Title

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

Journal

Developmental Biology, 445(1)

ISSN

0012-1606

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Publication Date

2019

DOI

10.1016/j.ydbio.2018.10.020

Peer reviewed

TITLE PAGE

MESP1 knock out in human iPSC inhibits early vascular smooth muscle cell differentiation after completed mesoderm specification

RUNNING TITLE

***MESP1*-KO inhibits VSMC differentiation**

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KEY WORDS

MESP1, iPSC, Vascular Smooth Muscle Cells (VSMC), CRISPR, differentiation

SUMMARY STATEMENT

Knock out of *MESP1* in human induced pluripotent stem cells inhibit the early stages of vascular smooth muscle cell differentiation after completed differentiation of mesoderm.

ABSTRACT (180 words)

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 smooth muscle (VSMC) specification is unclear. Here, we used inducible CRISPRi knock down of MESP1 to analyze the molecular processes of the early differentiation stages of human induced pluripotent stem cells into mesoderm and subsequently VSMC progenitor cells.

We found that expression of the mesodermal marker, BRACHYURY (encoded by *T*) was unaffected in *MESP1*-KO cells as compared to wild type cells, and although another mesodermal marker MIXL1 was slightly decreased these data suggest timely mesodermal development. In contrast, the expression of the vascular cell surface marker KDR was delayed and CD31 and CD34 expression were substantially reduced in *MESP1*-KO cells supporting delay of VSMC specification.
In addition, mRNA array data revealed several other altered genes including the transcription factors *SNAI1* and *TWIST1*, that regulate EMT were both significantly decreased indicating that *MESP1*-KO cells are less likely to undergo EMT during VSMC differentiation.

Our study demonstrates that while leaving primitive streak and mesoderm markers unaffected, MESP1 expression is required for timely VSMC progenitor specification. Thus, MESP1 expression is essential for the molecular features behind both CM and VSMC early lineage specification.

INTRODUCTION

MESP1 is fundamentally important for early cardiovascular development, and *MESP1+* cells give rise to all heart cells. Multiple studies address the importance of *MESP1* in CMs, however its precise role has been difficult to study due 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 *MESP1* 'on' and 'off'. To understand the molecular basis of early VSMC specification, we used *MESP1* CRISPRi guide-RNA to generate an inducible MESP1 knock out system in human induced Pluripotent Stem cells (iPSC). *MESP1* is transiently expressed in early mesoderm (E6.5 to E7.5), and is considered the earliest marker of cardiovascular development (Saga et al., 1999, Wu, 2008). Lineage tracing studies of MESP1 in mouse embryos demonstrate that most cardiac cells especially CM, endothelial cells and some vascular cells arise from cells that have expressed *Mesp1* (Saga et al., 1999). *Mesp1-/-* embryos do generate cardiac mesoderm, however their heart tubes fail to fuse, leading to cardia bifida and embryonic lethality (Saga et al., 1999). A recent study of *Mesp1-/-* cardiac progenitor cells showed that MESP1 is required for the exit from the pluripotent state and the induction of the cardiovascular gene expression program (Lescroart et al., 2018). In *Mesp1/2* double-deficient embryos there is an accumulation of cells in the primitive streak and complete failure of mesoderm 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; VEGF or FLK1) *(Bondue et al., 2008, den Hartogh et al., 2016, Wu, 2008)*. 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 (Soibam 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 (Lindsley 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 VSMC differentiation remains unknown. Using CRISPRi knock out of *MESP1*, we describe the early differentiation stages of human iPSCs into mesoderm and subsequently vascular progenitor cells. Interestingly, our data suggest that MESP1 inhibits VSMC progenitor specification while leaving primitive streak and mesoderm markers unaffected, thus

giving new insights to the VSMC differentiation process.

RESULTS AND DISCUSSION

Doxycycline induced MESP knock out cell line

We previously generated iPSC lines with doxycycline (Dox) –inducible knock out 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 out 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 perfectly inhibited in the *MESP1* CRISPRi g1 (from hereon called *MESP1*-KO) (Fig 1B,C). The following analyses of *MESP1*-KO 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 1µM Dox was sufficient to inhibit MESP1 expression, without affecting other measured cell parameters (Fig 1D, E, F, G).

Characterization of mesoderm and vascular smooth muscle progenitors derived from *Mesp1*-KO human iPSCs

We next performed a time course experiment to compare the expression of primitive streak, mesoderm and VSMC markers (MESP1, MIXL1, BRACHYURY, KDR, CD31 and CD34) in differentiation conditions with and without Dox-induced *MESP1*-KO (Fig 2A,B). Cells were analyzed at day 0, 2 4 and 6 during differentiation of VSMC progenitors.

As expected 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 (Figure 2B). Importantly *MESP1* expression was verified as being 98.6 % repressed in Dox treated cells at day 2 of VSMC differentiation (Fig 2B).

Interestingly, *MIXL1* decreased by 8% in the *MESP1*-KO whereas *T* was not affected. MIXL1 and BRACHYURY are both 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 (Bondue et al., 2011, Lindsley et al., 2008, Lescroart et al., 2018). Thus, our data suggest that *MESP1*-KO does not change the movement through the primitive streak and the mesodermal specification during iPSC differentiation into VSMCs. This observation corresponds to previous *in vivo* studies showing that *Mesp1*-/- embryos can generate cardiac mesoderm, but fail to fuse their heart tubes, leading to cardia bifida and embryonic lethality (Saga et al., 1999, Lescroart et al., 2018).

We next investigated VSMC progenitor specification at day 4 and 6. As shown in figure 2B, 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, suggesting a MESP1 KO mediated delay of KDR expression 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, 2003).

Interestingly, the expression of the cell surface markers, CD31 and CD34 were reduced significantly by 47 % and 46 % respectively at day 6 (Fig 2B). The decreased expression of CD31 and CD34 was verified by immunocytochemistry of $CD31^+$ and $CD34^+$ cells (Fig 2C). These results show that MESP1 has a major promoting effect on CD31 and CD34 expression, and that MESP1 knock out inhibits early vascular smooth muscle differentiation. 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 out of MESP1 disrupts the VSMC specification downstream from/after 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 (Bondue et al., 2011, Lindsley et al., 2008). However, the effects of *MESP1*-KO on EMT genes during vascular differentiation have not been investigated. We found that the transcription factors that regulate EMT, *SNAI1* and *TWIST1* were both significantly decreased in the absence of MESP1 (Fig 3A) indicating that *MESP1*-KO cells are less likely to undergo EMT during VSMC differentiation than cells expressing MESP1. Moreover it has been suggested that MESP1 promotes cardiac development through EMT, but independently of WNT-signaling (Lindsley et al., 2008). To this end, using mRNA array data and verified by qRT-PCR, we found that the Dickkopf WNT signaling pathway inhibitor 4 (*DKK4*) was significantly higher expressed in *MESP1*-KO 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). Our data suggest that DKK4 has a previously unrecognized role in the vascular smooth muscle development, potentially as an inhibitor of vascular differentiation. This notion 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)

Surprisingly, the mRNA expression data showed that only few mRNAs were differentially expressed in the *MESP1*-KO and control cells at day 2 (Fig 3B, Fig S1 and Table S1), suggesting either that MESP1 regulation is limited to fewer pathways than suspected or more likely, that the *MESP1*-KO

effects emerge after the early time point of primitive streak and mesoderm development. However, both cell cycle and proliferation were affected at day 6 in *MESP1*-KO cells. The cell cycle marker *CDKN1a* was increased, whereas the proliferation marker *PCNA* was slightly but significantly decreased in *MESP1*-KO cells (Fig 3A). This could indicate the existence of two VSMC progenitor populations that grow and differentiate asynchronously depending of *MESP1*-KO.

Taken together, the *MESP1*-KO iPS cell line we have developed represents an elegant and robust *in vitro* model for analyzing MESP1's role in induced pluripotent stem cell differentiation into vascular smooth muscle cells and other biological processes. 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 overexpression 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 smooth muscle cell determination downstream from mesodermal commitment.

Importantly, additional studies are required to map the molecular pathways that MESP1 is exerting during cardiovascular differentiation, especially in WNT-signaling, cell division and proliferation.

MATERIALS AND METHODS

hiPSC lines culture, differentiation into vascular smooth muscle cell progenitors and doxycycline induced *MESP1* knock out.

Previously described, *MESP1* CRISPR interference human induced Pluripotent Stem Cells (hiPSC) (Mandegar et al., 2016) were cultured in E8 medium (Life Technologies). VSMC were induced in a monolayer on ECM matrigel (Thermo Scientific) coated plates in ultraglutamine DMEM supplemented with 6 μM CHIR99021 and 100 μg/ml ascorbic acid. After 48h of differentiation, CHIR99021 was retracted from the differentiation media and further differentiated until day 6 (Figure 2A). Optimal knock out conditions of *MESP1* were found using multiple CRISPRi cell lines and dose-response verification of Doxycycline Hyclate (Sigma-Aldrich) induced *MESP1* knock out. Details on VSMC conditions and differentiation are provided in the supplementary Materials and Methods.

Gene expression analysis and mRNA array

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.

Total RNA samples of 500 ng were reverse-transcribed followed by *in vitro* transcription into biotin-labelled cRNA using the GeneChip 3' IVT Express Kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instruction. Purified and fragmented biotin-labeled cRNA was hybridized to Affymetrix GeneChip HG_133_2+ arrays and subsequently stained, washed and scanned using the GeneChip Fluidics station and Affymetrix Scanner.

Differential gene expression analysis between Dox treated and untreated human iPSC was conducted on the gene expression measured by the microarrays. Paired Significance analysis of microarrays (SAM) (Tusher et al., 2001) was performed using the *samr* package (cran.r-project.org/web/packages/samr/index.html) in R. Genes with Q-value ≤ 0.05 were considered as being significantly differentially expressed between Dox-treated and untreated human iPSC.

Cell imaging and counting

Images 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 Z2 and counting was performed in independent experiments, each comprising triplicate measurements.

Immunofluorescence

Cells were fixed for 10min in 4% paraformaldehyde (PFA) in PBS, permeabilized for 10min in 0.5% TX100 (Sigma-Aldrich) and blocked for 10min in 2% BSA (Calbiochem) in TBS. Cell 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). For more information se the supplementary Materials and Methods

Statistics

All analyses comprised independent experiments and two-way ANOVA or paired *t*-test was performed as indicated (GraphPad Prism 7) to test significant levels. A value of $p \leq 0.05$ was considered statistically significant. All error bars indicate mean ± s.d.

ACKNOWLEDGEMENT

We thank Bruce R. Conklin and Mohammed A. Mandegar for generating the *MESP1* CRISPRi hiPSC,

generously sharing the cell line and stimulating discussions.
We also thank Charlotte Nielsen, Tonja Lyngse Jorgensen (Odense University Hospital) and Edward Shin (Gladstone Institutes, University of California San Francisco) for technical assistance.

COMPETING INTERESTS

NA

FUNDING

This project was funded by: The Lundbeck Foundation (R181-2014-2920) and the Augustinus Foundation.

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FIGURE LEGENDS

Figure 1

A) A schematic presentation of the *MESP1* CRISPRi human induced pluripotent cells generated using the nuclease-deactivated version of Cas9 (dCas9) that blocks transcription in cells when treated with doxycycline. The dCas9 is fused to a Krüppel-associated box (KRAB) repression domain (dCas9-KRAB), producing a more efficient transcriptional interference. In this drawing the dCas9-KRAB is called CRISPRi for simplicity.

For visualization, we used mCherry fluorophore reporter as indicator of doxycycline activated Tet-on system.

B) We generated three different *MESP1* CRISPRi hiPSC lines each with a different guideRNA (gRNA 1-3) to target different sequences in the *MESP1* transcription start site. Each *MESP1* CRISPRi hiPSC line g1, g2 and g3 was tested in triplicates for inhibition of *Mesp1* expression using 0 ( ), 1 and 2 µM Dox ( ). Clearly the *MESP1* CRISPRi hiPSC g1 cell line exhibited the highest repression of *Mesp1*.

C) Four independent clones of the *MESP1* CRISPRi hiPSC g1 cell lines (from hereon simply called *MESP1*-KO) was tested in triplicates for dose-dependent effects of Dox using 0 ( ), or 0.1, 0.5, 1, 2 and 5 µM Dox ( ), showing 94 % repression at 0.1 µM Dox and ≥ 98 % repression at 0.5 - 5 µM Dox.

D) The mCherry fluorophore reporter (indicating activated Tet-on system) was visualized at Dox concentrations ranging from 0 ( ) and 0.1 - 5 µM ( ). Images were acquired by phase-contrast microscopy and by fluorescence of the dCas9-KRAB activated mCherry indicator. 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 250 µm.

E) The dose-dependent effects on *MESP1*-KO cell number and size was measured using Beckman Coulter Multisizer Z2 and counting was performed in four independent experiments, each comprising triplicate measurements.

F-G) qRT-PCR expression analysis of the pluripotent, apoptotic, cell cycle and proliferation markers showing normal expression at the range of 0-1 µM Dox.

E-G) All results of dose-response experiments are represented as mean ± s.d. The analyses comprised four independent experiments each comprising triplicate measurement. Dox concentration: 0 ( ) or 0.1, 0.5, 1, 2 and 5 µM ( ). One-way ANOVA were performed and with Dunnet post-tests (GraphPad Prism software version 7.0) to test significant levels. Differences were considered to be significant at $P < 0.05$. Abbreviations used: OCT4 (Octamer-binding transcription factor 4), SOX2 (sex determining region – box2), BAX (BCL associated X), CDKN1a (cyclin-dependent kinase inhibitor 1a), CCND3 (cyclin D3), PCNA (proliferating cell nuclear antigen).

Figure 2

A) Schematic presentation of the differentiation strategy to direct human induced pluripotent stem cells towards primitive streak/mesoderm and vascular smooth muscle cell progenitors. Chemically defined conditions using DMEM with ultraglutamine added 100 µg/mL ascorbic acid and 6 µM CHIR99021 for 48 h have been used to generate the primitive streak mesodermal cells. Hereafter the CHIR99021 have been retracted and cells differentiation into vascular smooth cell 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*-KO 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 smooth cell progenitor markers at day 6. *MESP1*-KO 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. Greaphs 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 immunofluorence 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.

Figure 3

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*-KO with ( ) or without ( ) Dox.

MESP1-KO 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) Heat map of 229 differentially regulated genes at day 2 of VSMC differentiation treated with or without Dox. The separation of Dox-treated and untreated human iPSC based on the significantly differentially expressed genes was visualized using a principal component analysis (PCA) plot (Fig S1) and this heatmap with associated sample- and gene-wise hierarchical clustering. The heatmap was created by the heatmap.2 function from the *gplots* R-package.

386**FIGURES**

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Figure 1

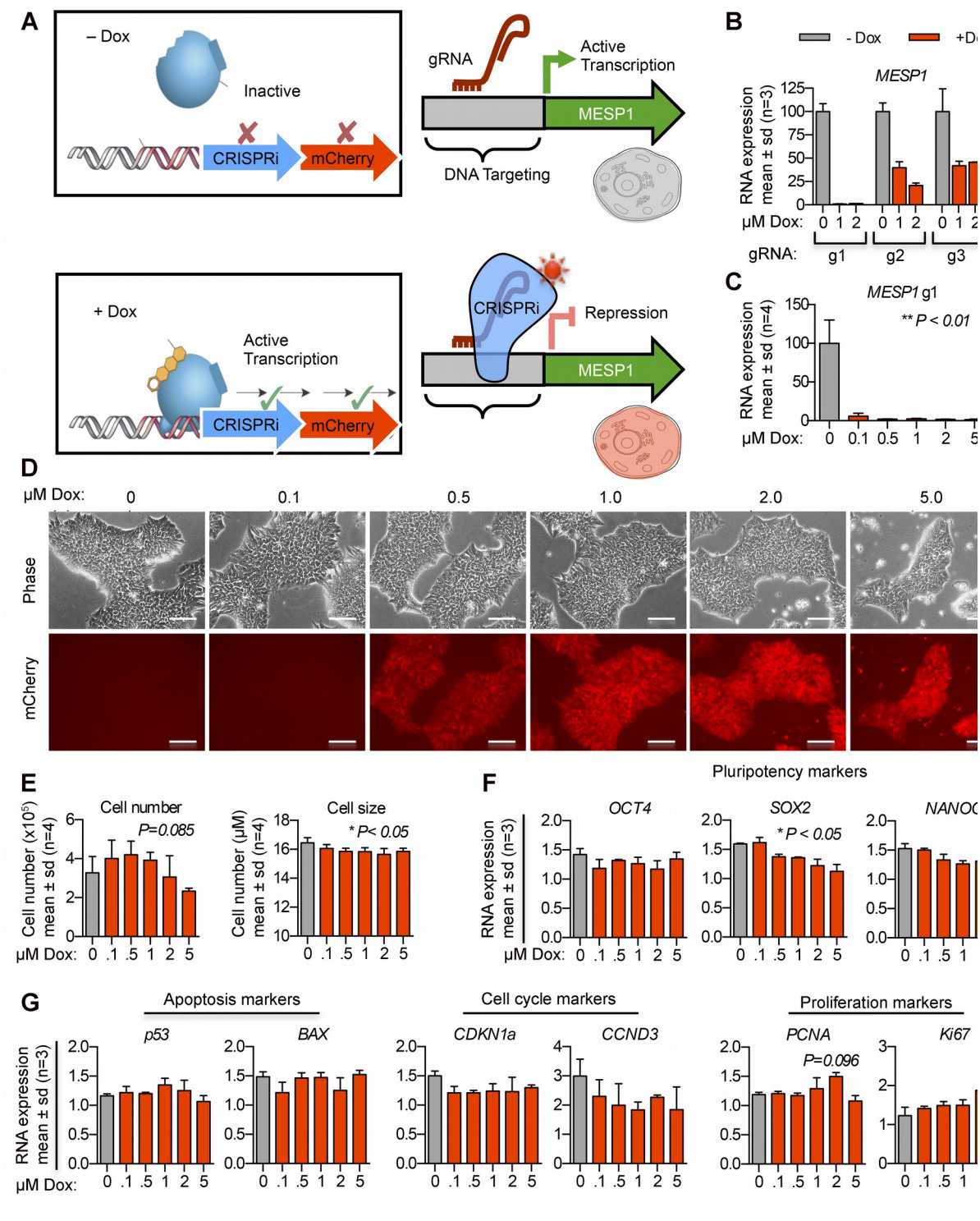


Figure 2

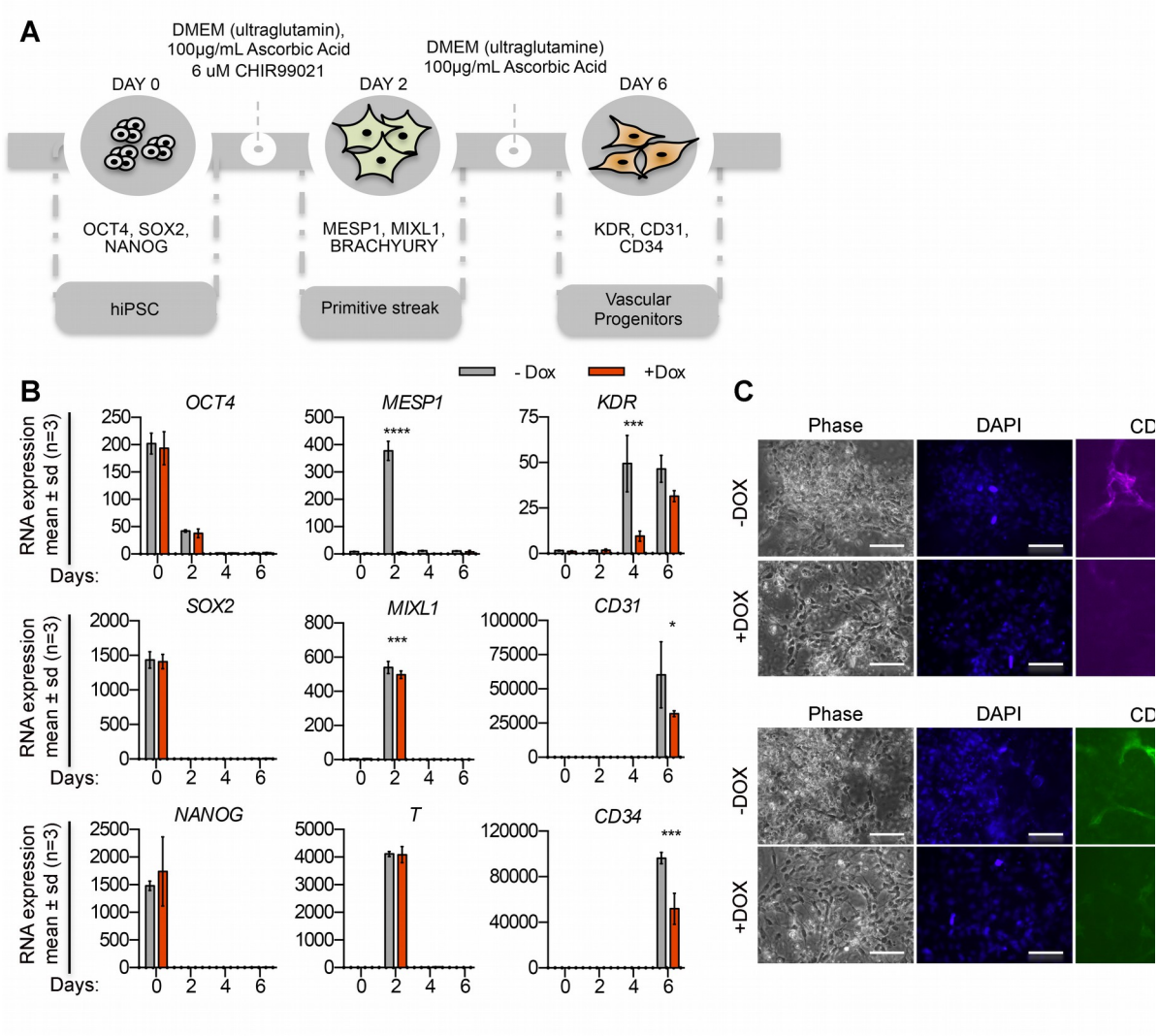
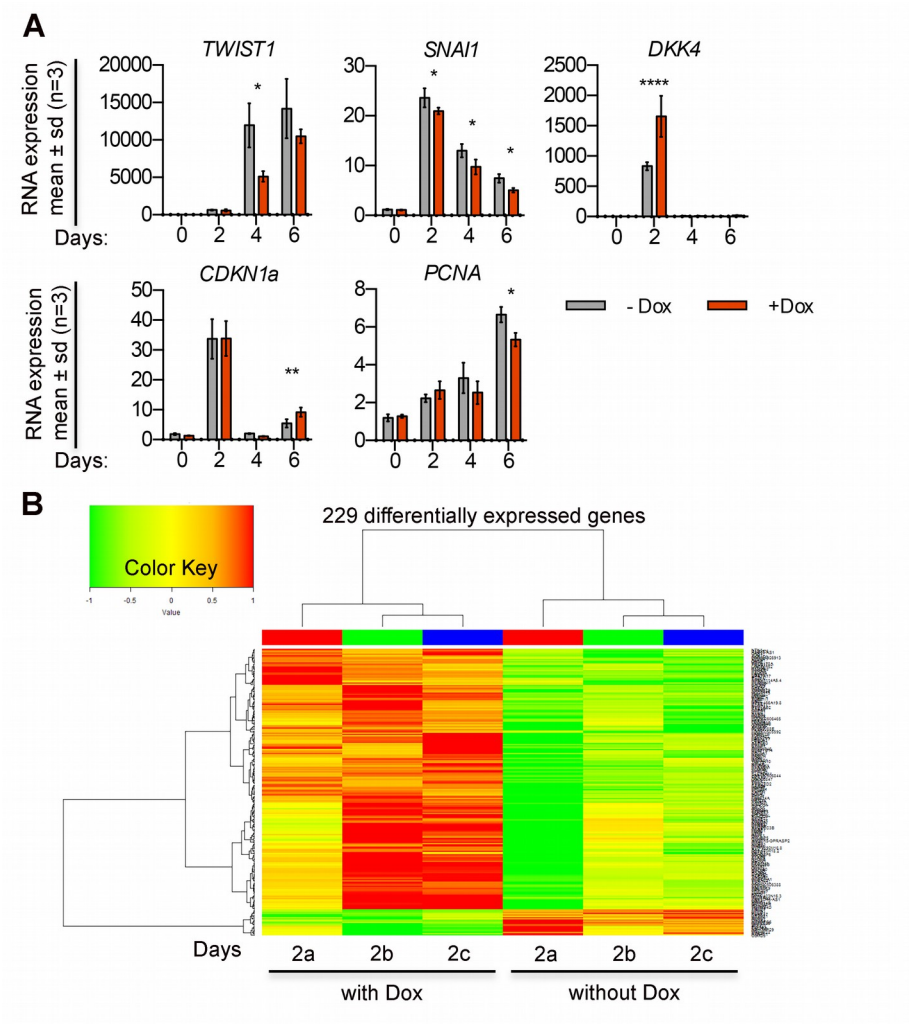


Figure 3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SNAI1
DKK4
CDKN1a
PCNA
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229 differentially expressed genes
Color Key
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2a
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2a
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with Dox
without Dox

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SUPPLEMENTARY

Cell lines, Dox testing and VSMC differentiation

Previously described *MESP1* CRISPR interference human induced Pluripotent Stem cells (hiPSC) (Mandegar et al., 2016) were cultured 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.

For testing of Dox effects 0, 0.1, 0.5, 1, 2 and 5 µM Dox (Doxycycline Hyclate, Sigma) was added to the media.

Vascular smooth muscle 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 6 µM CHIR99021 in DMEM (ultraglutamine) and 100 µg/ml ascorbic acid. After 48h of differentiation, CHIR99021 was retracted from the differentiation media (DMEM (ultraglutamine) and 100 µg/ml ascorbic acid) and further differentiated until day 6. Gene knockout of *MESP1* was induced by addition of 1 µM doxycycline.

Immunofluorescence

CD31 positive cells were identified using an Alexa 647 conjugated mouse anti-human CD31 antibody at 1µg/ml (BD Biosciences cat no: 561654) while CD34 positive cells were recognized using mouse anti-human CD34 at 2µg/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/bright-ness and color balance by Photoshop CS5) were applied equally to all images

Figure S1: PCA plot

The separation of Dox-treated and untreated human iPSC based on the significantly differentially expressed genes was visualized using a principal component analysis (PCA) plot and a heatmap with associated sample- and gene-wise hierarchical clustering (Fig 3B). The PCA-plot was created using the plot.ca function from the *ca* R-package.

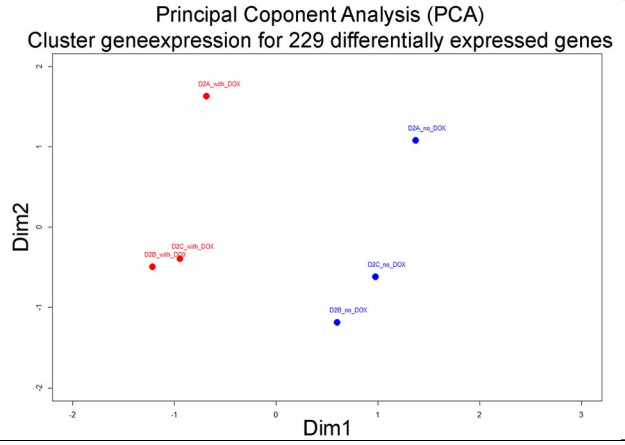

Table S1: Publication of the full array list…. 23.000 gene names

Genes with Q-value ≤ 0.05 were considered as being significantly differentially expressed between Dox-treated and untreated human iPSC.

Table S2

Gene	qRT-PCR primer sequence	Amplification efficiency	Standard Error
OCT4	(FW) 5'- CAGTGCCCGAAACCCACAC -3' (RV) 5'- GGAGACCCAGCAGCCTCAAA -3'	2.106	0.003
SOX2	(FW) 5'- CCCT GTGGTTACCTTTTCCT -3' (RV) 5'- AGTGCTGGGACATGTGAAGT -3'	2.107	0.014
NANOG	(FW) 5'- CGAAGAATAGCAATGGTGTGACG -3' (RV) 5'- TTCCAAAGCAGCCTCCAAGTC -3'	2.020	0.033
MESP1	(FW) 5'- TCGAAGTGGTTCCTTGGCAGAC -3' (RV) 5'- CCTCCTGCTTGCCTCAAAGTGTC -3'	1.875	0.012
MIXL1	(FW) 5'- CAGAGTGGGAAATCCTTCCA -3' (RV) 5'- TGAGTCCAGCTTTGAACCAA -3'	2.139	0.017
T	(FW) 5'- AAGAAGGAAATGCAGCCTCA -3' (RV) 5'- TACTGCAGGTGTGAGCAAGG -3'	1.998	0.021
KDR	(FW) 5'- GTGACCAACATGGAGTCGTG -3' (RV) 5'- TGCTTCACAGAAGACCATGC -3'	1.999	0.019
CD31	(FW) 5'- GCTGACCCTTCTGCTCTGTT -3' (RV) 5'- TGAGAGGTGGTGCTGACATC -3'	2.201	0.177

CD34	(FW) 5'- CCTAAGTGACATCAAGGCAGAA -3' (RV) 5'- GCAAGGAGCAGGGAGCATA -3'	2.368	0.178
SNAI1	(FW) 5'- CGAGTGGTTCTTCTGCGCTA -3' (RV) 5'- CTGCTGGAAGGTAAACTCTGGA -3'	1.972	NA
TWIST1	(FW) 5'- GCCGGAGACCTAGATGTCATTGT -3' (RV) 5'- CGCCCTGTTTCTTTGAATTTGGA -3'	2.085	NA
p53	(FW) 5'- AGCACTGTCCAACAACACCA -3' (RV) 5'- CTTCAGGTGGCTGGAGTGAG -3'	1.788	0.022
BAX	(FW) 5'- CCCTTTTGCTTCAGGGTTTCAT -3' (RV) 5'- GGAAAAAGACCTCTCGGGGG -3'	1.902	0.018
CDKN1a	(FW) 5'- TGCCGAAGTCAGTTCCTTGT -3' (RV) 5'- GTTCTGACATGGCGCCTCC -3'	1.917	0.038
CCND3	(FW) 5'- GACCGAAACTTGGCTGAGCA -3' (RV) 5'- CACATACCTCCTCGTCAGGTG -3'	2.004	0.222
PCNA	(FW) 5'- CGGTTACTGAGGGCGAGAAG -3' (RV) 5'- GGCTGAGACTTGCGTAAGGG -3'	1.981	0.042
Ki67	(FW) 5'- GAGCGGTGGTTCGACAAGT -3' (RV) 5'- TGAGCTTTCTCATCAGGGTCAG -3'	1.663	0.030
DKK4	(FW) 5'- TAGTGGAAGCTCTGCTCTGG -3' (RV) 5'- TACAAACATCGTTCACACAGAGT -3'	1.796	0.055
HPRT1	(FW) 5'- GGCTCCGTTATGGCGACCCG -3' (RV) 5'- CCCCTTGAGCACACAGAGGGCTA -3'	1.961	0.015
GAPDH	(FW) 5'- GCCACATCGCTCAGACACCATGG -3' (RV) 5'- TCCCGTTCTCAGCCTTGACGGT -3'	2.059	0.040
PGK1	(FW) 5'- GTCGGCTCCCTCGTTGACCGAA -3' (RV) 5'- GGGACAGCAGCCTTAATCCTCTGGT -3'	1.974	0.010

Table S2: Primers used for qRT-PCR. AE: Amplification efficiency is calculated by dilution curve and SE: Standard Error is calculated for each primer pair's AE by the qBase^{+} software. All qRT-PCR data has been normalized against two stably expressed control genes (i.e. *GAPDH*, *HPRT1* and *PGK1*).