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MESP1 knock-down in human iPSC attenuates early vascular progenitor cell differentiation after completed primitive streak specification

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\textbf{ABSTRACT}

\textit{MESP1} is a key transcription factor in development of early cardiovascular tissue and it is required for induction of the cardiomyocyte (CM) gene expression program, but its role in vascular development is unclear. Here, we used inducible CRISPRi knock-down of \textit{MESP1} to analyze the molecular processes of the early differentiation stages of human induced pluripotent stem cells into mesoderm and subsequently vascular progenitor cells.

We found that expression of the mesodermal marker, BRACHYURY (encoded by \textit{T}) was unaffected in \textit{MESP1} knock-down cells as compared to wild type cells suggesting timely movement through the primitive streak whereas another mesodermal marker MIXL1 was slightly, but significantly decreased. In contrast, the expression of the vascular cell surface marker KDR was decreased and CD31 and CD34 expression were substantially reduced in \textit{MESP1} knock-down cells supporting inhibition or delay of vascular specification.

In addition, mRNA microarray data revealed several other altered gene expressions including the EMT regulating transcription factors SNAI1 and \textit{TWIST1}, which were both significantly decreased indicating that \textit{MESP1} knock-down cells are less likely to undergo EMT during vascular progenitor differentiation.

Our study demonstrates that while leaving primitive streak markers unaffected, \textit{MESP1} expression is required for timely vascular progenitor specification. Thus, \textit{MESP1} expression is essential for the molecular features of early CM, EC and VSMC lineage specification.

\textbf{1. Introduction}

\textit{MESP1} is fundamentally important for early cardiovascular development, and \textit{MESP1+} cells give rise to all heart cells. Multiple studies address the importance of \textit{MESP1} in CMs, however its precise role has been difficult to study due to the lack of model systems and poor survival of knock out model organisms. Especially, it will be important to develop cellular models with temporal induction systems to turn \textit{MESP1} ‘on’ and ‘off’. To understand the molecular basis of early vascular progenitor specification, we used \textit{MESP1} CRISPRi guide-RNA to generate an inducible \textit{MESP1} knock-down system in human induced Pluripotent Stem cells (iPSC). \textit{MESP1} is transiently expressed in early mesoderm (mouse E6.5 to E7.5), and is considered the earliest marker of cardiovascular development (Saga et al., 1999; Wu, 2008). Lineage tracing studies of \textit{MESP1} in mouse embryos demonstrate that most cardiac cells especially CM, endothelial cells and some vascular cells arise from cells that have expressed \textit{MESP1} (Saga et al., 1999). \textit{MESP1-/−} embryos do generate cardiac mesoderm, however their heart tubes fail to fuse, leading to cardiac bифida and embryonic lethality (Saga et al., 1999). A recent study of \textit{MESP1-/−} cardiac progenitor cells showed that \textit{MESP1} is required for the exit from the pluripotent state and the induction of the cardiovascular gene expression program (Lescroart et al., 2018). In \textit{MESP1/2} double-deficient embryos there is an accumulation of cells in the primitive streak and complete failure

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of mesodermal specification (Tam and Loebel, 2007; Kitajima et al., 2000).

MESP1 transcription is controlled by canonical WNT and transcription factors such as BRACHYURY and EOMES (Costello et al., 2011; David et al., 2011). Recent studies have shown that MESP1 accelerates expression of CD31, CD34 and KDR (also known as Vascular Endothelial Growth Factor Receptor 2; VEGFR2 or FLK1) ((Wu, 2008); (Bondue et al., 2008); (den Hartogh et al., 2016). Moreover, MESP1 is suggested to regulate its own expression and form a self-regulatory network together with BRACHYURY and EOMES that successfully drives the formation of mesendoderm and subsequent cardiovascular differentiation (Solbam et al., 2015). During this process, MESP1 regulates transcription factors important for EMT and cardiovascular commitment including SNAI1, TWIST1, and SLUG (Lindsley et al., 2008). These transcription factors repress promoters of epithelial markers and activate mesenchymal markers resulting in morphological changes as well as cell migration (Lindsay et al., 2008).

The early embryonic lethality of MESP1−/− embryos and the general lack of MESP1 knock out cell lines, including pluripotent stem cell lines, has prevented molecular characterization of the specific role and molecular and cellular mechanisms by which MESP1 acts during cardiac and vascular development. Moreover, mainly CM specification has been studied, whereas the question of MESP1’s influence on vascular progenitor differentiation remains unknown. Using CRISPRi knock-down of MESP1, we describe the very early differentiation stages of human iPSCs into primitive streak, mesoderm and subsequently vascular progenitor cells. Interestingly, our data suggest that lack of MESP1 inhibits mesoderm markers and vascular progenitor specification while leaving primitive streak markers unaffected, thus supporting previous findings and giving new insights to the vascular progenitor differentiation process.

2. Results and discussion

2.1. Doxycycline induced MESP1 knock-down cell line

We previously generated iPSC lines with doxycycline (Dox) – inducible knock-down of MESP1, which contains a mCherry reporter as indicator of an activated Tet-on system and thus repression of the MESP1 transcription start site (Mandegar et al., 2016). This allows timing of the appearance of MESP1 knock-down cells during growth and differentiation (Fig. 1A). By analyzing MESP1 inhibition in three different MESP1 CRISPRi guide-RNA (gRNA) cell lines (g1–3) we found that MESP1 expression was significantly inhibited in the MESP1 CRISPRi g1 (further referred to as MESP1 knock-down) (Fig. 1B,C). The following analyses of MESP1 knock-down cell growth (cell number and size) and thrive as verified by pluripotency-, apoptosis-, cell cycle- and proliferation markers during treatment with increasing Dox concentrations, showed that 1 μM Dox was sufficient to inhibit MESP1 expression, without affecting the majority of measured cell parameters (Fig. 1D-G). Of the many parameters analyzed only cell size and SOX2 expression was slightly but significantly affected by treatment at 1, 2 and 5 and 0.5 and 1 μM Dox respectively (Fig. 1E and F).

2.2. Characterization of mesodermal and vascular progenitors derived from knock-down of MESP1 in human iPSCs

We next performed a time course experiment to compare the expression of primitive streak, mesoderm and vascular progenitor markers (MESP1, MIXL1, BRACHYURY, KDR, CD31 and CD34) in differentiation conditions with and without Dox induced knock-down of MESP1 (Fig. 2A,B). Cells were analyzed at day 0, 2, 4 and 6 during differentiation of vascular progenitors. Indeed, MESP1 expression was verified as being 98.6% repressed in Dox treated cells at day 2 of vascular differentiation (Fig. 2B). Also, the pluripotent markers, OCT4, SOX2 and NANOG, which rapidly decrease when differentiation is initiated and thus should be absent when MESP1 is peaking, were not affected by the Dox treatment (Fig. 2B). Interestingly, MIXL1 decreased by 8% in the MESP1 knock-down cells whereas T (encoding BRACHYURY) was not affected. Both MIXL1 and BRACHYURY are among the major molecular determinants in the patterning and induction of mesoderm. BRACHYURY has been shown to be important for proper specification of mesoderm and correct movement through the primitive streak, whilst MIXL1 is important in endoderm differentiation and acts as a negative regulator of BRACHYURY (Izumi et al., 2007; Pereira et al., 2011). These data are in line with previous findings of MESP1 being an accelerator of early mesoderm and a regulator of the transcription factors involved in cardiovascular development (Lescroart et al., 2018; Lindsay et al., 2008; Bondue et al., 2011). Thus, our data suggest that MESP1 knock-down does not change the movement through the primitive streak nor the commitment to mesodermal specification during iPSC differentiation into vascular progenitors. These observations correspond to previous in vivo studies showing that MESP1−/− embryos can generate cardiac mesoderm, but fail to fuse their heart tubes (Saga et al., 1999; Lescroart et al., 2018).

We next investigated vascular progenitor specification at day 4 and 6. Absence of MESP1 significantly decreased the expression of the vascular cell surface marker KDR by 80% at day 4, and by 32% at day 6 (Fig. 2B), suggesting a MESP1 knock-down mediated decrease or delay of KDR expression at day 4 of vascular progenitor differentiation in these cells. KDR is a common marker of mesodermal precursors and its presence or absence can direct the mesodermal cells in several directions determined by co-expression of other lineage-specific transcription factors. Cells that retain KDR activity have endothelial potential whereas cells that lose the transient KDR expression but gain expression of other transcription factors can become VSMC (Ema and Rossant, 2005). In line with this, the mRNA expression of the cell surface markers, CD31 and CD34 were reduced significantly by 47% and 46% respectively at day 6 (Fig. 2B). Expression of CD31 and CD34 was verified by immunocytochemistry of CD31+ and CD34+ cells (Fig. 2C). These results show that MESP1 promotes CD31 and CD34 expression, and that MESP1 knock-down affects early vascular progenitor differentiation negatively. Our data are supported by a recent study investigating endothelial differentiation showing that sorted MESP1+ and MESP1− cells express 18.5% and 1.5% CD31 respectively (Zhang et al., 2017). Taken together, we suggest that knock-down of MESP1 disrupts the vascular progenitor specification after completed primitive streak and mesodermal commitment.

It has been described that MESP1 induces Epithelial-Mesenchymal Transition (EMT) genes in Embryonic Stem Cells (ESC) during mesodermal to cardiovascular differentiation including induction of differentiation into endothelial cells, cardiomyocytes and smooth muscle cells (Lindsay et al., 2008; Bondue et al., 2011). However, the effects of MESP1 knock-down on EMT genes during vascular differentiation have not been investigated. We found that the transcription factors that regulate EMT, such as SNAI1 and TWIST1 both were significantly decreased in the absence of MESP1 (Fig. 3A) indicating that MESP1 knock-down cells are less likely to undergo EMT during vascular differentiation than cells expressing MESP1. Moreover, it has been suggested that MESP1 promotes cardiac development through EMT, but independently of WNT-signaling (Lindsay et al., 2008). To this end, using mRNA microarray data and verified by qRT-PCR, we found that the Dickkopf WNT signaling pathway inhibitor 4 (DKK4) was significantly higher expressed in MESP1 knock-down cells as compared to control cells (Fig. 3A,B). DKK4 is a negative regulator of WNT/β-catenin signaling pathway and known to
be transiently expressed during embryonic development (Kawano and Kypta, 2003). It is interesting to speculate on the role of DKK4 during vascular progenitor specification as it could possibly act as an inhibitor of early vascular differentiation. This is supported by data showing that the differentiation of iPSC into vascular smooth muscle cells is enforced by CHIR99021, a small molecule that potently inhibit the GSK3β-pathway and thus functions as a WNT activator (Lian et al., 2014; Ayoubi et al., 2017). Further studies may confirm this. Overall, the mRNA expression data showed that very few mRNAs were differentially expressed in the MESP1 knock-down and control cells at day 2 (Fig. 3B, Fig. S1 and Table S1). This suggests that MESP1 regulation is limited to few pathways or that the effects of MESP1 knock-down only

(caption on next page)
emerge after the early time point of primitive streak and mesoderm development around day 2. Indeed, proliferation was affected at day 6 in MESP1 knock-down cells where CDKNIa was increased, whereas PCNA was slightly but significantly decreased in MESP1 knock-down cells (Fig. 3A). This may indicate the existence of two VSMC progenitor populations that grow and differentiate asynchronously depending of MESP1 knock-down.

Taken together, the MESP1 knock-down iPSC cell line we have developed represents a robust in vitro model for analyzing MESP1’s role in induced pluripotent stem cell differentiation into vascular progenitor cells, but may also be used for other purposes. The CRISPRi system allows for repression and re-induction of MESP1 expression in a very strict timeframe mimicking the transient expression of MESP1 physiologically and thus represents a unique and improved model to study MESP1 influence on vascular development which have not yet been possible in the existing tracing and over-expression studies in ESC.

In conclusion, we observed that lack of MESP1 during hiPSC differentiation into vascular progenitors does not affect the primitive streak mesoderm but rather decreases the expression of genes specific for vascular progenitor cell determination downstream from primitive streak specification and mesodermal commitment.

3. Materials and methods

3.1. hiPSC lines culture, differentiation into vascular progenitor cells and doxycycline induced MESP1 knock-down

Previously described, MESP1 CRISPR interference human induced Pluripotent Stem Cells (hiPSC) (Mandegar et al., 2016) were cultured in E8 medium (Life Technologies) under feederfree conditions on growth factor-reduced Matrigel and fed daily with E8 medium (Life Technologies). To promote cell survival during enzymatic passaging, cells were passaged with the p160-Rho-associated coiled-coil kinase (ROCK) inhibitor Y-27632. Vascular progenitor cells were induced in a monolayer on ECM matrigel (Thermo Scientific) coated plates in E8 with 10 μM ROCKi 3 days prior to differentiation. Differentiation was initiated by 6μM CHIR99021 in DMEM (ultraglutamine) and 100 μg/ml ascorbic acid. After 48 h of differentiation, CHIR99021 was retracted from the differentiation media (DMEM (ultraglutamine) and 100 μg/ml ascorbic acid) and further differentiated until day 6. (Fig. 2A). Gene knock-down of MESP1 during vascular differentiation was induced by addition of 1 μM doxycycline.

For testing of Dox effects 0, 0.1, 0.5, 1, 2 and 5 μM Dox (Doxycycline Hyclate, Sigma) was added to the media. Optimal knock-down conditions of MESP1 were found using multiple CRISPRi cell lines and dose-response verification of Doxycycline Hyclate (Sigma-Aldrich) induced MESP1 knock-down.

3.2. Gene expression analysis and mRNA microarray

Total RNA was extracted using TriReagent protocol (Molecular Research Center, Inc.), and RNA purity and quantity was examined by nanodrop (NanoDrop® Technologies). Relative quantitative mRNA PCR was performed on reverse transcribed cDNA (High Capacity cDNA RT kit; Applied Biosystems). Amplification and detection were performed using 7900HT Fast Real-Time PCR System (Applied Biosystems). As recommended (Vandesompele et al., 2002; Hellemans et al., 2007), we used the qBase+ software to normalize all qRT-PCR data against stably expressed control genes. Primers are listed in Table S2.

One-way ANOVA were performed and with Dunnett’s post-tests (GraphPad Prism software version 7.0) to test significant levels. Differences were considered to be significant at P < 0.05. At least three biological replicates were used for testing of Dox effects. The separation of Dox-treated and untreated human iPSC, based on the fluorescent of the dCas9-KRAB activated mCherry indicator, was visualized using a differential expression genes, was visualized using a principal component analysis (PCA) plot (Fig. S1) and a heatmap with associated sample- and gene-wise hierarchical clustering. The heatmap was created by the heatmap.2 function from the ggplot2 package.

Imagery were acquired after 3 days in culture by phase-contrast microscopy or by fluorescence of the dCas9-KRAB activated mCherry fluorophore as indicator of activated Tet-on system. In all experiments, exposure (camera settings) and picture processing (brief adjustment of contrast/brightness and color balance by Photoshop CS5) were applied equally to all images. The cell number was measured using Beckman Coulter Multisizer II and counting was performed in independent experiments, each comprising triplicate measurements.
3.4. Immunofluorescence

Cells were fixed for 10 min in 4% paraformaldehyde (PFA) in PBS, permeabilized for 10 min in 0.5% TX100 (Sigma-Aldrich) and blocked for 10 min in 2% BSA (Calbiochem) in TBS. Cells were incubated with primary antibodies for 2 h in 1% BSA/TBS (CD31 conjugated to Alexa 647 and CD34 detected using Alexa Flour 488-conjugated secondary antibodies). CD31 positive cells were identified using an Alexa 647 conjugated mouse anti-human CD31 antibody at 1 µg/ml (BD Biosciences cat no: 561654) while CD34 positive cells were recognized using mouse anti-human CD34 at 2 µg/ml (BD Biosciences cat no: 562383) followed by Alexa 488 Donkey anti-mouse IgG (Invitrogen cat:no: A21202, 1:200). Isotype controls (BD Biosciences cat no: 557714 (isotype for CD31) and 562292 (isotype for CD34)) were used at the same concentration as their respective primary antibody.

Slides were mounted with mounting medium (Vectorshield, Vector Lab, UK) containing DAPI for staining of nuclei, and images were acquired using a Leica DMI4000B Cool Fluo Package instrument equipped with a Leica DFC340 FX Digital Camera. In all experiments, exposure (camera settings) and picture processing (brief adjustment of contrast/brightness and color balance by Photoshop CS5) were applied equally to all images. Scale bar indicates 150 µm.

Fig. 2. : MESP1 knock-down affect development of vascular progenitors. A) Schematic presentation of the differentiation strategy to direct human induced pluripotent stem cells towards primitive streak/mesoderm and vascular progenitor cells. Chemically defined conditions using DMEM with ultraglutamine, 100 µg/ml ascorbic acid and 6 µM CHIR99021 added for 48 h is used to generate the primitive streak mesodermal cells. Hereafter the CHIR99021 was retracted and cells differentiated into vascular progenitors. Figure is made in PowerPoint and Photoshop. B) qRT-PCR expression analysis of the pluripotent, mesodermal and vascular progenitor markers over the time course of differentiation, verifying that control cells (MESP1 knock-down not treated with Dox) express differentiation markers as expected ( ) with a rapid decline in pluripotent markers after induction of differentiation, a transient increase in primitive streak mesodermal markers at day 2 and an increase in vascular progenitor markers at day 6. MESP1 knock-down cells treated with 1 µM Dox ( ) show absence of MESP1 at day 2, reduced expression of MIXL1, KDR, CD31 and CD34 at day 2, 4 and 6 respectively. The analyses comprised three independent experiments each comprising triplicate measurement. Graphs are presented as mean ± s.d. Two-way ANOVA was performed (GraphPad Prism software version 7.0) to test significant levels. Differences were considered to be significant at $P < 0.05$. C) Representative phase images together with corresponding immunofluorescence analysis of DAPI (blue), CD31 (purple) and CD34 (green) at day 6 in wells treated with and without Dox. Images were acquired by phase-contrast microscopy and by fluorescence. In all experiments, exposure (camera settings) and picture processing (brief adjustment of contrast/brightness and color balance by Photoshop CS5) were applied equally to all images. Scale bar indicates 150 µm.

3.5. Statistics

All analyses comprised at least three independent experiments and two-way ANOVA or one-way ANOVA was performed as indicated (GraphPad Prism 7) to test significant levels. Dunnett’s post-test was performed following one-way ANOVA. A value of $p \leq 0.05$ was considered statistically significant. All error bars indicate mean ± s.d.

For more information on reagents and resources please see Key Resource Table (KRT-table).
Fig. 3. Regulation of EMT and WNT-signaling pathway in MESP1 knock-down cells. A) qRT-PCR expression analysis of EMT markers (TWIST1 and SNAI1), DKK4, cell cycle marker (CDKN1a), proliferation marker (PCNA) over the time course of differentiation of MESP1 knock-down with (□) or without (○) Dox. MESP1 knock-down cells treated with 1 μM Dox (□) show decreased EMT, increased DKK4 expression, slightly increased cell cycle and slightly decreased cell proliferation. The analyses comprised three independent experiments each comprising triplicate measurement. Graphs are presented as mean ± s.d. Two-way ANOVA was performed (GraphPad Prism software version 7.0) to test significant levels. Differences were considered to be significant at P < 0.05. B) Heatmap of the 229 differentially regulated genes at day 2 of vascular progenitor differentiation treated with or without Dox. This heatmap shows associated sample- and gene-wise hierarchical clustering.
Key resources table

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Competing interests

None.

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Summary statement

Knock down of MESPI in human induced pluripotent stem cells inhibits the early stages of vascular progenitor differentiation after completed specification of the primitive streak.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2018.10.020.

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Hellemanes, J., et al., 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 8 (R), R19.


