Angiogenesis (2016) 19:1–7
DOI 10.1007/s10456-015-9484-3

ORIGINAL PAPER

Matrix Gla protein regulates differentiation of endothelial cells derived from mouse embryonic stem cells

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Received: 11 May 2015 / Accepted: 7 September 2015 / Published online: 12 September 2015

Abstract Matrix Gla protein (MGP) is an antagonist of bone morphogenetic proteins and expressed in vascular endothelial cells. Lack of MGP causes vascular abnormalities in multiple organs in mice. The objective of this study is to define the role of MGP in early endothelial differentiation. We find that expression of endothelial markers is highly induced in *Mgp* null organs, which, in wild type, contain high MGP expression. Furthermore, *Mgp* null embryonic stem cells express higher levels of endothelial markers than wild-type controls and an abnormal temporal pattern of expression. We also find that the *Mgp*-deficient endothelial cells adopt characteristics of mesenchymal stem cells. We conclude that loss of MGP causes dysregulation of early endothelial differentiation.

Keywords Bone morphogenetic proteins · Endothelial cells · Differentiation · Embryonic stem cells · Matrix Gla protein

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Introduction

Normal differentiation of vascular endothelial cells (ECs) is essential for maintaining vascular stability and integrity. EC differentiation requires tight regulation of both intrinsic genetic determinants and microenvironmental factors, which provide extracellular signals for ECs to adapt to the vascular bed. Many extracellular factors have been shown to regulate EC differentiation, such as vascular endothelial growth factor (VEGF), Wnt, activin, fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs) [1]. In cross talk with both Wnt and Notch signaling, BMPs regulate EC proliferation, specification and cell motility [1]. In particular, BMP-4 has been shown to be critical for determining the EC fate [2].

Matrix Gla protein (MGP) is an efficient inhibitor of BMP-2, 4 and 7 [3–6]. MGP is best known for its ability to prevent calcification [7, 8], but is highly expressed in tube-forming ECs [9, 10] and acts as an inhibitor of angiogenesis [6, 11]. We previously demonstrated that excessive MGP in mice inhibits pulmonary vascular growth by limiting BMP-4 activity and VEGF expression [6, 12]. *Mgp* gene mutations in humans cause multiple developmental defects (the so-called Keutel syndrome) [13], which include abnormal growth of vasculature [14]. Several studies have shown that deletion of the *Mgp* gene in mice causes vascular abnormalities, including arterial calcification and arteriovenous malformations (AVMs) in lungs, kidneys and brain [6, 15]. In addition, the studies suggest altered EC differentiation and the presence of endothelial–mesenchymal transitions (EndMTs) in *Mgp* null mice [8].

However, the role of MGP in early endothelial differentiation is unclear. This study examines whether lack of MGP disrupts endothelial differentiation in endothelial progenitor cells derived from embryonic stem cells (ESCs).

Methods

Animals

$Mgp^{+/-}$ mice on C57BL/6J background were obtained from the Jackson laboratory (strain: B6.129S7-Mgptm1Kry/KbosJ). Genotypes were confirmed by PCR [16], and experiments were performed with generation F4–F6. Littermates were used as wild-type controls. All mice were fed a standard chow diet (Diet 8604, HarlanTeklad Laboratory). The studies were reviewed and approved by the Institutional Review Board and conducted in accordance with the animal care guideline set by the University of California, Los Angeles. The investigation conformed to the National Research Council, Guide for the Care and Use of Laboratory Animals, Eighth Edition (Washington, DC: The National Academies Press, 2011).

Tissue culture and EC differentiation

Wild-type ESCs (C57BL/6 J background) were obtained from ATCC (SCRC-1002). Mouse ESCs were cultured and maintained as previous described [17]. The derivation of ECs from ESCs was performed by a previously published protocol [18]. BMP-4, activin A, FGF-2, Noggin and VEGF (all from R&D Systems), anti-Jagged 1 antibodies (Abcam) and anti-Jagged 2 antibodies (Abgent) were added to StemPro-34® medium prior to use. The process of derivation lasted 14 days.

RNA analysis

Real-time PCR analysis was performed as previously described [19]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control gene [19]. Primers and probes for mouse MGP, fetal liver kinase 1 (Flk1), fms-like tyrosine kinase 1 (Flt1), cluster of differentiation (CD) 31, CD34, VE-cadherin, von Willebrand factor (vWF), tyrosine-protein kinase Kit (c-kit), CD90, Snail, N-cadherin, octamer-binding transcription factor 3/4 (Oct3/4), Nanog homeobox (Nanog), Sry-box2 (Sox2) and stage-specific embryonic antigen (SSEA) 1 and 3 were obtained from Applied Biosystems as part of Taqman® Gene Expression Assays.

Immunofluorescence

Immunofluorescence was performed as previously described in detail [8]. We used specific antibodies for CD34 and Flk1 (both from Cell Signaling Technology). The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich).

Flow cytometric analysis

Fluorescence-activated cell sorting analysis was done as previously described [8]. The cells were stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or Alexa Fluor 488 (AF-488)-conjugated antibodies against CD34, Flk1 and VE-cadherin. Nonspecific fluorochrome- and isotype-matched IgGs (BD Pharmingen) served as controls.

Statistical analysis

Data were analyzed for statistical significance by ANOVA with post hoc Tukey's analysis. The analyses were performed using GraphPad Instat®, version 3.0 (GraphPad Software). Data represent mean ± SD. P values less than 0.05 were considered significant, and experiments were repeated a minimum of three times.

Results

Enhanced expression of EC markers in multiple organs of $Mgp^{-/-}$ mice

To assess how MGP expression relates to endothelial differentiation, we examined the expression of MGP and the endothelial-specific markers Flk1, VE-cadherin and vWF in $Mgp^{+/+}$ and $Mgp^{-/-}$ organs, including lungs, kidneys, brain, aorta, heart, stomach, intestine, bone, muscle and liver by real-time PCR. MGP was highly expressed in lungs, kidneys, aorta, heart, stomach and intestine (Fig. 1a). In $Mgp^{-/-}$ mice, EC markers were strongly induced in several of these organs, including lungs, kidneys and aorta (Fig. 1b–d). These are the same organs where AVMs or vascular calcification has been documented [6, 8]. The EC markers were also highly induced in $Mgp^{-/-}$ brain (Fig. 1b–d), although the total MGP expression in wild-type brain was relative low (Fig. 1a). The data were consistent with the previous findings of AVMs in $Mgp^{-/-}$ brains [15] and suggested that MGP expression may be limited to the brain ECs. The endothelial markers were mildly increased in heart, stomach, intestine, bone and muscle of $Mgp^{-/-}$ mice, which had relatively lower levels of MGP expression (Fig. 1a–d). There were no significant changes in endothelial markers in $Mgp^{-/-}$ liver (Fig. 1b–d), which had very low MGP expression (Fig. 1a) and no detectable phenotype in $Mgp^{-/-}$ mice [7].

Derivation of $Mgp^{-/-}$ embryonic stem cells

To determine whether MGP regulates early endothelial differentiation, we generated $Mgp^{-/-}$ mouse ESCs by

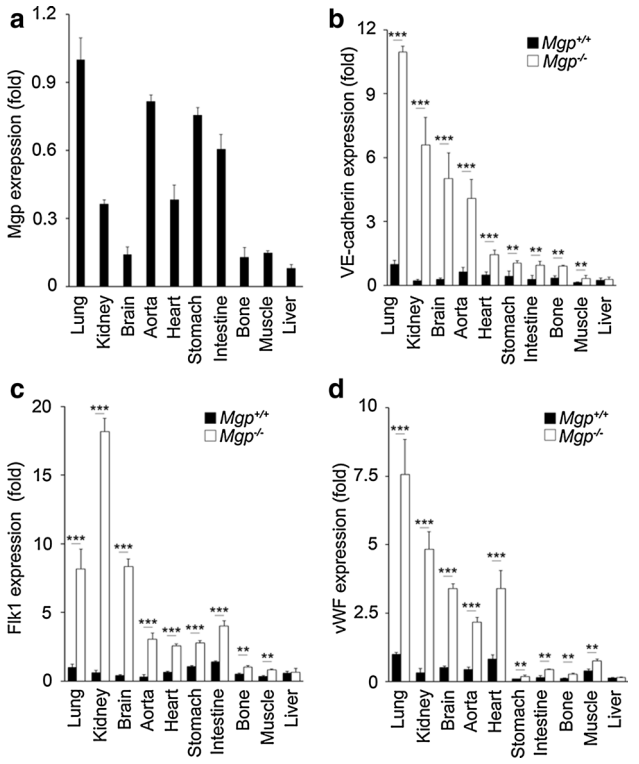

Fig. 1 Expression of endothelial markers in tissues and organs of $Mgp^{-/-}$ mice. **a** Expression of MGP in the lungs, kidneys, brain, aorta, heart, stomach, intestine, bone, muscle and liver of wild-type mice. Expression of MGP was determined by real-time PCR with normalization to GAPDH expression. The MGP expression in the different organs is calculated as fold change compared to the MGP expression in the lungs of wild-type mice. **b**, **c** Expression of VE-cadherin (**b**), Flk1 (**a**) and von Willebrand factor (vWF) (**c**) in the lungs, kidneys, brain, aorta, heart, stomach, intestine, bone, muscle and liver of wild-type ($Mgp^{+/+}$) and $Mgp^{-/-}$ mice. ***$P < 0.0001$; **$P < 0.01$; *$P < 0.05$

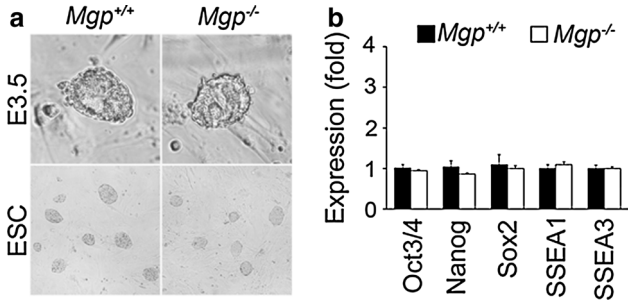

Fig. 2 Characteristics of $Mgp^{-/-}$ ES cells. **a** Bright-field images of wild-type ($Mgp^{+/+}$) and $Mgp^{-/-}$ embryoblast (*top*) and derived $Mgp^{+/+}$ and $Mgp^{-/-}$ ES cells (*bottom*). **b** Expression of Oct3/4, Nanog, Sox2, SSEA1 and SSEA3 in $Mgp^{+/+}$ and $Mgp^{-/-}$ ES cells

using a previously published protocol [17]. We isolated $Mgp^{-/-}$ embryoblast at E3.5 days and cultured them on $Mgp^{-/-}$ mouse embryonic fibroblast feeders (Fig. 2a, top). After 7–9 days of culture, the ESC colonies were disaggregated and expanded (Fig. 2a, bottom). We compared the expression of pluripotency markers to wild-type ESCs by real-time PCR and found that the expression of Oct3/4, Nanog, Sox2, and SSEA 1 and 3 was unchanged in $Mgp^{-/-}$ ESCs (Fig. 2b).

We also performed karyotypic analysis of $Mgp^{-/-}$ ESCs, which showed that 49 of 50 spreads had 40 chromosomes and the cell line was 98 % euploid. The $Mgp^{-/-}$ ESCs were then expanded and used for further experiments.

Enhanced EC induction in $Mgp^{-/-}$ ESCs

To assess the effect of MGP on endothelial differentiation, $Mgp^{-/-}$ and wild-type ESCs were driven into EC lineage by established protocols [18]. We first examined the expression of MGP at different time points of derivation from day 1 to day 14. In wild type, expression of MGP increased slightly on day 3 and kept increasing from day 6 to day 14 (Fig. 3a). No MGP expression was detected in $Mgp^{-/-}$ ESCs. Then, we examined the expression of endothelial markers Flk1, Flt1, CD31, CD34 and VE-cadherin. In wild-type cells, expression of Flk1 showed an early peak on day 6 and continued high expression from day 6 to day 14 (Fig. 3a). The expression of Flt1, CD31 and VE-cadherin peaked on day 9, whereas expression of CD34 peaked on day 12. The results suggested that, under these conditions of derivation, different EC markers had different patterns of expression, with Flk1 being the earliest marker.

We then examined the expression of EC markers in cells derived from $Mgp^{-/-}$ ESCs. We found that expression of all EC markers was higher in $Mgp^{-/-}$ cells compared to wild-type controls (Fig. 3a). We also observed that the pattern of expression of EC markers was altered. To better visualize this, we compared the expression pattern in cells derived from wild-type ESCs and $Mgp^{-/-}$ ESCs by combining the curves from Fig. 3a in two graphs using arbitrary units (Fig. 3b). In cells derived from $Mgp^{-/-}$ ESCs, expression of Flk1 was increased a second time on day 14 (Fig. 3b, bottom). Furthermore, in $Mgp^{-/-}$ cells, the induction of Flt1, CD31 and VE-cadherin occurred earlier, and the expression of CD34 and VE-cadherin peaked earlier, compared to wild-type controls (Fig. 3b, bottom). Overall, the progression of the EC differentiation was less organized in the $Mgp^{-/-}$ cells than in the wild-type controls.

To determine whether inhibition of BMP improved the $Mgp^{-/-}$ EC differentiation, we added Noggin on day 3 of the EC derivation from $Mgp^{-/-}$ ESCs and examined the expression of EC markers. The results showed that Noggin decreased induction of EC markers in cells derived

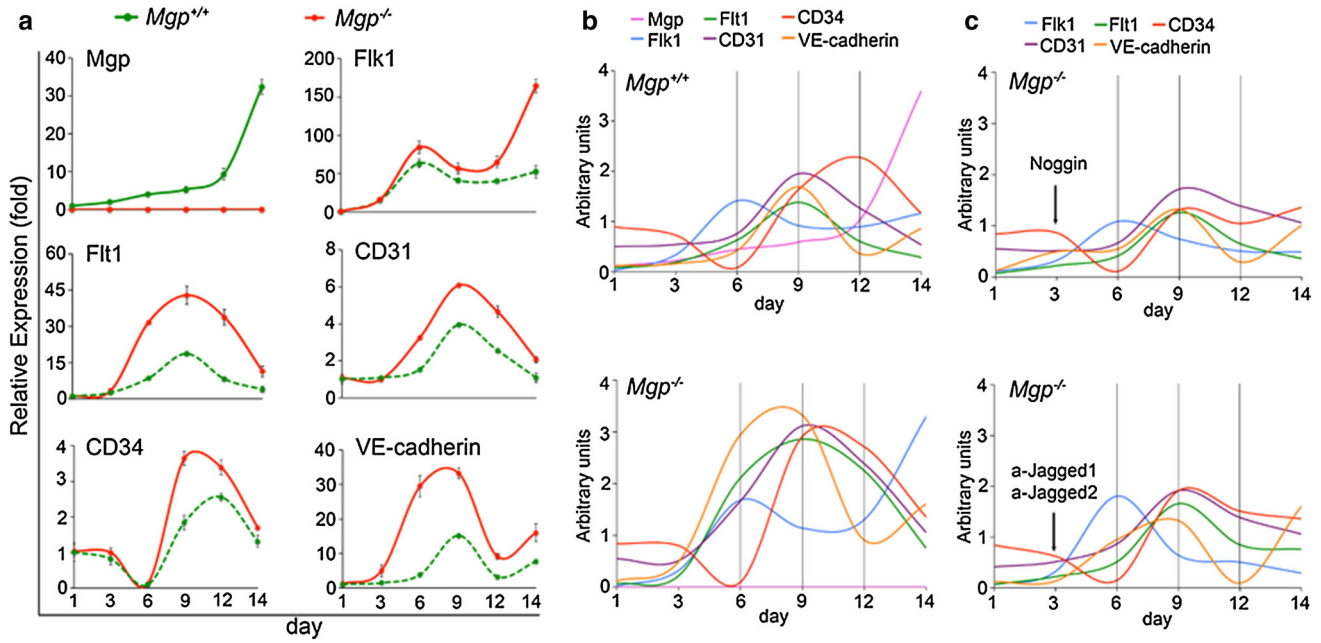

Fig. 3 Expression of endothelial markers increase during $Mgp^{+/+}$ and $Mgp^{-/-}$ EC derivation. **a** Time course of expression of MGP, Flk1, Flt1, CD31, CD34 and VE-cadherin as determined by real-time PCR during EC derivation from $Mgp^{+/+}$ and $Mgp^{-/-}$ ESCs. Gene expression is calculated as fold change compared to the expression of $Mgp^{+/+}$ control cells on day 1. **b** Comparison of expression pattern of MGP, Flk1, Flt1, CD31, CD34 and VE-cadherin in ECs derived from $Mgp^{+/+}$ and $Mgp^{-/-}$ ESCs. **c** Comparison of expression pattern of Flk1, Flt1, CD31, CD34 and VE-cadherin in ECs derived from $Mgp^{-/-}$ ESCs after adding noggin (*top*), or anti-jagged 1 and 2 antibodies (*bottom*)

from $Mgp^{-/-}$ESCs (Fig. 3c, top), confirming that increased BMP activity contributed to abnormal EC differentiation.

We previously showed that the Notch ligands Jagged 1 and Jagged 2 were abnormally induced in $Mgp^{-/-}$ cerebral vasculature [15]. To determine whether limiting Jagged 1 and 2 affected $Mgp^{-/-}$ EC differentiation, we added anti-Jagged 1 and 2 neutralizing antibodies on day 3 in the EC derivation from $Mgp^{-/-}$ ESCs. We examined the expression of EC markers and found that inhibition of Jagged 1 and 2 reduced induction of EC markers in cells derived from $Mgp^{-/-}$ ESCs, confirming that Notch signaling is involved in abnormal $Mgp^{-/-}$ EC differentiation.

We confirmed the enhanced expression of EC markers on day 9 during the EC derivation by flow cytometric analysis using specific antibodies to CD34, Flk1 and VE-cadherin. The analysis showed that the populations of CD34, Flk1 or VE-cadherin positive cells in cells derived from $Mgp^{-/-}$ ESCs were about double the size of those from wild-type ESCs (Fig. 4a). We also costained ECs derived from $Mgp^{-/-}$ ESCs with antibodies to CD34 and Flk1 on day 9. The CD34 and Flk1 staining was more intense in ECs derived from $Mgp^{-/-}$ ESCs than in wild-type controls (Fig. 4b), consistent with the flow cytometric analysis.

Enhanced mesenchymal stem cell characteristic in ECs derived from $Mgp^{-/-}$ ESCs

We previously showed that lack of MGP caused stem cell characteristic to emerge in the aortic endothelium [8]. To investigate whether ECs derived from $Mgp^{-/-}$ ESCs exhibited stem cell characteristics, we first sorted Flk1-positive ECs derived from $Mgp^{-/-}$ and wild-type ESCs by flow cytometry. We then examined the expression of the stem cell markers c-kit, CD90 and N-cadherin, and the mesenchymal marker Snail by real-time PCR in the Flk1-positive cells. The results showed that the expression of all these markers increased in ECs derived from $Mgp^{-/-}$ ESCs on days 6, 9 and 12, compared to wild-type cells (Fig. 5a). CD90 showed a significant increase already on day 3 (Fig. 5a). We further examined whether lack of MGP affected the expression pattern of stem cell markers in ECs derived from ESCs. We combined the curves from Fig. 5a in graphs using arbitrary units (Fig. 5b). In ECs from wild-type and $Mgp^{-/-}$ ESCs, CD90 peaked on day 3, whereas c-kit peaked on day 6 in $Mgp^{-/-}$ ECs and on day 9 in wild-type ECs. Snail and N-cadherin increased progressively from day 3 to day 12 in the $Mgp^{-/-}$ ECs. The results suggested that ECs derived from $Mgp^{-/-}$ ESCs maintain expression of stem cell markers and may start to undergo EndMTs.

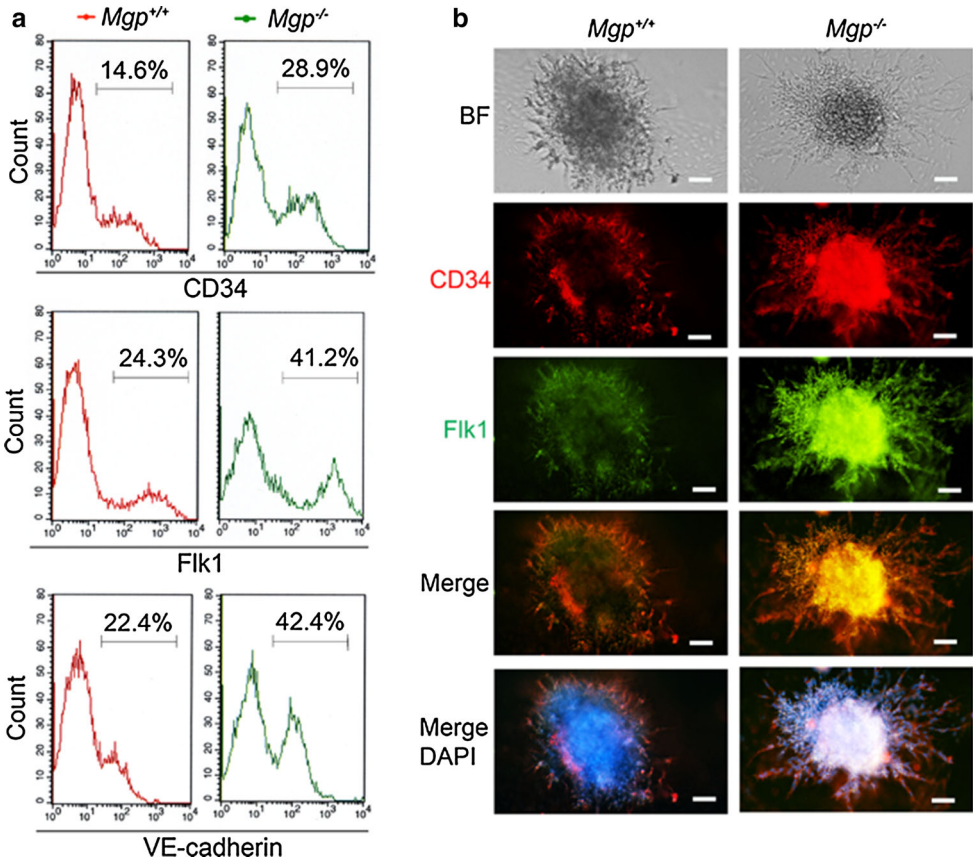

Fig. 4 Enhanced number of EC-like cells derived from $Mgp^{-/-}$ ESCs. **a** Flow cytometry analysis of ECs derived from $Mgp^{+/+}$ and $Mgp^{-/-}$ ESCs using specific antibodies to CD34, Flk1 and VE-cadherin ECs at day 9. **b** Costaining of CD34 and Flk1 in ECs derived from $Mgp^{+/+}$ and $Mgp^{-/-}$ ES cells on day 9. *Scale bars* 50 μm

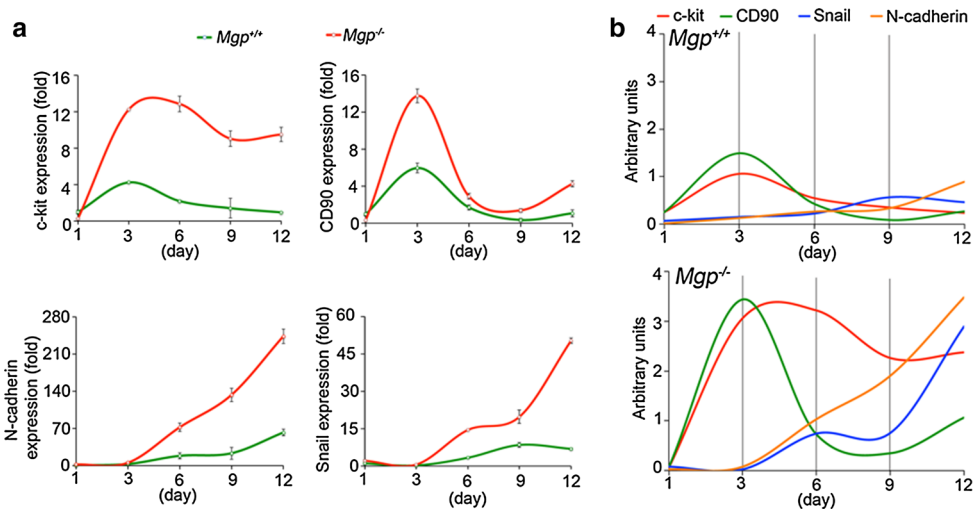

Fig. 5 Expression of mesenchymal stem cell markers in $Mgp^{-/-}$ ECs. **a** Expression of c-kit, CD90, N-cadherin and Snail in ECs derived from $Mgp^{+/+}$ and $Mgp^{-/-}$ ESCs, which were presorted using specific antibodies to Flk1, as shown by real-time PCR. **b** Comparison of expression patterns of c-kit, CD90, N-cadherin and snail in ECs derived from $Mgp^{+/+}$ and $Mgp^{-/-}$ ESCs

Together, our results suggested that lack of MGP caused dysregulation of early EC differentiation

Discussion

In this study, we found enhanced expression of endothelial markers in multiple organs of $Mgp^{-/-}$ mice. The induction of endothelial markers in MGP deficiency was often strong in organs that normally expressed a lot of MGP. Moreover, lack of MGP dysregulated differentiation of ECs when derived from ESCs and caused characteristics of mesenchymal stem cells to emerge.

MGP is normally highly expressed in ECs [20, 21], and deletion of the *Mgp* gene in mice causes a number of vascular abnormalities including AVMs and irregular vessel caliber in the lungs, kidneys and brain, an increased number of glomeruli in the kidneys, and calcification in the elastic arteries

[6, 8, 15, 22]. The dysregulation of endothelial differentiation and appearance of stem cell characteristics when MGP is reduced, nonfunctioning or missing are likely to form the basis for such vascular abnormalities.

Our results showed that $Mgp^{-/-}$ and wild-type ESCs had identical pluripotency while still undifferentiated and that differentiation of $Mgp^{-/-}$ ECs turned abnormal between day 3 to day 6 when MGP usually begins to express. It is consistent with previous reports that did not detect expression of MGP in the mesenchymal epithelial interphase until E10.5 in mice [23], and supports a role for MGP once the initial vasculature has been established.

Our results further suggested that MGP regulates EC differentiation by inhibiting BMP activity. Lack of MGP increases BMP activity in ECs of multiple organs, such as aorta, lung, brain and kidneys [6, 15, 20]. Here, we show that lack of MGP promotes EC differentiation, whereas Noggin reduces the EC induction. We argue that MGP regulates EC differentiation, both the level of expression and the timing of induction, in ESCs by controlling the BMP activity.

BMP-4 is known to induce expression of MGP, which provides negative feedback inhibition by binding and inhibiting BMP-4 [12]. BMP activity is important for both maintaining stem cell characteristics and promoting EC differentiation [24, 25] and has been shown to induce EndMTs in ECs [8, 26]. In published protocol and our experiments, BMP-4 acts as a critical exogenous factor when ECs are derived from ESCs [18]. The loss of MGP is likely to dysregulate the activity of BMP-4 and potentially other BMPs, triggering the abnormal progression of the endothelial differentiation.

In our experiments, lack of MGP increased both EC and mesenchymal stem cell markers, suggesting that MGP helps differentiate endothelial lineage from early mesenchymal differentiation [27]. The results showed that lack of MGP increased the expression of the mesenchymal stem cell markers CD90 and c-kit as well as the duration of the expression, indicating an enhanced mesenchymal state in MGP-deficient conditions. Also, we showed that Snail and N-cadherin increased simultaneously with EC markers, suggesting that $Mgp^{-/-}$ ECs adopt stem cell characteristics, previously noted in the $Mgp^{-/-}$ aortic ECs [8]. Overall, the results are consistent with our previous results, showing that stem cell and EC markers coexist in $Mgp^{-/-}$ ECs.

Acknowledgments Funding for this work was provided in part by NIH grants NS79353, HL30568, HL81397 and HL112839, and the American Heart Association (Western Affiliate).

Compliance with ethical standards

Conflict of interest The authors have declared that no conflict of interest exists.

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