LETTER

doi:10.1038/nature12207

EndMT contributes to the onset and progression of cerebral cavernous malformations

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Cerebral cavernous malformation (CCM) is a vascular dysplasia, mainly localized within the brain and affecting up to 0.5% of the human population. CCM lesions are formed by enlarged and irregular blood vessels that often result in cerebral haemorrhages. CCM is caused by loss-of-function mutations in one of three genes, namely *CCM1* (also known as *KRIT1*), *CCM2* (*OSM*) and *CCM3* (*PDCD10*), and occurs in both sporadic and familial forms[1]. Recent studies[2–7] have investigated the cause of vascular dysplasia and fragility in CCM, but the *in vivo* functions of this ternary complex remain unclear[8]. Postnatal deletion of any of the three *Ccm* genes in mouse endothelium results in a severe phenotype, characterized by multiple brain vascular malformations that are markedly similar to human CCM lesions[9]. Endothelial-to-mesenchymal transition (EndMT) has been described in different pathologies, and it is defined as the acquisition of mesenchymal- and stem-cell-like characteristics by the endothelium[10–12]. Here we show that endothelial-specific disruption of the *Ccm1* gene in mice induces EndMT, which contributes to the development of vascular malformations. EndMT in CCM1-ablated endothelial cells is mediated by the upregulation of endogenous BMP6 that, in turn, activates the transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP) signalling pathway. Inhibitors of the TGF-β and BMP pathway prevent EndMT both *in vitro* and *in vivo* and reduce the number and size of vascular lesions in CCM1-deficient mice. Thus, increased TGF-β and BMP signalling, and the consequent EndMT of CCM1-null endothelial cells, are crucial events in the onset and progression of CCM disease. These studies offer novel therapeutic opportunities for this severe, and so far incurable, pathology.

To study the role of CCM1 *in vivo*, we generated endothelial-specific tamoxifen-inducible *Ccm1* loss-of-function (iCCM1) mice. *Ccm1* deletion resulted in the development of vascular lesions within the central nervous system. The lesions were mostly concentrated in the cerebellum (Supplementary Fig. 1a), and were composed of dilated vessels with multiple lumens, sometimes with signs of vascular leakage as haemosiderin accumulation (Supplementary Fig. 1a, b). We also observed a marked inflammatory reaction, characterized by infiltration of leukocytes and increased expression of the adhesion molecules intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule-1 (VCAM1) (Supplementary Fig. 1c–h). As in patients with CCM[13], iCCM1 mice presented venous malformations at the periphery of the retinal vascular plexus (Supplementary Fig. 1i). Enhanced yellow fluorescent protein (eYFP) staining confirmed the high gene recombination efficiency (Supplementary Fig. 1j). To investigate the phenotype of endothelial cells lining the vascular lesions, we analysed the expression and the localization of junctional proteins. In these cells the adherens junction protein VE-cadherin was strongly disorganized whereas N-cadherin was markedly upregulated (Fig. 1a, b) as compared with cadherin distribution in endothelial cells of the normal vessels surrounding the lesions. This type of cadherin switch has been associated with EndMT[14,15]. To test the hypothesis of the occurring EndMT, we evaluated the expression of typical mesenchymal markers, such as SLUG (also known as SNAI2), inhibitor of DNA binding 1 (ID1) and α-smooth muscle actin (αSMA)[16,17]. We found that these proteins were strongly upregulated in the endothelial cells forming the vascular malformations (Fig. 1c–e). The stem-cell markers stem cell antigen 1 (SCA1) and CD44 were also strongly expressed by cells lining the lesions, but not by endothelial cells in the surrounding normal vessels (Fig. 1f, g). KLF4, a transcription factor involved in cell differentiation[18], was the only investigated marker that was upregulated both in the lesions and the normal vasculature of the iCCM1 mice (Fig. 1h, i). In iCCM1 malformations, endothelial expression of mesenchymal and stem-cell markers increased with the size of the lesion, with the exception of KLF4, SLUG and ID1 (Fig. 1j). Furthermore, the upregulation of mesenchymal and stem-cell markers and leukocyte adhesion molecules was confirmed in freshly isolated mouse brain endothelial cells (Fig. 1k) and in the retina of iCCM1 mice (Supplementary Fig. 2a–f). Importantly, the acquisition of the mesenchymal and stem-cell phenotype occurs only in endothelial cells expressing eYFP, confirming *Ccm1* recombination (Supplementary Fig. 2g for KLF4). Thus, *Ccm1* ablation *in vivo* confers EndMT traits to endothelial cells.

To investigate whether EndMT in CCM lesions is a cell-autonomous process, we treated cultured endothelial cells from $Ccm1^{fl/fl}$ mice with Tat-Cre recombinase. CCM1 downregulation in lung (CCM1 knockout) and primary brain microvascular endothelial cells (BMECs) (CCM1 knockdown) (Supplementary Fig. 3a, e) caused a switch in phenotype, which recapitulates the findings observed *in vivo* (Supplementary Fig. 3b–g). Cell transition to a mesenchymal state is accompanied by increased proliferation and invasiveness[12]. We found that loss of CCM1 caused a significant enhancement of endothelial cell proliferation and a greater invasive/sprouting capacity (Supplementary Fig. 4).

Because the TGF-β pathway is a major inducer of EndMT[10], we investigated whether the EndMT switch in CCM1-knockout endothelial cells is due to a greater sensitivity of these cells to this factor. TGF-β signalling is mediated by phosphorylation of receptor-activated SMAD proteins, which, once phosphorylated (pSMADs), translocate to the nucleus and regulate the transcription of target genes. Phosphorylation of SMAD1 and SMAD3 was enhanced after loss of CCM1 (Fig. 2a, quantification in Supplementary Fig. 11). In addition, TGF-β1 stimulation of CCM1-knockout endothelial cells showed a stronger and more prolonged SMAD phosphorylation and transcriptional activity compared to control endothelial cells (Fig. 2b, c). The higher

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