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Skip is essential for Notch signaling to induce Sox2 in cerebral arteriovenous malformations

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Abstract

Notch signaling and Sry-box (Sox) family transcriptional factors both play critical roles in endothelial cell (EC) differentiation in vascularization. Recent studies have shown that excessive Notch signaling induces Sox2 to cause cerebral arteriovenous malformations (AVMs). Here, we examine human pulmonary AVMs and find no induction of Sox2. Results of epigenetic studies also show less alteration of Sox2-DNA binding in pulmonary AVMs than in cerebral AVMs. We identify high expression of ski-interacting protein (Skip) in brain ECs, a Notch-associated chromatin-modifying protein that is lacking in lung ECs. Knockdown of Skip abolished Notch-induction of Sox2 in brain ECs, while restoration of Skip in lung ECs enabled Notch-mediated Sox2 induction. The results suggest that Skip is a key factor for induction of Sox2 in cerebral AVMs.

INTRODUCTION

The members of the Sox family of transcriptional factors, which are sharing a DNA-binding high mobility group (HMG) box domain (Sarkar and Hochedlinger, 2013; Wegner, 1999), are of key importance in vascularization. Sox7, Sox17, and Sox18 constitute important factors in the specification of arterial-venous endothelial cells (ECs) and direct the development of lymphatic vasculature (Herpers et al., 2008; Park et al., 2013; Pendeville et

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AUTHOR CONTRIBUTIONS

Y.Y., and K. I. B. supervised the experiments, analyzed data, and wrote the manuscript. D.Z., X.Q., L.W., L.Z., J. Y., X.W., T.Y. performed experiments and data analysis.

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al., 2008; Sakamoto et al., 2007). Sox2, referred to as one of the Yamanaka factors, participates in the induction of pluripotency in somatic cells (Takahashi and Yamanaka, 2006). Sustained expression of Sox2 is observed along the ectoderm (Sarkar and Hochedlinger, 2013) to regulate the differentiation of cell lineages (Amador-Arjona et al., 2015; Basu-Roy et al., 2010; Clavel et al., 2012; Ochieng et al., 2014; Pispa and Thesleff, 2003) and cell fate transitions (Luo et al., 2013; Mandalos et al., 2014). Recent studies show that Sox2 is essential in endothelial differentiation and altered Sox2 expression impacts endothelial integrity (Yao et al., 2019b). Furthermore, ECs double positive for endothelial marker fetal liver kinase 1 (Flk1) and Sox2 can be identified adjacent to Sox2 positive brain cells, suggesting that these ECs and brain cells are derived from the same progenitor cells (Bostrom et al., 2018). Knockdown of Sox2 changes the alternating temporal coordination between neuronal and endothelial differentiation (Yao et al., 2019b). Excess Sox2 signaling is also found to disrupt the transcriptional landscape of cerebral-endothelial differentiation and cause cerebral arteriovenous malformation (AVMs) (Yao et al., 2019a).

Notch signaling is essential for angiogenesis and vascular homeostasis. The Notch ligands interact with the Notch receptors to generate Notch intracellular domains (NICD), which translocate into the nuclei. The NICDs work together with recombination signal binding protein for immunoglobulin kappa J (RBPJκ) to recruit other Notch-associated chromatin-modifying proteins and form complexes that regulate expression of target genes. Previous studies have identified a number of Notch-associated chromatin-modifying proteins, including Mastermind (MAM) (Wu et al., 2000), Silencing mediator of retinoic acid and thyroid hormone receptor (Kao et al., 1998), nuclear receptor co-repressor (Kao et al., 1998), CBF1 interacting corepressor (Hsieh et al., 1999), SMRT/HDAC1 associated repressor protein (Oswald et al., 2002), LIM-only protein (Taniguchi et al., 1998), and Ski-interacting protein (Skip) (Zhou et al., 2000). These Notch-associated proteins act as either a co-activator or suppressor of transcriptional activation of Notch targets (Hsieh et al., 1999; Kao et al., 1998; Oswald et al., 2002; Taniguchi et al., 1998; Wu et al., 2000; Zhou et al., 2000).

Previous studies show that bone morphogenetic protein (BMP) 6 specifically induces the Notch 1 receptor and the Notch ligands Jagged 1 and 2 in brain ECs (Wu et al., 2019). The induction of Notch signaling, in turn, increases Sox2 expression in cerebral AVMs (Wu et al., 2019). Here, we determine the specificity of endothelial Sox2 induction by examining cerebral and pulmonary AVMs, and show a distinct role of Skip in the expression of Sox2 in cerebral AVMs.

RESULTS:

Induction of Sox2 in cerebral AVMs but not in pulmonary AVMs

In a previous study, we showed that the induction of Sox2 by Notch signaling gave rise to endothelial-mesenchymal transitions and caused lumen disorder in cerebral AVMs (Yao et al., 2019a). To determine whether Sox2 also plays a role in AVM formation in other organs, we examined the expression of Sox2 and components of the Notch pathway in lesions of human cerebral and pulmonary AVMs. Real-time PCR revealed that the expression of Sox2 and the Notch ligands Jagged 1 and 2 was increased in cerebral AVMs, but not in pulmonary AVMs (Figure 1a–b, left). Undetectable Sox2 in pulmonary AVMs was confirmed by

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immunostaining, which showed that Sox2 was only induced in cerebral ECs (Figure 1a–b, right). The results suggest that Sox2 induction occurs specifically in cerebral AVMs in response to excessive Notch signaling.

Limiting Sox2 improves cerebral AVMs but not pulmonary AVMs

We previously used matrix Gla protein ($Mgp^{-/-}$) mice as models for AVMs. In these mice, severe AVMs are observed in many organs including the brain and the lungs (Yao et al., 2011; Yao et al., 2013b), and induction of Sox2 is central in causing lumen disorder and cerebral AVMs (Yao et al., 2019a). However, when we limited Sox2 expression in endothelium of $Mgp^{-/-}$ mice, we found that it only reduced cerebral AVMs without affecting the AVMs in the $Mgp^{-/-}$ lungs (Yao et al., 2019a). In this study, we isolated ECs from $Mgp^{-/-}$ brain and lungs and examined the expression of Sox2 and Jagged 1 and 2. We also determined the expression of VEGF in the brain and pulmonary tissues. The results showed a specific induction of Sox2 in the $Mgp^{-/-}$ brain ECs with an increase in Jagged 1 and 2, but no induction of these three factors in the $Mgp^{-/-}$ lung ECs (Figure 2a–c). On the contrary, VEGF was induced only in the $Mgp^{-/-}$ lungs, and not in the brain (Figure 2d). The results support the specificity of Sox2 induction in cerebral AVMs.

Less alteration of Sox2 DNA-binding in pulmonary AVMs

To determine the difference in Sox2 transcriptional activity in lungs and brain, we performed chromatin immunoprecipitation with parallel DNA sequencing (ChIP-seq) in the brain and lung ECs isolated from $Mgp^{+/+}$ and $Mgp^{-/-}$ mice. The genomic DNA that bound to Sox2 was immunoprecipitated by using specific Sox2 antibodies, applied to the library preparation and sequenced. We analyzed data using $Mgp^{+/+}$ cells as control. The results revealed a distinct difference in Sox2 DNA-binding between the brain and the lungs. The Sox2-enriched DNA-binding changed significantly in the $Mgp^{-/-}$ brain ECs, but not in the $Mgp^{-/-}$ lung ECs (Figure 2e–f). The pattern of Sox2 DNA-binding in $Mgp^{-/-}$ pulmonary ECs is also similar to $Mgp^{+/+}$ pulmonary ECs (Figure 2f), supporting that the transcriptional activity of Sox2 was not altered in the pulmonary AVMs.

Skip is essential for Notch to induce Sox2 in cerebral AVMs

We found that Sox2 and Jagged 1 and 2 were increased in cerebral AVMs, but not in pulmonary AVMs (Figure 1). To determine if lack of Sox2 response in pulmonary AVMs was due to lack of induction of Notch signaling, we treated human pulmonary arterial endothelial cells (HPAECs) and human brain microvascular endothelial cells (HBMECs) with Jagged 1 and 2. The results showed an induction of Sox2 in HBMECs, but not in HPAECs as determined by real-time PCR and immunoblotting (Figure 3a–b). The results reveal that Sox2 does not respond to excess Notch ligands in lung ECs, suggesting that additional factors may be required to trigger Sox2 induction in brain ECs.

Therefore, we examined the expression of Notch-associated chromatin-modifying proteins in ECs of $Mgp^{-/-}$ lungs and brain. We found that the Notch-associated chromatin-modifying protein Skip was highly expressed in brain ECs, but exceptionally low in lung ECs (Figure 4a). Using co-immunoprecipitation, we examined interactions between NICDs and the Notch-associated chromatin-modifying proteins RBPJκ, MAM and Skip in ECs of $Mgp^{-/-}$

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lungs and brain. The results revealed that NICD1 bound to RBPJκ and MAM in both ECs of *Mgp*$^{-/-}$ lungs and brain (Figure 4b). However, the complex of RBPJκ and MAM was only able to recruit Skip in *Mgp*$^{-/-}$ brain ECs, not in lung ECs (Figure 4b).

To further determine the role of Skip in transcriptional regulation of Sox2 in brain versus lung ECs, we examined HBMECs and HPAECs and again found the extremely low Skip expression in HPAECs (Figure 5a). We then preformed two experiments. First, we treated HBMECs and HPAECs with Jagged 1 or 2 and reduced Skip by using siRNA (Figure 5b–c). Second, we treated HBMECs and HPAECs with Jagged 1 or 2 and restored Skip expression by using Skip-expressing lentivirus (Figure 5d–e). The result showed that knockdown of Skip abolished Jagged1 or 2-induced Sox2 in HBMECs, while restoration of Skip in HPAECs enabled Jagged1 or 2 to induce Sox2 expression (Figure 5f–g). The results suggest that Skip is required to interact with other Notch-associated chromatin-modifying proteins for Sox2 induction to occur in response to Notch signaling (Figure 6).

DISCUSSION

AVMs appear in many organs, such as lung, kidneys, liver, intestines, skin, retina, and brain (Shovlin; Yao et al., 2019a; Yao et al., 2011; Yao et al., 2013b). Different mechanisms appear to drive AVM formation in different organs (Yao et al., 2019a; Yao et al., 2011). For example, excess VEGF induced by BMPs contributes to AVMs in lungs and kidneys (Yao et al., 2011) whereas dysregulated Notch signaling induces brain AVMs (Murphy et al., 2008; Yao et al., 2019a). In patients with hereditary hemorrhagic telangiectasia (HHT), AVMs can occur in any locations that may differ between individuals with same gene mutations. This suggests that the local conditions is another driving force in the AVM formation (Govani and Shovlin, 2009; Shovlin).

Here, we show that Sox2 expression induced by Notch signaling occurs in cerebral AVMs, but not in pulmonary AVMs. The abnormal emergence of Sox2 causes endothelial-mesenchymal transitions and lumen disorder in cerebral AVMs (Yao et al., 2019a). Normally, Sox2 is expressed in endothelial progenitors in early brain development (Bostrom et al., 2018), and co-ordinate endothelial and neuronal differentiation (Yao et al., 2019b). The expression of endothelial markers alternates with neuronal markers in an on-off-on-off pattern during the neuronal differentiation (Yao et al., 2019b). Disruption of the Sox2 expression changes this pattern to on-on-off-off and derails the ordination between endothelial and neuronal differentiation (Yao et al., 2019b). Previous studies also support a connection between Sox2 and Notch signaling, both of which regulate cell fate or modulate cell-cell transitions (Mandalos et al., 2014; Zakharova et al., 2012), and interact in developmental process, such as specification of the sensory progenitors (Pan et al., 2013), differentiation of neural stem cells (Ehm et al., 2010), and cochlear development (Liu et al., 2012).

We find that the regulation of Sox2 expression in brain ECs has a unique mechanism compared with lung ECs. We identify the Notch-associated protein Skip as a key component for Notch to induce Sox2, and show that the presence of Skip determines the specificity of Sox2 induction. Skip is co-transcription factor that is involved in various signaling

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pathways. Skip binds to SMADs in TGFβ/BMP pathway (Xu et al., 2000), interacts with poly(A)-binding protein 2 in muscle development (Kim et al., 2001), acts as a repressor with FoxN3 (Scott and Plon, 2005), and functions as a splicing factor to control apoptosis (Bres et al., 2005). Our results added more functions to Skip and show that recruitment of Skip together with other Notch-associated proteins is essential for the induction of Sox2 in cerebral AVMs. The expression of Skip is detected uniquely in the brain ECs, but not in lung ECs, which points to differences in the mechanisms of cerebral AVMs and lung AVMs (Figure 6). It also opens the possibility of targeting cerebral AVMs without disrupting the pulmonary circulation.

METHODS

Animals

Mgp$^{+/-}$ mice (B6.129S7-Mgptm1Kry/KbosJ) on C57BL/6J background were obtained from the Jackson's laboratory. Genotypes were confirmed by PCR (Yao et al., 2012), and experiments were performed with generations F4-F6. Littermates were used as wild type controls. All mice were fed a standard chow diet (Teklad Rodent Diet 8604, Envigo, Placentia, CA). The use of animals and all the experimental procedures were reviewed and approved by University of California Los Angeles (UCLA) Chancellor's Animal Research Committee and conducted in accordance with the animal care guidelines set by UCLA. 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).

The lesions of human cerebral AVMs

De-identified specimens will be obtained from the Department of Pathology, David Geffen School of Medicine at UCLA. The specimens were not obtained specifically for this research, and none of the investigators involved in the research were able to ascertain the identities of the subjects. Human Subjects Research Exemptions were approved by Office of the Human Research Protection Program at UCLA.

Tissue culture

HBMECs were obtained from ScienCell Research Laboratories (San Diego, CA). HPAECs were obtained from ThermoFisher Scientific (Waltham, MA). Both cell lines were cultured as per manufacturer's protocol. For treatment, Jagged1 and 2 (R&D Systems, Minneapolis, MN) were added as indicated in the results section. Transient transfections with Skip siRNA (Silencer® predesigned siRNA, Applied Biosystem, Foster City, CA) were optimized and performed as previously described (Yao et al., 2011). When compared with unrelated control siRNA and scrambled siRNA, the selected siRNAs resulted in an 90%–95% decrease in mRNA and protein levels, as determined by real-time PCR and immunoblotting, respectively.

RNA analysis

Real-time PCR analysis was performed as previously described (Bostrom et al., 2004). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control gene (Bostrom

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et al., 2004). Primers and probes for mouse or human Jagged 1, Jagged 2, VEGF, Sox2, RBPJκ, MAM and Skip were obtained from Applied Biosystems as part of TaqMan Gene Expression Assays.

Immunoblotting

Immunoblotting and immunoprecipitation were performed as previously described (Yao et al., 2013a). Equal amounts of tissue lysates were used for immunoblotting. Blots were incubated with specific antibodies to Sox2, RBPJκ and MAM (Abcam, Cambridge, England). β-Actin (Sigma-Aldrich, St. Louis, MO) was used as a loading control.

ChIP-seq

ChIP was performed as previous described (Yao et al., 2019a). Specific anti-Sox2 antibodies (Abcam) were used to perform ChIP in order to enrich the genomic DNA. ChIP DNA was sequenced by Technology Center for Genomics & Bioinformatics at UCLA. Reads from each sample were mapped to human genome by using Bowtie2. The Homer tool was used to detect significant enrichment of peaks with 5% false discovery rate and more than four folds over input. Motif occurrences in peaks were identified by the homer motif discovery function. Peak annotation was performed to associate peaks with nearby genes, and calculate tag densities.

Statistical analysis

The analyses were performed using GraphPad Instat®, version 3.0 (GraphPad Software). Data were analyzed by either unpaired 2-tailed Student's t test or one-way ANOVA with Tukey's multiple-comparisons test for statistical significance. Data represent mean ± SD. P-values less than 0.05 were considered significant, and experiments were repeated a minimum of three times.

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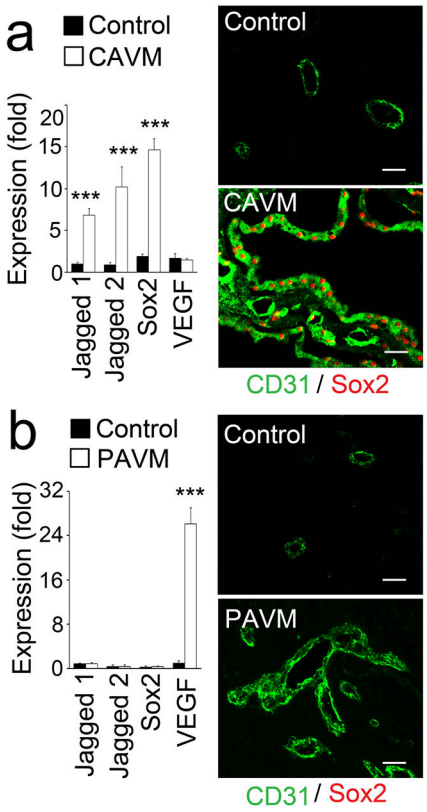

Figure 1. Increased Sox2 in cerebral AVMs but not in pulmonary AVMs

(a-b) Gene expression of Jagged 1 and 2, Sox2, and VEGF in human cerebral AVM (CAVM) and pulmonary AVM (PAVM), as determined by real-time PCR (left). The immunostaining of Sox2 and the endothelial marker CD31 shows that Sox2 emerges in the ECs of cerebral AVM, but not in pulmonary AVM (right).

***, P<0.001. Scale bar: 50 μm.

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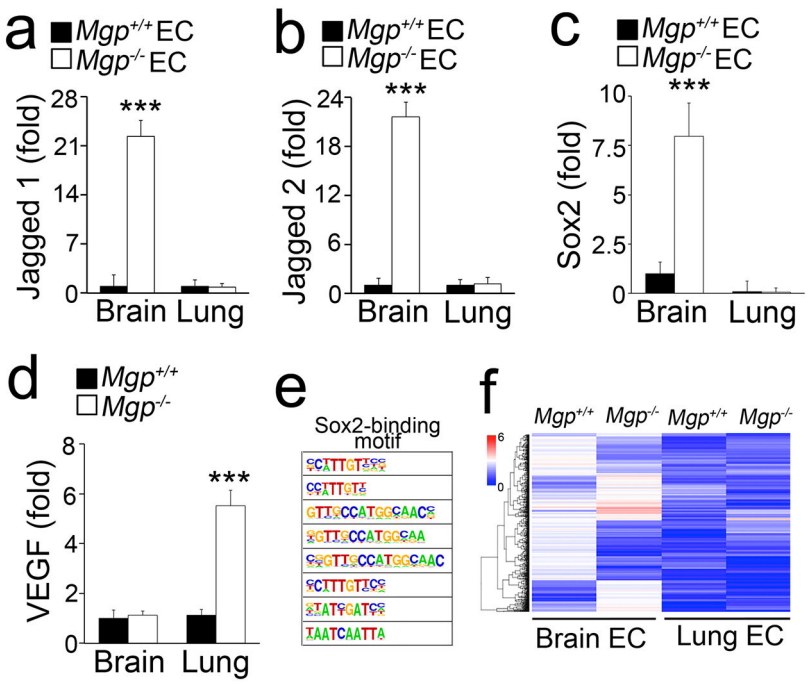

Figure 2. Notch and Sox2 are induced in brain ECs but not in lung ECs in Mgp$^{-/-}$ mice, leading to different alterations in Sox2 DNA-binding

(a-c) Expression of Jagged1 and 2 and Sox2 in ECs isolated from *Mgp*$^{-/-}$ brain and lungs.

(d) Expression of VEGF in *Mgp*$^{-/-}$ brain and lungs.

(e) The motifs used for the analysis of Sox2 ChIP-seq.

(f) Heat map of genes with alteration of Sox2 DNA-binding in the ECs isolated from *Mgp*$^{-/-}$ brain and lungs.

***, P<0.001.

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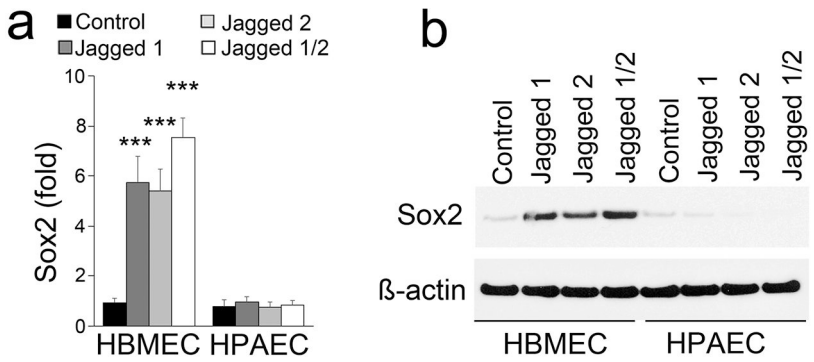

Figure 3. Notch does not induce Sox2 in HPAECs

(a-b) Sox2 expression in HBMECS and HPAECs after treatment with Jagged 1, or 2, or the combination of both detected by real-time PCR (left) and immunoblotting (right).

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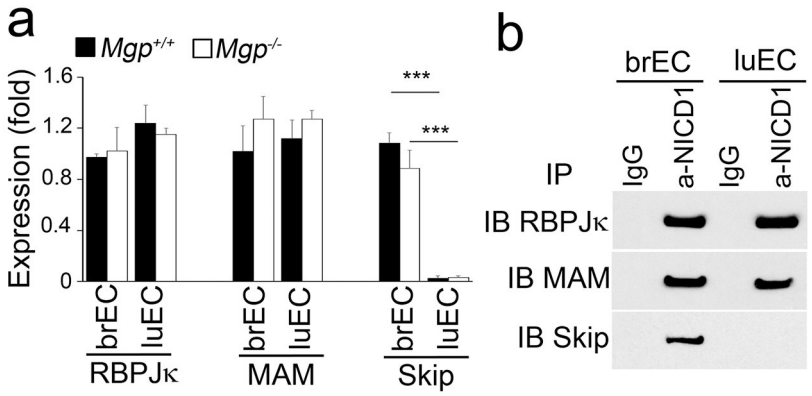

Figure 4. Low expression of Skip identified in ECs isolated from brain and lungs

(a) Expression of RBPJκ, MAM and Skip in the ECs isolated from *Mgp*$^{-/-}$ brain and lungs. ***, P<0.001.

(b) Immunoprecipitation of lysed ECs isolated from *Mgp*$^{-/-}$ brain and lungs using anti-NICD1 antibodies followed by immunoblotting using anti- RBPJκ, MAM and Skip antibodies.

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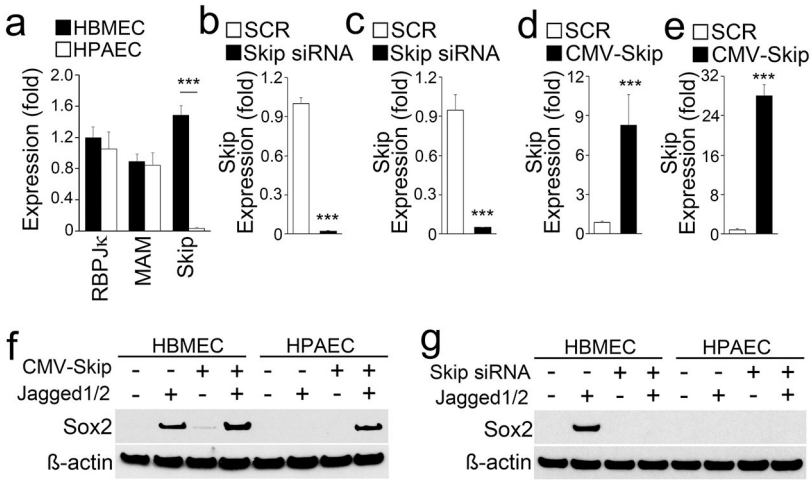

Figure 5. Skip is essential for Notch to induce Sox2

(a) Expression of RBPJκ, MAM and Skip in HBAECs and HPAECs.

(b-e) Expression of Skip in HBAECs transfected with Skip siRNA (b) or infected with lentiviral vector expressing Skip (d), and HPAECs transfected with Skip siRNA (c) or infected with lentiviral vector expressing Skip (e).

(f) Immunoblotting of Sox2 in HBAECs and HPAECs after transfected with Skip siRNA and treated with or without Jagged1 and 2.

(g) Immunoblotting of Sox2 in HBAECs and HPAECs after infected with lentiviral vector expressing Skip and treated with or without Jagged1 and 2.

***, P<0.001.

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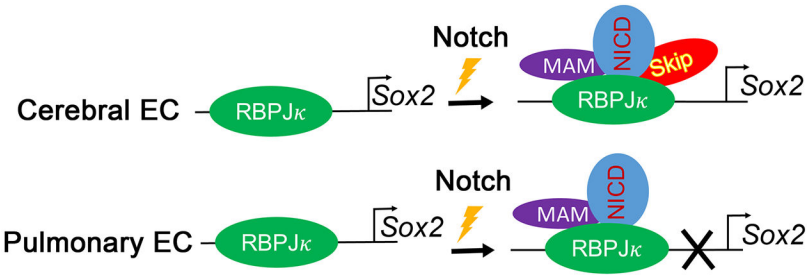

Figure 6.
Schematic diagram showing the role of Skip in the regulation of Sox2 induction by Notch