation of CD144 during EC differentiation. Additional experiments have been conducted to address the important question whether QKI-5 is able to initiate EC differentiation from iPS cells. When QKI-5 was overexpressed in undifferentiated iPS cells, the cells started expressing the arterial marker Ephrin B2 3 days later, while venous, lymphatic or markers of other cell lineages, such as Nestin and SMA, were not expressed (Supplementary Fig. S4A). Importantly, EC markers were highly induced upon QKI-5 overexpression in early stages of EC differentiation (Supplementary Fig. S4B). Finally, to shed light on the upstream signaling, which activates QKI-5 expression in early stages of EC differentiation from iPS cells, transcription factor binding analysis on the QKI-5 promoter has predicted a potential binding site for the transcription factor ETS1. ETS factors, and in particular ETS1 and Etv2, have been reported as key factors that regulate endothelial development [43]. Interestingly, ETS1 is induced during EC differentiation from iPS cells in a time dependent manner (Supplementary Fig. S4C), while overexpression of ETS1 in early stages of EC differentiation has led to induction of QKI-5 (Supplementary Fig. S4D). These results demonstrate that QKI-5 is able to initiate EC differentiation from iPS cells, and ETS1 may be implicated in the activation of QKI-5 in the early stages of EC differentiation.

QKI-5 induced VEGF Receptor 2 (VEGFR2) activation and VEGF secretion through direct binding of the 3'UTR region of STAT3

Formation of vascular networks are highly dependent on VEGF as a critical regulator of EC differentiation and vasculogenesis during development. Overexpression of QKI-5 by lentiviral gene transfer induced the secretion of VEGF on day 6 during the EC differentiation process (Fig. 3A). Likewise, QKI-5 overexpression led to activation of VEGFR2 during EC differentiation from iPS cells (Fig. 3B). In order to shed light on the signaling cascade initiated by the VEGFR transcriptional activation, the expression of the transcription factors STAT3, JAK-1 and AP-1 was assessed. Over-expression of QKI-5 leads to enhanced transcriptional activation of the VEGFR2 signaling pathway, which seems to be related to increased expression of VEGFA and autocrine stimulation of its cognate receptor (Fig. 3C). Notably, overexpression of QKI-5 induced the expression of EC markers, CD144 and CD31, in parallel with the induction of JAK-1, STAT3 and phosphorylation of STAT3 (Fig. 3D, and quantification in 3E). These results revealed that QKI-5 is implicated in the activation of the VEGFR2-regulatory binding sites AP1, STAT3 as well as STAT3 phosphorylation. Additional experiments were performed to investigate the nature of QKI-5-mediated activation of VEGFR. When STAT3 was knocked down by shRNA during EC differentiation, the previously observed upregulation of EC

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markers (CD144, CD31, eNOS) and VEGFA, mediated by QKI-5, was ablated (Fig. 3F). Furthermore, luciferase assays revealed that knockdown of STAT3 by shRNA ablated the QKI-5-mediated activation of VEGFR2 (Supplementary Fig. S5A). In agreement with the above results, when the differentiated ECs were treated with inhibitors to block the JAK-1, and STAT3 pathways, QKI-5 did not activate the EC markers CD31, CD144, eNOS, FLK-1 (VEGFR2) and VEGFA (Supplementary Fig. S5B). Taken together, these data demonstrate that QKI-5 modulates the activation of VEGFR and EC differentiation through the transcription factor STAT3. Interestingly, the KH DOMAIN-mutant QKI-5 constructs did not induce the expression of the EC markers, and STAT3 signalling when it was overexpressed in the iPS cells (Supplementary Fig S6A-D). These results suggest that QKI-5 binds candidate RNAs in the JAK1-STAT3 signalling pathway and regulates EC differentiation. Importantly, luciferase assays have shown that QKI-5 activated the 3'UTR of STAT3. Remarkably, QKI-5 was unable to activate the 3'UTR of STAT3 when the QKI motif was deleted (Figure 3G). Moreover, RNA binding assays have confirmed that QKI binds directly to the 3'UTR of STAT3 (Figure 3G, lower panel). Moreover, when differentiated ECs were treated with Actinomycin D in a time point experiment from 0 to 24 hours STAT3 expression was stabilized as a decay curve is shown in Figure 3H**.** These results clearly demonstrate that the RNA binding protein QKI-5 induced VEGF Receptor 2 (VEGFR2) activation and VEGF secretion through direct binding of the 3'UTR of STAT3.

QKI-5 has a critical role during EC differentiation and vascular tube formation

When QKI-5 expression was suppressed by shRNA during EC differentiation, this resulted in a significant suppression of the EC markers CD144 and CD31 (Fig. 4A and 4B) and the transcription factor STAT3 (Fig. 4B). Moreover, knockdown of QKI-5 caused inhibition in the transcriptional activation of VEGFR (Fig. 4C). Furthermore, knockdown of QKI-5 by shRNA resulted in inhibition of angiogenesis in Matrigel plugs *in vivo* (Fig. 4D), which further reinforced the notion that QKI-5 plays a critical role during EC differentiation and subsequent angiogenic function of the derived cells.

QKI-5 induced angiogenesis in both *in vitro* and *in vivo*

QKI-5 appears to play an important role in iPS cell-derived ECs during the formation of vascular networks *in vitro* and *in vivo*. These processes are greatly dependent on the presence of VEGF, although these experiments were conducted in the absence of exogenous VEGF. Notably, overexpression of QKI-5 by lentiviral gene transfer induced vascular tube formation *in vitro* (Fig. 4E, quantified in the right panel as total tube length /field (μM)) and vessel formation *in vivo* (Fig. 4F, quantified in the right panel as capillary density) when compared to controls. CD144 immunofluorescence staining verified the presence of differentiated cells in *in vivo* vascular tubes and demonstrated that QKI-5 over-expressing iPS cells displayed well-formed vascular structures and enhanced engraftment ability when compared to the control cells (Fig. 4G). Moreover, the nascent vessels in vivo (matrigel plugs) were stabilised by pericytes/vascular smooth muscle cells in the presence of QKI-5 as smooth muscle alpha- actin (SMA) staining is shown in Supplementary Figure S7. These results demonstrate that QKI-5 induced the angiogenesis in both *in vitro* and *in vivo* Matrigel plug assays.

QKI-5 significantly improved neovascularization and blood flow recovery in the hind limb ischemic model

ECs derived from mouse iPS cells (iPS-ECs CTL) or iPS ECs overexpressing QKI-5 (iPS- ECs OE-QKI-5) were labelled with Vybrant and injected intramuscularly immediately after induction of hind limb ischemia in mice. PBS was also used as an additional control. After 7 and 14 days, delivery of iPS- ECs OE-QKI-5 improved neovascularization and promoted significantly higher blood flow in the ischemic limbs compared to the non-modified, iPS-ECs controls or PBS controls (Fig. 5A-D). Limbs receiving iPS- ECs OE-QKI-5 also displayed significantly higher capillary numbers in the musculature in comparison to the controls (Fig. 5E, F) as shown by staining of adductor muscle sections with CD144 (Fig. 5G) and CD31 (Supplementary Fig. S8A) in immunopositive vessel profiles. Notably, engrafted iPS- ECs OE-QKI-5 displayed a typical vascular architecture while in the PBS control experiments a random pattern and no vascular structures were observed (Fig. 5E, F).

Finally, when adductor muscle sections from iPS-ECs CTL or iPS- ECs OE-QKI-5 injected animals were stained and quantified with Vybrant and EC markers, iPS- ECs OE-QKI-5 were found to display an enhanced engraftment ability compared to controls (Fig. 5H and Supplementary Fig. S8B). These results indicate that iPS- ECs OE-QKI-5 display characteristic endothelial functions when tested *in vivo*. Importantly, no tumours have been detected in any animals during the duration of these experiments highlighting that these pluripotent stem cells were fully differentiated into ECs.

QKI-5 has a key role in EC differentiation derived from human iPS cells

Generation of high-fidelity and stable EC populations, derived from human pluripotent cells, is a major limitation that the EC differentiation and cell reprogramming field are facing at the moment. Thus, we investigated whether QKI-5 has a role in EC differentiation derived from human iPS cells. Human iPS cells were generated and differentiated in low attachment plates using StemPro serum free media supplemented with BMP4, Activin A, FGF and VEGF for 5 days. Then, a KDR positive population was selected and cultured in Fibronectin

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coated plates supplemented with EGM-2 media for 3 to 9 days. The derived human iPS-ECs displayed a typical pattern of EC specific markers such as CD144, CD31, eNOS, and vWF (Fig. 6A). The efficiency of EC differentiation from human iPS cells was 62% as shown by FACs analysis for the positive cells for the EC marker CD144 (Fig 6B). QKI-5 was expressed during EC differentiation from human iPS cells in parallel with VE-cadherin expression (Fig. 6C, D). QKI-5 overexpression by lentiviral gene transfer significantly induced the expression of the EC markers CD144, CD31 and eNOS (Fig. 6E, F, and signaling of VEGFR (KDR), VEGFA and STAT3 during EC differentiation (Fig. 6G). Further data from luciferase assays also confirmed that QKI-5 induced the transcriptional activation of CD144 and VEGFR in human cells (Fig. 6H, I). In contrast, knockdown of QKI-5 by shRNA suppressed the transcriptional activation of both CD144 and VEGFR (Fig. 6H, I). Importantly, QKI-5 only induced the expression of arterial specific EC marker and not the expression of venous and lymphatic markers (Fig. 6J). This data reveal that QKI-5 is implicated in EC differentiation from human iPS cells towards an arterial EC specification.

Human iPS-ECs overexpressing QKI-5 induced angiogenesis in vivo

Since iPS-ECs in the presence of QKI-5 maintained a stable phenotype for many passages without drifting to various non-endothelial phenotypes (as it has already been shown in Fig 2), we further investigated whether QKI-5 had a similar role in mature ECs. For these experiments, QKI-5 was overexpressed in Human Umbilical Vein Endothelial Cells (HUVECs) by lentiviral gene transfer. These lentiviral constructs expressed mCherry, and the efficiency of infection was monitored by *in vivo* imaging using a fluorescence microscope (Fig. 7A). Indeed, QKI-5 was highly overexpressed in these mature ECs (Fig. 7B) and was able to induce further the expression of CD144 and VEGFA in both protein and mRNA levels (Fig. 7C, D). Importantly, QKI-5 overexpression did not induce the expression of non-EC markers such as SMA and SM22 (Fig. 7C). These results highlight that QKI-5 induces EC differentiation from human iPS cells and, in parallel, has an important role in the maintenance of the EC phenotype in both differentiated and mature ECs. Additional experiments with Matrigel plus assays in vivo provided further support to the above findings. Human iPS-ECs overexpressing QKI-5 significantly induced angiogenesis only 7 days after subcutaneous injection of ECs OE- QKI-5- iPS cells labeled with Vybrant cell surface marker in SCID mice, in comparison to the iPS-ECs CTL expressing an empty lentiviral vector (HE staining in Fig. 7E). CD31 immunofluorescence staining confirmed the presence of differentiated cells in *in vivo* vascular tubes and demonstrated that QKI-5 overexpressing iPS cells displayed well-formed vascular structures (Fig. 7G) and enhanced engraftment ability when co-stained with CD144 and Vybrant compared to the control cells (Fig. 7F, H). In addition, a human specific antibody for CD31 was used to stain exclusively the human cells in the Matrigel plugs and co-stained with the Vybrant in order to confirm the present of the human cells and the specificity of the Vybrant labelling (Fig. 7I). These results clearly demonstrate that QKI-5 induced angiogenesis *in vivo* Matrigel plug assays highlighting its pivotal role in EC differentiation derived from human iPS cells.

Discussion

iPS cells represent an attractive cellular approach for regenerative medicine today since they can be used to generate therapeutic cells of almost any type and, importantly, can be harnessed as patient-specific cells towards autologous cell therapy. In this study, using the model of iPS cells differentiation towards ECs, the RNA-binding protein QKI-5 was found to be an important regulator of VE-cadherin stabilisation and VEGFR2 transcriptional activation during the EC differentiation process. Notably, QKI-5 overexpression induced the activation of EC markers, whilst knockdown by shRNA suppressed EC differentiation. In the present study, the role of QKI-5 has been elucidated in EC differentiation from iPS cells. iPS cells have been generated based on a highly efficient approach, fully characterized and forced to differentiate towards ECs. QKI-5 was found to be induced during EC differentiation from iPS cells, and its expression was shown to be maintained at high levels in mature ECs. It has been demonstrated that QKI-5 plays a role in the induction and stabilisation of CD144 and activation of VEGFR-regulatory binding sites AP1 and STAT3 and induction of STAT3 phosphorylation. Importantly, QKI-5 modulated the activation of VEGFR through direct binding of the 3' UTR region of STAT3. The notion that QKI-5 indeed played an important role during EC differentiation was further supported from additional data which clearly demonstrated that knockdown of QKI-5 resulted in inhibition of angiogenesis *in vivo*. Remarkably, ECs derived from iPS overexpressing QKI-5 improved neovascularization and blood flow recovery (almost 100%) in a hind limb ischemia model by showing an enhanced engraftment capacity when compared to non-modified iPS-ECs or PBS control groups. Notably, human iPS cells overexpressing QKI-5 induced angiogenesis in Matrigel plug assays in vivo only seven days after subcutaneously injection in SCID mice, highlighting a clear functional benefit of QKI-5 in neovascularization, blood flow recovery and angiogenesis.

RNA-binding proteins add an additional layer of complexity to a series of events, which include processing and splicing of pre-mRNA, export to the cytoplasm, quality control assessment of mRNA through translation, message decay and stabilization, and translational repression and de-repression [44]. All of these events, from initiation of transcription by key transcription factors, to the stability and effective translation of the message, are regulated by the presence of specific

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nucleotide sequences which are bound by specific RNA-binding proteins [45].

CD144 is a strictly endothelial specific adhesion molecule and is of vital importance in maintaining and controlling the endothelial phenotype through appropriate cell-cell contacts [16]. CD144 is essential during embryonic angiogenesis and, thus, the elucidation of the underlying mechanisms that regulate CD144 are important in understanding the functions permeating vascular permeability, cell proliferation, apoptosis and modulation of VEGFR. A recent study has shown that QKI directly binds to the 3' UTR of CD144 [46]. In our study, we provide further support to the above notion that QKI-5 induced the mRNA stabilisation of CD144.

VEGF-A is one of the earliest markers for the endothelial lineage during development [47, 48] and this growth factor is also critical for vascular repair and neovascularization [49]. The data obtained in this study indicates that QKI-5 regulates EC expression of VEGF-A and autocrine stimulation of VEGFR and this pathway is controlled, at least in part, through STAT3 activation and phosphorylation. Interestingly, knockdown of STAT3 by shRNA ablated QKI-5-mediated activation of the VEGFR and subsequent expression of EC markers. It has been reported that constitutive STAT3 activity upregulates VEGFR [50], while STAT3 protein binds to the VEGFR promoter inducing VEGFR promoter activity [50]. Interestingly, QKI-5 regulates the expression of STAT3 through direct binding in the 3'UTR leading to mRNA stabilisation. Notably, the KH DOMAIN-mutant QKI-5 construct did not induce the expression of the EC markers, STAT3 signalling when it was overexpressed in iPS cells. Importantly, QKI-5 was unable to activate the 3'UTR of STAT3 when the QKI motif was deleted. These results demonstrate that QKI-5 binds to the 3'UTR of STAT3 in the JAK1-STAT3 signalling pathway and regulates EC differentiation towards an arterial lineage.

It has previously been reported that QKI-5 knockout mice display a range of blood vessel defects during development which result in embryonic lethality [39]. Additional studies of the extraembryonic yolk sac have also reported that QKI regulates visceral endoderm differentiated function at the cellular level, including the local synthesis of retinoic acid (RA [29]), which then exerts paracrine control of ECs within the adjacent mesoderm. QKI is also highly expressed during normal cardiac development, particularly in the outflow tract, suggesting potentially unique functions in the developing heart [51] and vascular smooth muscle cell development [52]. Taken together, this is strong evidence that QKI is highly conserved in the early embryo throughout the evolution of non-vertebrate and vertebrate organisms [29]. The role of QKI in the adult organism is less well-appreciated, although there is evidence that it could have a role in vascular smooth muscle cell phenotypic plasticity and can ameliorate pathogenic, fibroproliferative responses to vascular injury [53].

The present study provides strong evidence that QKI-5 is capable of inducing differentiation towards ECs and that, in parallel, regulates angiogenesis in both *in vitro* and *in vitro* Matrigel plus assays, even in the absence of exogenous VEGF. At the moment, the potential of iPS cells to differentiate towards therapeutic cells is only based on directed empiricism, while they are totally dependent on combinations of growth factors, media, and matrices to favour the desired lineage. In regards to vascular regeneration, it is important to understand the key regulatory pathways such as epigenetic alterations, transcriptional activity, and RNA-binding patterns associated with the differentiation processes. In particular, although there has been significant progression in the field [54], there are not any fully defined protocols to generate high-fidelity and stable endothelial cells from human pluripotent stem cells at the moment. This is a major limitation of generating pure populations of rejuvenated EC cells to be used for drug screening and cell-based therapies. Indeed, QKI-5 is a very interesting candidate, which holds the potential to derive stable populations of functional ECs. Specifically, the transcription factor ETS1 is implicated in the induction of the expression of QKI-5 during early stages of EC differentiation. QKI-5 is also acting as a splicing factor involved in the regulation of numerous signalling pathways. Data from our lab also showed that QKI-5 induced the splicing factor SF3B1 during EC differentiation (Supplementary Figure S9). Importantly, SF3B1 is induced in a time dependent manner during EC differentiation from iPS cells (Supplementary Fig. S10), indicating that QKI-5 is likely to be an important splicing regulator of EC differentiation. Notably, a number of QKI splicing isoforms have been reported, although their function(s) are not clearly defined. Additional studies are clearly required to shed light on the precise functions of the different QKI splicing isoforms. Interestingly, a recent paper has reported that QKI plays a remarkably dynamic role in regulating hundreds of circular RNAs (circRNAs) during human epithelial to mesenchymal transition (EMT), whilst QKI itself is regulated during EMT [16]. Therefore, it is tempting to speculate that precise regulation of QKI-5 during EC differentiation will provide fully defined experimental protocols, which could reproducibly guide iPS cells to a vascular lineage [55, 56] and, thereby, enable clinical application [57-59]. In addition, it would be very interesting to investigate the role of QKI and its mRNA splicing isoforms in disease models, such as atherosclerosis and diabetes, where the endothelial function is impaired.

In summary, our data strongly supports the notion that QKI-5 is induced during EC differentiation, resulting in stabilisation of STAT3 expression through direct binding to the 3' UTR region of STAT3, modulation of VEGFR transcriptional activation and VEGF secretion. Markedly, iPS-ECs overexpressing QKI-5 significantly improved angiogenesis and neovascularization and blood flow recovery in experimental hind limb ischemia (Supplementary Fig. S9) highlighting a clear functional

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benefit. This study provides support to the growing consensus that elucidating the molecular mechanisms underlying EC differentiation will ultimately advance stem cell regenerative therapy towards treating cardiovascular disease.

Author Information

The authors declare no competing financial interests.

Acknowledgments

This work was supported by Grants from British Heart Foundation and BBSRC.

Author Contributions

Amy Cochrane: Conception and design, Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing. Sophia Kelaini: Conception and design, Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing; Marianna Tsifaki: Collection and/or assembly of data; James Bojdo: Collection and/or assembly of data; Marta Vilà-González: Collection and/or assembly of data; Daiana Drehmer: Collection and/or assembly of data; Rachel Caines: Collection and/or assembly of data; Corey Magee: Collection and/or assembly of data; Magdalini Eleftheriadou: Collection and/or assembly of data; Yanhua Hu: Collection and/or assembly of data; David Grieve: Provision of study material, Final approval of manuscript; Alan W Stitt: Provision of study material, Final approval of manuscript; Lingfang Zeng: Provision of study material, Final approval of manuscript; Qingbo Xu: Provision of study material, Final approval of manuscript; Andriana Margariti: Conception and design, Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing, Financial support, Final approval of manuscript.

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Figure 1. Induced Pluripotent Stem Cell Differentiation towards endothelial cells. Mouse iPS cells were cultured on 0.02% gelatin with media supplemented with LIF (Leukaemia inhibitory factor) to maintain pluripotency. To induce EC differentiation iPS cells were seeded onto plates coated with collagen IV and differentiation media supplemented with 25ng/ml VEGF (DM+V). (A) Schematic diagram showing differentiation process. (B) Images show morphology of iPS cells (left panel) and of their differentiated EC counterparts (right panel) Scale bar: 50µm. (C) FACs analysis showing expression of CD144 (left panel) and CD31 (right panel) of ECs derived from iPS cells. (D,F) EC marker expression increased in a time dependent manner during differentiation at both mRNA and protein level, whilst the pluripotent marker OCT4 decreased during EC differentiation in a time-dependent manner (data are mean ±SEM (n=3), *p<0.05, *** p<0.001). (E) Immunofluorescence confocal image showing that the 6 days differentiated ECs express the EC specific marker CD144, Scale bar: 50µm. (G) iPS-ECs expressed EC markers CD144, FLK-1, eNOS, and VWF as shown by confocal immunofluorescent images, Scale bar: 50µm. (H) The transcription factor STAT3 was progressively expressed during EC differentiation, (data are mean ±SEM (n=3), *p<0.05).

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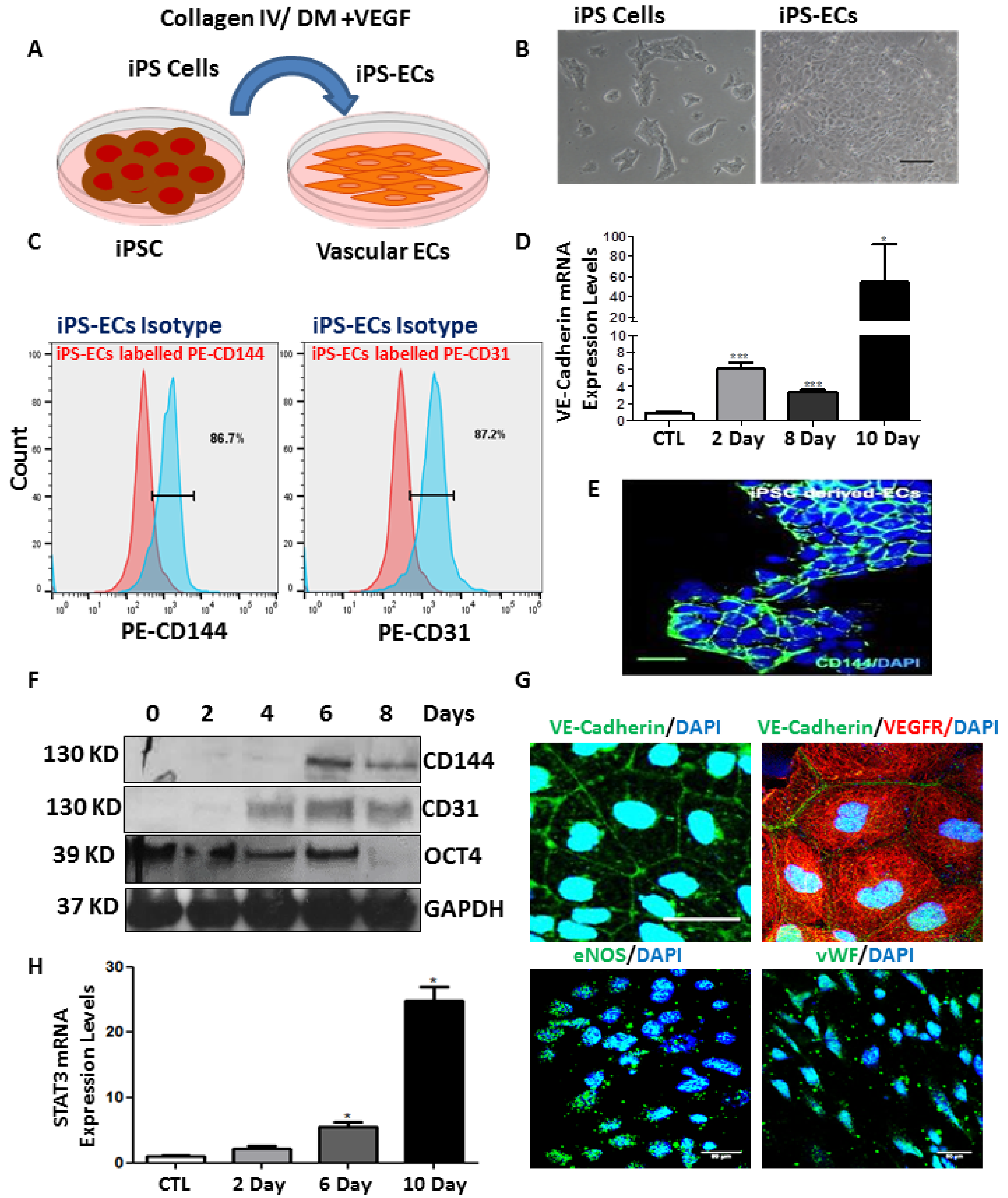

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Figure 2. The RNA binding protein QKI-5 is induced during EC differentiation from iPS cells. (A) RNA binding protein QKI-5 was induced during EC differentiation in parallel with the EC markers CD144 and CD31.The expression levels of QKI-6 and QKI-7 were not changed or decreased, respectively. (B) Western Blots showing that QKI-5 protein was progressively expressed in parallel with the EC marker CD144 and the transcription factor STAT3 in time point experiments from 0 to 10 days during EC differentiation. (C) Immunofluorescence staining showing that QKI-5 is localized in the nucleus and in parallel with CD144 in differentiated ECs (D), (Scale bar, 50 µm) and (E) with CD31 in mature ECs (HUVECs), Scale bar, 25 µm. (F) iPS-ECs were infected or transfected on day 4 with QKI-5 or an empty vector. 48 h later QKI-5 overexpression induced mRNA expression of endothelial markers CD144, CD31, eNOS, VEGFA, and the transcription factor STAT3. (G) QKI-5 induced protein expression of CD144. (H) iPS-ECs were differentiated for 3 days and transfections with the reporter of CD144 (VE-cadherin) construct (pGL3-Luc-CD144) in the presence or absence of QKI-5 were performed. Cells were harvested on day 5 of EC differentiation when luciferase assays demonstrated that QKI-5 induced transcriptional activation of the VE-cadherin promoter. (I) iPS-ECs were differentiated for 4 days and transfected with QKI-5 or an empty vector. 48 hours later QKI-5 overexpression directed differentiation towards arterial ECs (Ephrin B2) specifically and not towards venous (CoupTFII) or lymphatic (Lyve1) ECs or other tissues such as nerve (Nestin), cardiac (Mef2c) or smooth muscle cell (SMA), (data are means ± SEM. (n=3), *p<0.05, ** p<0.01, *** p<0.001).

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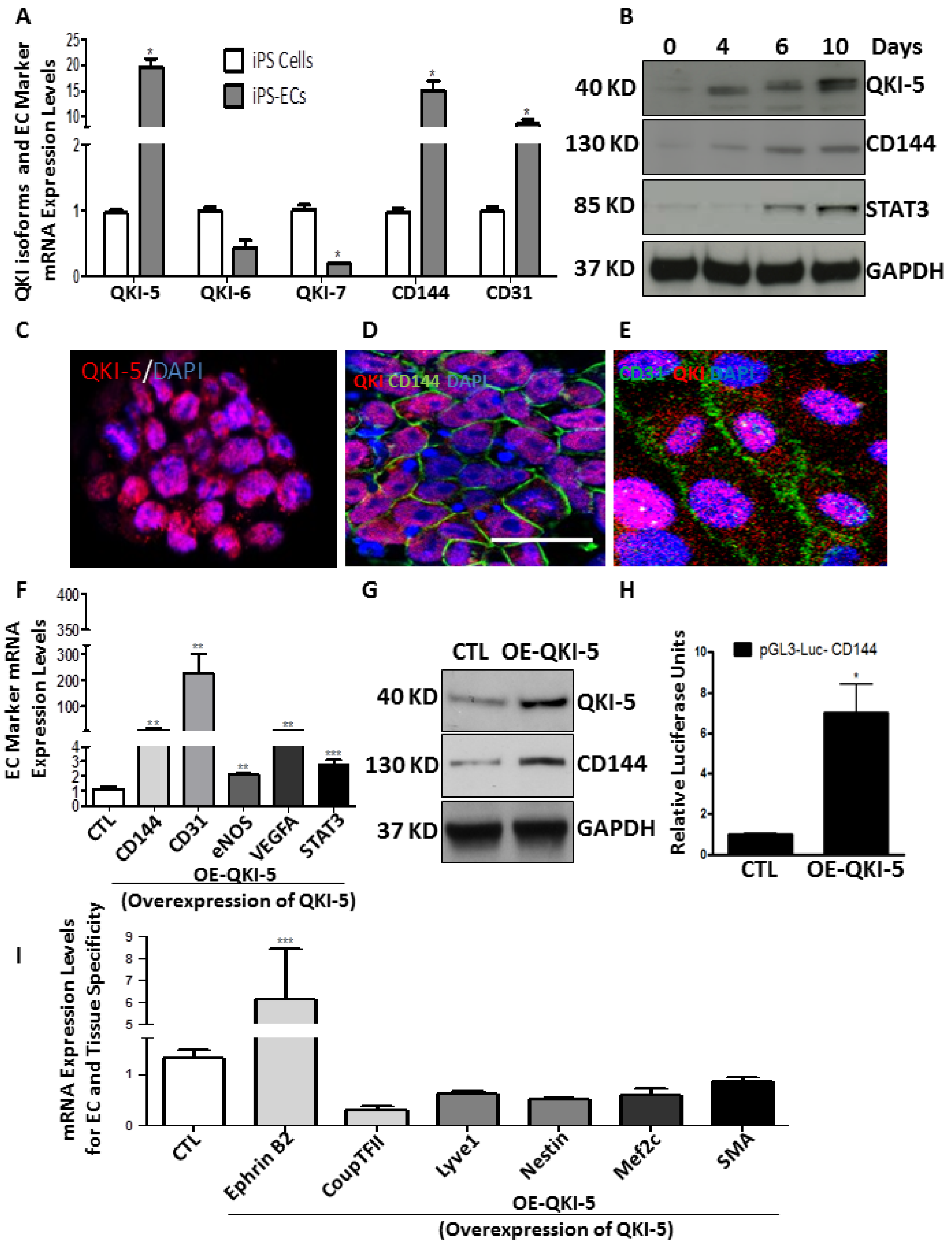

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Figure 3. QKI-5 induced VEGF Receptor 2 (VEGFR2) activation and VEGF secretion through direct binding of the 3'UTR region of STAT3

Overexpression of QKI-5 by lentiviral gene transfer induced the secretion of VEGF on day 6 of the EC differentiation process (A) and the transcriptional activation of the VEGFR2 (B). (C) Real time PCR data showing that QKI-5 leads to activation of VEGFA, JAK-1, STAT3 and AP1. (D) Western blots showing that overexpression of QKI-5 induced the expression of EC markers CD144, and CD31 in parallel to induction of JAK-1, STAT3 and phosphorylation of STAT3 (quantification in E). (F) STAT3 was knocked down by shRNA on day 3 of EC differentiation, and QKI-5 was overexpressed next day. Real data PCR data reveal that STAT3 knocked down ablated activation of EC markers CD31, CD144, eNOS, and VEGFA mediated by QKI-5. The cells were harvested on day 6 of EC differentiation. (G) Luciferase assays have shown that QKI-5 activated the 3'UTR of STAT3. QKI-5 was unable to activate the 3'UTR of STAT3 when the QKI motif was deleted. (G, lower panel) RNA binding assays have confirmed that QKI binds directly to the 3'UTR of STAT3. (H) When differentiated ECs were treated with Actinomycin D in a time point experiment from 0 to 24 hours STAT3 expression was stabilised as a decay curve is shown (data are means ± SEM. (n=3), $^{*}p<0.05$).

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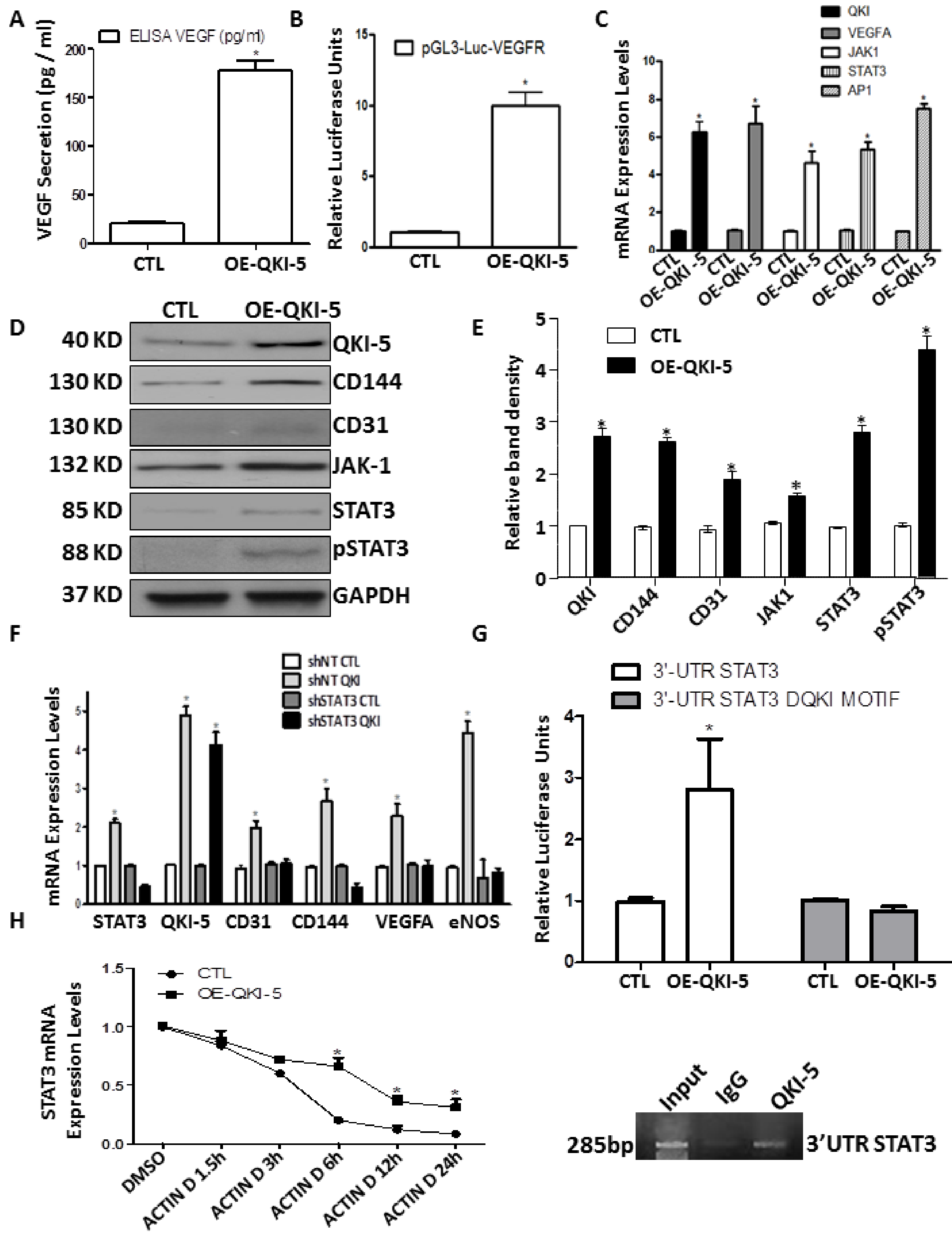

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Figure 4. QKI-5 plays a critical role during EC differentiation and vascular tube formation both *in vitro* and *in vivo*. QKI-5 knockdown by shRNA in iPS-ECs at day 4 of differentiation resulted in suppression of (A) mRNA expression of the EC markers CD144 and CD31 (data are means ± SEM. (n=3), *p<0.05), and (B) protein expression of CD144 and the transcription factor STAT3, when assessed on day 6. (C) shRNA knockdown of QKI-5 resulted in suppression of the transcriptional activity of the VEGFR2 during EC differentiation, as shown by luciferase assays. Non targeting (NT) control for knockdown experiments using shRNA. These experiments were performed on day 3 and analysed on day 5 of EC differentiation (data are means ± SEM. (n=3), *p<0.05). (D) shRNA knockdown of QKI-5 during EC differentiation suppressed the formation of vascular-like tubes *in vivo* in Matrigel plug assays. iPS-ECs were infected with QKI-5 shRNA on day 4 of EC differentiation, and 48 h later, mixed with Matrigel and subcutaneous injected in to mice, prior to analysis of Matrigel plugs 7 days later; Scale bar, 50 µm. iPS cells were seeded on collagen IV-coated plates and cultured in differentiated media in the absence of VEGF for 4 days when QKI-5 or control plasmids were introduced by lentiviral gene transfer. Two days later, cells were mixed with Matrigel for *in vitro* plug assays. (E) QKI-5 formed vascular structures within a few hours *in vitro* in comparison to the control where less defined vascular structures were observed (e, left panel, with right panel showing quantification as total tube length/field (µm) (data are means ± SEM. (n=3), *p<0.05). (F) similarly, cells were subjected to Matrigel plug assays *in vivo* which showed that QKI-5-expressing cells form well defined vascular structures at 7 days in comparison to the control where less well-formed vascular structures were observed (f, left panel, with quantification of capillary density (number / mm [2]) in the right panel), (data are means ± SEM. (n=3), *p<0.05). (G) Frozen sections from the *in vivo* Matrigel plugs were stained for CD144 to demonstrate that over-expression of QKI-5 induced the formation of vascular structures and enhanced engraftment ability compared to the control cells (data are means ± SEM. (n=3), *p<0.05). Scale bar, 50 µm.

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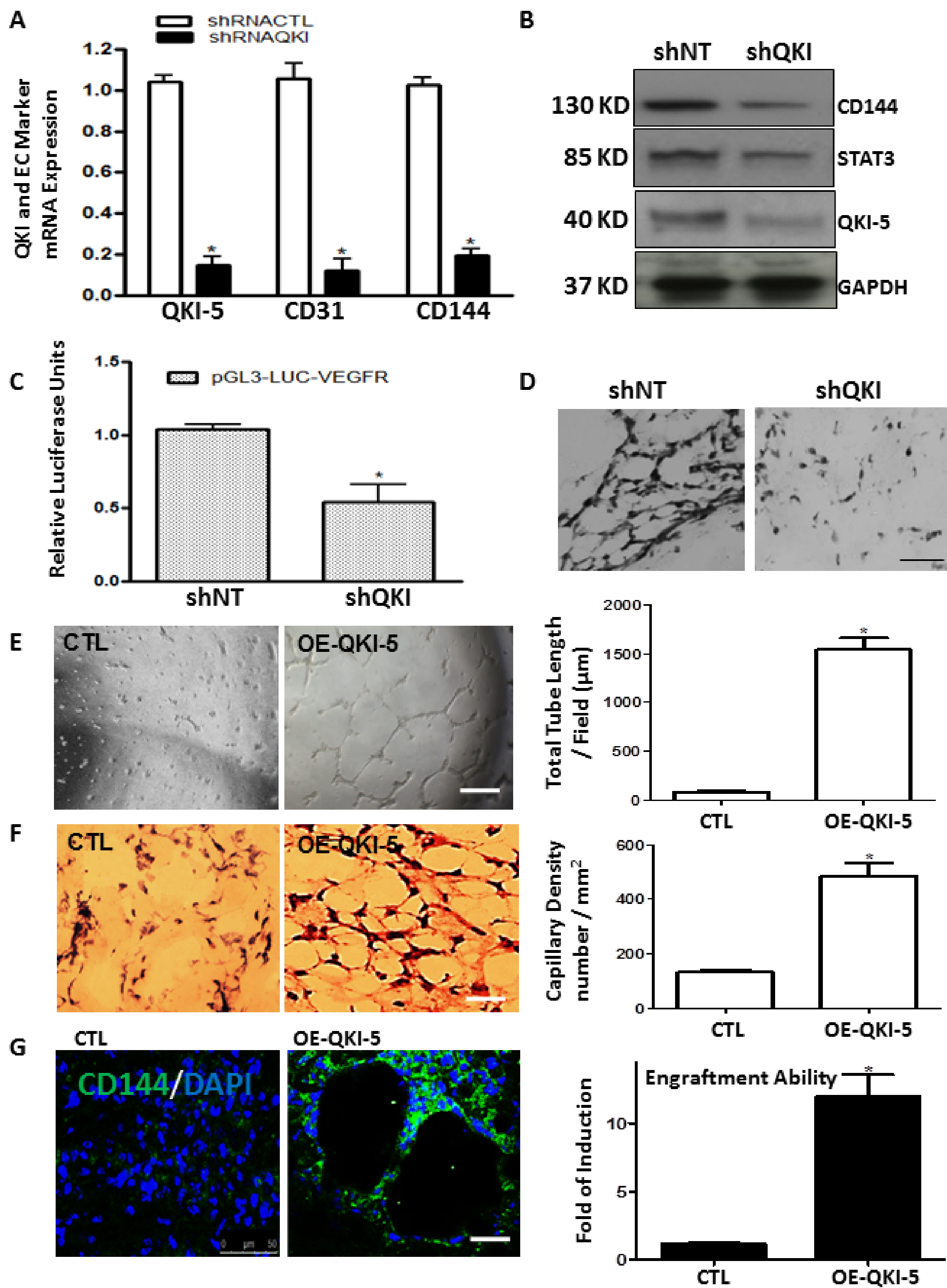

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Figure 5. QKI-5 significantly improved neovascularization and blood flow recovery in experimental hind limb ischemia. ECs derived from iPS cells (iPS-ECs), iPS-ECs overexpressing QKI-5 (iPS-QKI-5-ECs) labelled with Vybrant, or PBS as a control were injected intramuscularly immediately after induction of hind limb ischemia. (A-C) Laser Doppler images of blood flow (BF) in the lower limbs of mice in prone position, with the ischemic leg highlighted by the white rectangle. (D) Time course of BF recovery in the ischemic foot (calculated as a percentage ratio between ischemic foot BF and the contralateral foot BF) for each of the three conditions. Statistical analysis shows significantly higher BF recovery in the iPS-EC QKI-5 treated mice at 7 and 14 days in comparison with PBS control and also comparing iPS-ECs to iPS-ECs- OE-QKI-5; Bonferroni post test (to one way anova) confirms significant difference after 14 days between iPS-ECs and iPS-ECs overexpressing QKI-5; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ [data are means ± SEM (n=3)] (E-F) Sections of adductor muscles were stained with CD144 or CD31 antibody and (G) capillary density expressed as capillary number per mm [2] , and (E,F) further co-stained with CD144 (green) and Vybrant (red) prior to (H) quantification, (and for CD31 please see Supplementary Figure S4A and B) * $p<0.05$, *** $p<0.001$ data are mean ± SEM, quantification from 4 random microscopic fields at x40, scale: 50µm).

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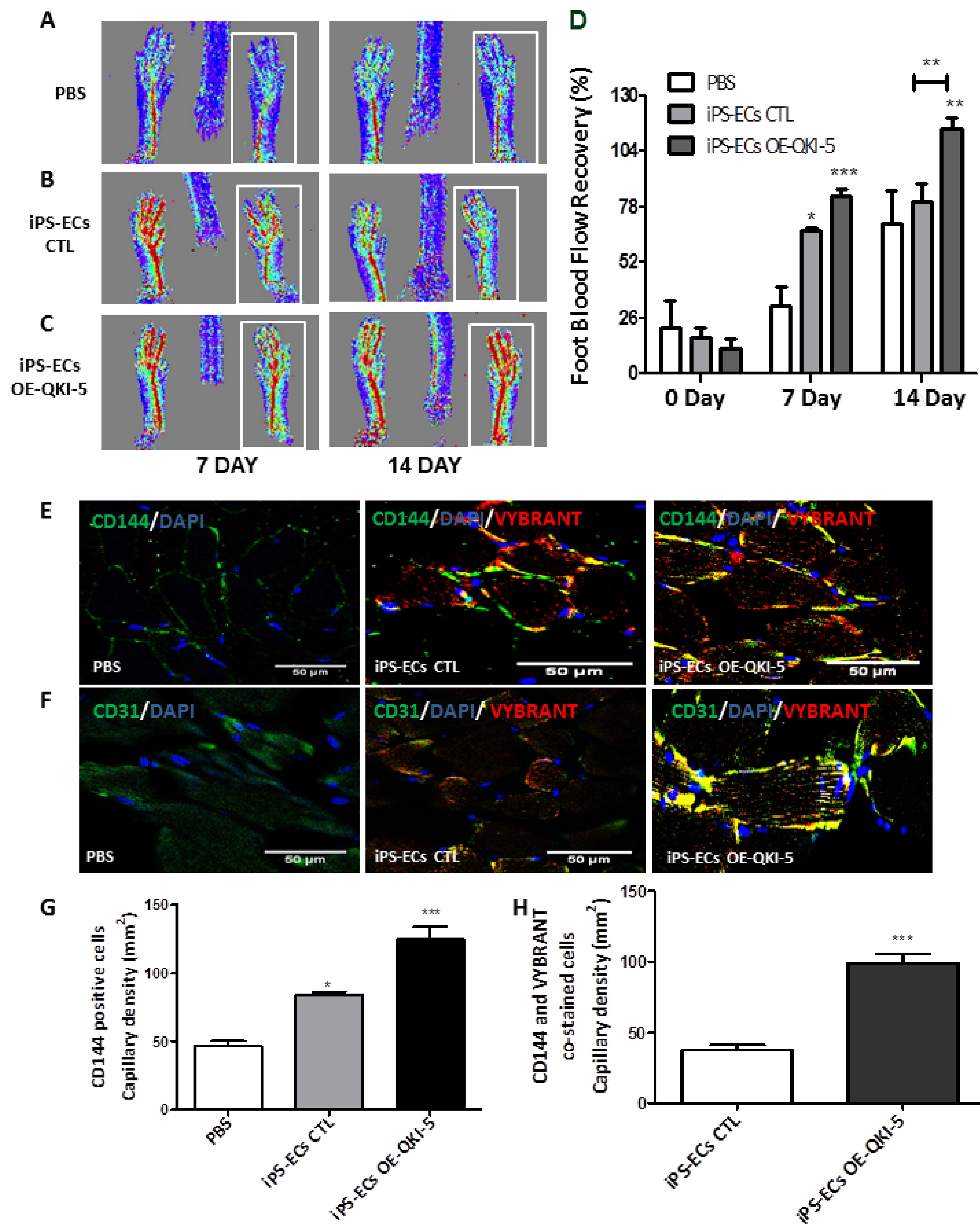
A
PBS
B
iPS-ECs
CTL
C
iPS-ECs
OE-QKI-5
7 DAY
14 DAY
D
Foot Blood Flow Recovery (%)
PBS
iPS-ECs CTL
iPS-ECs OE-QKI-5
0 Day
7 Day
14 Day
E
CD144/DAPI
CD144/DAPI/VYBRANT
PBS
iPS-ECs CTL
iPS-ECs OE-QKI-5
50 µm
F
CD31/DAPI
CD31/DAPI/VYBRANT
G
CD144 positive cells
Capillary density (mm^2)
H
CD144 and VYBRANT
co-stained cells
Capillary density (mm^2)

Figure 6. QKI-5 has a key role in EC differentiation derived from human iPS cells. (A) Human iPS cells were generated and differentiated on low attachment plates using StemPro serum free media supplemented with BMP4, Activin A, FGF and VEGF for 5 days. KDR (VEGFR) positive population was selected using MicroBeads and cultured on Fibronectin coated plates supplemented with EGM-2 media for 3 to 9 days. iPS-ECs derived cells expressed a typical pattern for EC specific markers CD144, CD31, eNOS, and vWF as immunofluorescence images shown. (B) FACs analysis showing cells positive for EC specific marker CD144 (C-D) QKI-5 was expressed during EC differentiation in parallel with VE-cadherin expression in mRNA level. (E-G) QKI-5 was overexpressed by lentiviral gene transfer in human iPS cells on day 3 after KDR selection and the cells were harvesting on day 6 of EC differentiation showing a significant induction of the EC markers CD144, CD31 and eNOS, and signalling of VEGFR (KDR), VEGFA and STAT3 as western blots and real time data shown. (H-I) Luciferase assays shown that QKI-5 induced the transcriptional activation of CD144 and VEGFR. (J) Overexpression of QKI-5 directs differentiation towards arterial EC as cells express specific marker Ephrin B2 and not venous (CoupTFII) or lymphatic (Lyve1) EC markers, (data are means ± SEM. (n=3), $*p<0.05$, $** p<0.01$).

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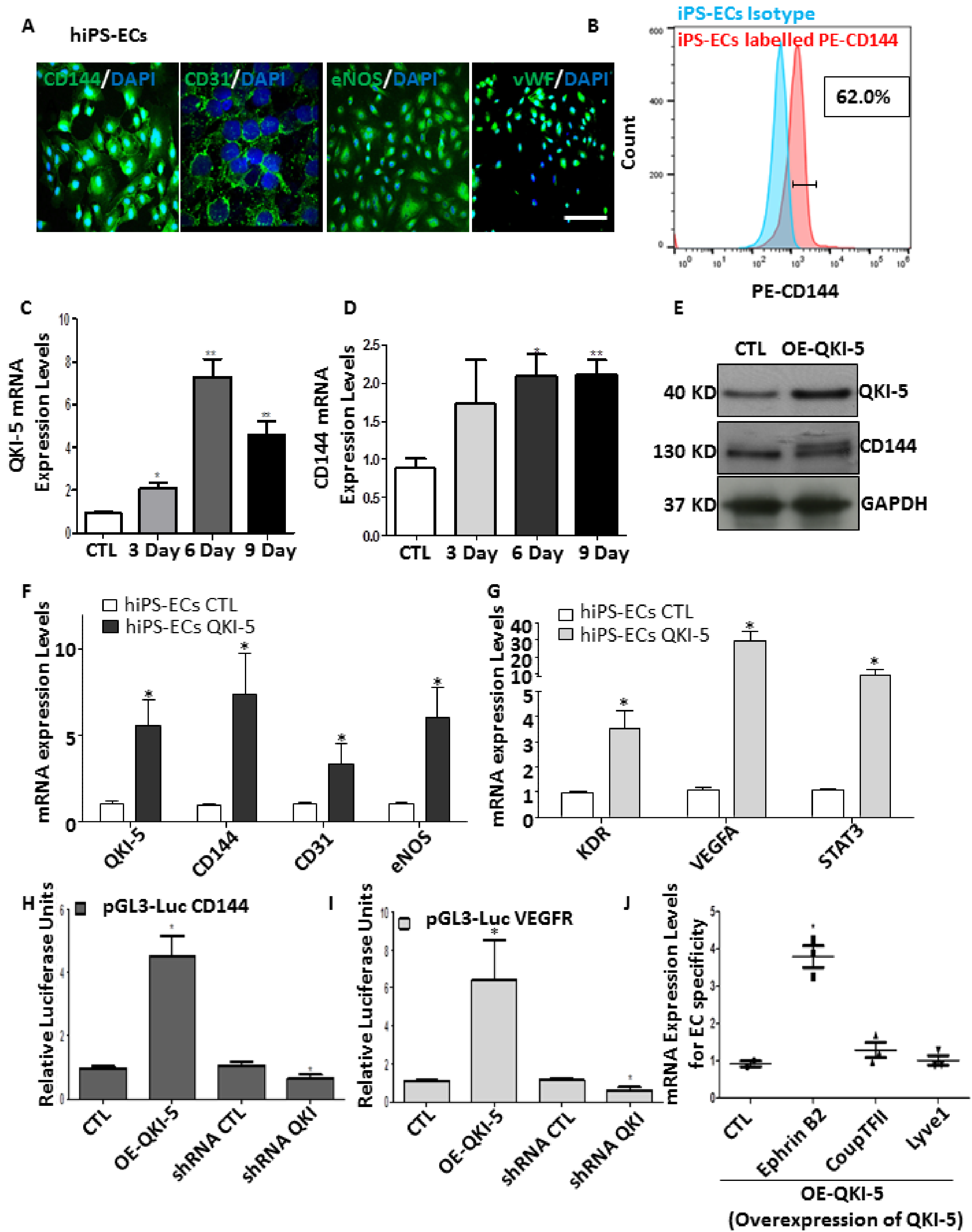

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Figure 7. QKI-5 induced CD144 and VEGFA expression in HUVECs and Human iPS-ECs overexpressing QKI-5 induced angiogenesis in vivo. (A) QKI-5 was overexpressed in HUVECs by lentiviral gene transfer. The efficiency of infection was monitored by the mCherry expression. (B-C) QKI-5 was highly overexpressed in mature ECs and induced further the expression of CD144 and VEGFA (C-D), but it did not induce non-EC markers such as SMA and SM22 in the mRNA level (D), (data are means ± SEM. (n=3), *p<0.05, ** p<0.01). Scale bar, 50 µm. Human iPS-ECs were forced to over-express QKI-5 by lentiviral gene transfer on day 3 of EC differentiation after KDR selection. On day 6 iPS-ECs overex-pressing an empty lentiviral vector or iPS-QKI-5-ECs were labelled with Vybrant Cell Labelling and subcutaneously injected in SCID mice. (E) iPS-QKI-5-ECs significantly formed well-defined vascular structures at 7 days in comparison to the control where less or none formed vascular structures were observed as H&E staining shown. (F) Frozen sec-tion of CD31 immunofluorescence staining confirmed the presence of differentiated cells in *in vivo* vascular tubes. (G-H) Enhanced engraftment ability of iPS-QKI-5-ECs was observed when co-stained with CD144 and Vybrant compared to the control iPS-ECs. Scale bar, 50 µm. (I) Further staining using human specific CD31 antibody confirms the pres-ence of human cells and specificity of the Vybrant in samples seen in (G-H).

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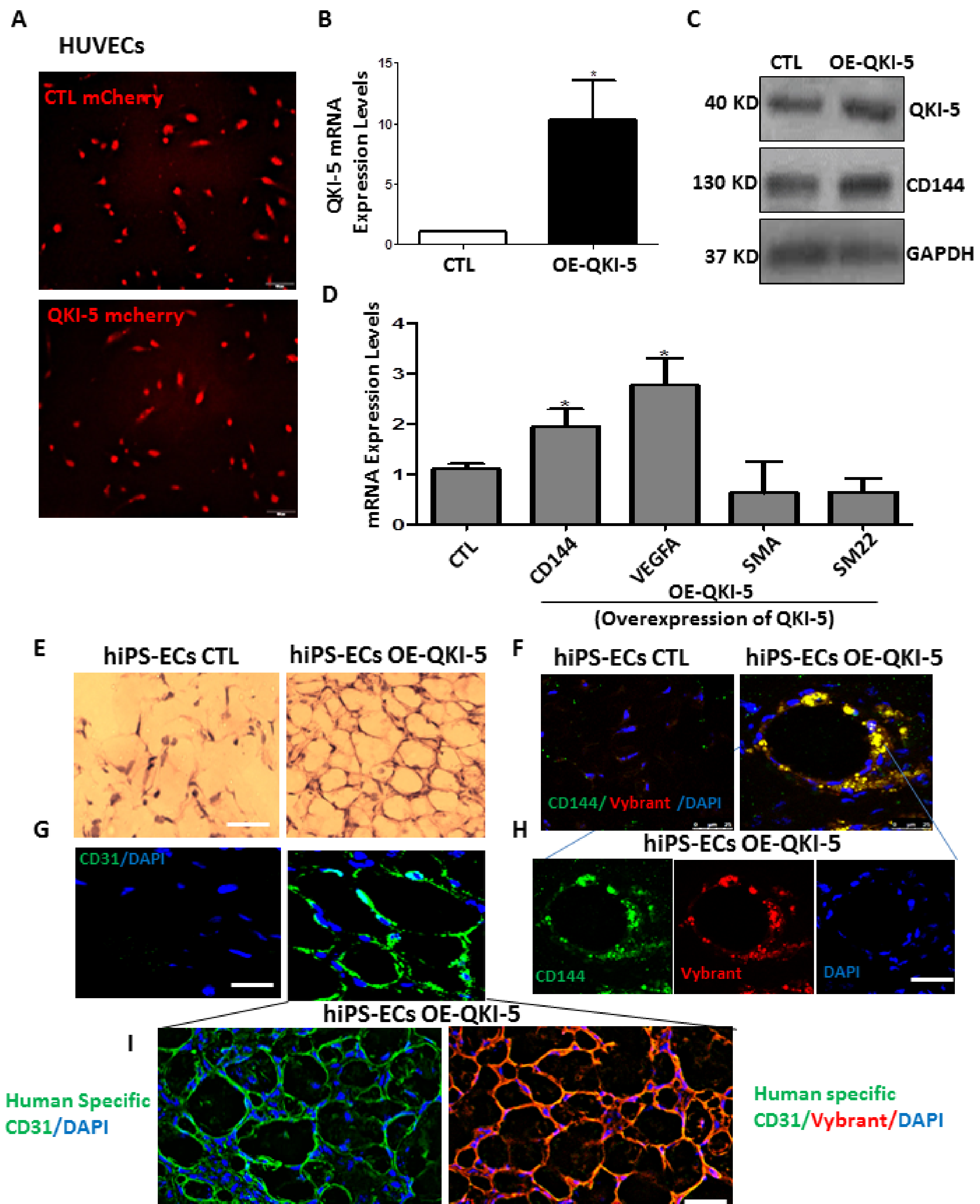

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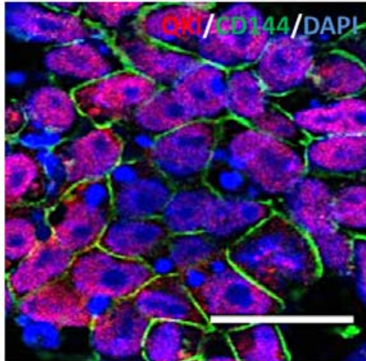
Graphical Abstract

The RNA binding protein QKI-5 is induced during EC differentiation from iPS cells. RNA binding protein QKI-5 was induced during EC differentiation in parallel with the EC marker CD144. Immunofluorescence staining showing that QKI-5 is localized in the nucleus and stained in parallel with CD144 in differentiated ECs, (Scale bar, 50 μm).



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