Spatiotemporal coordination of trophoblast and allantoic Rbpj signaling directs normal placental morphogenesis

Jinhua Lu 1,2, Weiwei Wu 3, Qiliang Xin 4, Chan Zhou 4, Jianqi Wang 1,2, Zhangli Ni 4, Dong Liu 1,2, Yingchun Xu 1,2, Yongqin Yu 1,2, Ningjie Yang 1,2, Yang Sun 1,2, Bo He 1,2, Shuangbo Kong 1,2, Shumin Wang 5, Chao Wang 4 and Haibin Wang 1,2

Abstract
The placenta, responsible for the nutrient and gas exchange between the mother and fetus, is pivotal for successful pregnancy. It has been shown that Rbpj, the core transcriptional mediator of Notch signaling pathway, is required for normal placentation in mice. However, it remains largely unclear how Rbpj signaling in different placental compartments coordinates with other important regulators to ensure normal placental morphogenesis. In this study, we found that systemic deletion of Rbpj led to abnormal chorioallantoic morphogenesis and defective trophoblast differentiation in the ectoplacental cone (EPC). Employing mouse models with selective deletion of Rbpj in the allantois versus trophoblast, combining tetraploid aggregation assay, we demonstrated that allantois-expressed Rbpj is essential for chorioallantoic attachment and subsequent invagination of allantoic blood vessels into the chorionic ectoderm. Further studies uncovered that allantoic Rbpj regulates chorioallantoic fusion and morphogenesis via targeting Vcam1 in a Notch-dependent manner. Meanwhile, we also revealed that trophoblast-expressed Rbpj in EPC facilitates Mash2’s transcriptional activity, promoting the specification of Tpbpα-positive trophoblasts, which differentiate into trophoblast subtypes responsible for interstitial and endovascular invasion at the later stage of placental development. Collectively, our study further shed light on the molecular network governing placental development and functions, highlighting the necessity of a spatiotemporal coordination of Rbpj signaling for normal placental morphogenesis.

Introduction
The placenta is vital to the survival and growth of the fetus before birth, since it provides the fetus with nutrients and gases during pregnancy. Any disturbance to placental development would lead to pregnancy-related complications, such as preeclampsia and intrauterine growth restriction 1–3. In mice, the mature placenta is composed of three distinct cellular layers: the outmost maternal decidua, derived from uterine stromal cells across which the maternal blood comes into the placenta through spiral arteries; the intermediate junction zone, through which the maternal vessels get in and out of the underlying labyrinth layer; the innermost labyrinth layer, highly branching to form large surface areas responsible for nutrient and gas exchanges between the mother and fetus 4. During placental morphogenesis in mice, the allantois derived from the extraembryonic mesoderm and the chorion developed from the extraembryonic ectoderm undergo chorioallantoic fusion at E8.5 to initiate
chorioallantoic branching morphogenesis\textsuperscript{5,6}, during which fetal vessels invaginate into the chorionic plate and branch with the underlying trophoblast cells to form placental labyrinth\textsuperscript{7}. Simultaneously, maternal spiral arteries (SPA) delivering blood to the placenta undergo intensive remodeling, during which the endothelial cells, even regions of the smooth muscle wall, are replaced by invasive trophoblast cells. Moreover, the glycogen trophoblast cells invade interstitially into maternal decidua and form wrapping layers around spiral arteries\textsuperscript{8}, contributing to spiral artery dilation\textsuperscript{9}. These dynamic cellular events coordinately decrease vascular resistance of spiral arteries, thus increasing placental perfusion by maternal blood to meet the increasing needs of the fetus\textsuperscript{10,11}. However, it remains largely unknown how these developmental processes are tightly coordinated to guarantee normal development and functions of the placenta.

Previous studies have demonstrated that a wide range of molecules in development-related signaling pathways is involved in regulation of placental development, including the molecules in Notch signaling\textsuperscript{12–14}. Notch signaling is initiated by the release of the Notch intracellular domain (NICD) when Notch receptors are bound by its specific ligands. When translocated to the nucleus, NICD interacts directly with Rbpj, together with Mastermind-like protein (MAML) to exert transcriptional activity\textsuperscript{15}. Rbpj, the nuclear transducer of Notch signaling, is an important transcription factor highly conserved from \textit{Drosophila} to human\textsuperscript{16}. It has been reported that systemic deletion of Rbpj led to defective chorioallantoic fusion during early placentaion\textsuperscript{17}. However, it remains elusive how Rbpj-mediated signaling directs normal chorioallantoic fusion. Moreover, it is still largely unknown about the precise function of Rbpj signaling during trophoblast specification, due to the early death of \textit{Rbpj}-null mutants.

Employing multiple mouse models, we provided herein molecular and genetic evidence that allantoic Rbpj regulates chorioallantoic fusion and branching morphogenesis via targeting \textit{Vcam1} in a Notch-dependent manner. Meanwhile, trophoblast Rbpj facilitates Mash2’s transcriptional activity to promote specification of \textit{Tpbpa}-positive trophoblasts, which differentiate into trophoblast cells responsible for interstitial and endovascular invasion at the later stage of placental development.

\textbf{Results}

\textbf{Rbpj deficiency derails normal placental development}

Our previous study has demonstrated that uterine Rbpj is essential for normal embryo development via instructing the initial embryonic–uterine orientation and ensuring normal decidual patterning in a stage-specific manner\textsuperscript{18}. To further explore the pathophysiological significance of Rbpj signaling during early placental development, we first performed in situ hybridization to examine the spatiotemporal expression profile of \textit{Rbpj} in extraembryonic tissues. At E8.0, \textit{Rbpj} mRNA expression was detected in the floating allantois and chorion, and also in the ectoplacental cone (EPC), as well as trophoblast giant cells (TGC) (Fig. 1a). With the initiation and progression of chorioallantoic fusion and branching morphogenesis, \textit{Rbpj} expression sustained in chorioallantoic cells at E8.5 followed by expression in placental labyrinth at E9.0–10.5, as well as in EPC and TGCs (Fig. 1a). By quantitative RT-PCR analysis, we confirmed \textit{Rbpj} expressing in isolated allantois and chorion plus EPC (Fig. S1a). The spatiotemporal \textit{Rbpj} expression indicates that Rbpj might play critical roles during chorioallantoic morphogenesis, as well as trophoblast differentiation at the fetal–maternal interface.

To determine the physiological significance of Rbpj during placental development, we employed mouse models with systemic \textit{Rbpj} deletion (\textit{Rbpj}\textsuperscript{−/−}) achieved by crossing \textit{Rbpj}\textsuperscript{f/f}\textit{Zp3Cre}\textsuperscript{−/−} female mice with \textit{Rbpj}\textsuperscript{f/f}\textit{PrmCre}\textsuperscript{−/−} male mice. At E9.5, \textit{Rbpj}\textsuperscript{−/−} embryos exhibited growth-arrested and possessed pale yolk sacs with disorganized blood vessels, as well as few fetal blood cells (Fig. S1b), consistent with previous observations\textsuperscript{17}. In \textit{Rbpj}\textsuperscript{f/f} conceptus, the allantois (Al, white arrowhead) attached to the chorion normally at E8.5 followed by formation of the umbilical artery and vein at E10.5. However, \textit{Rbpj}\textsuperscript{−/−} mutants exhibited defective chorioallantoic attachment and fusion with the allantois (red arrowheads) failing to fuse with the chorion and appearing as a bud at E8.5 and even E9.5, leading to fetal death (Fig. 1b). HE and laminin staining further revealed that Rbpj deletion resulted in defective chorioallantoic fusion and shallow invagination of allantoic blood vessels into the chorionic ectoderm, leading to impaired chorioallantoic branching morphogenesis and labyrinth development, with no or less fetal blood vessels appearing in the placental labyrinth at E10.5 (Fig. 1c and S1c, d). These findings indicate that Rbpj deficiency derails normal chorioallantoic fusion and branching morphogenesis during placentaion.

\textit{Rbpj} in allantois is indispensable for normal chorioallantoic fusion

Chorioallantoic attachment and branching morphogenesis require intimate interaction between chorionic trophoblast and allantoic cells\textsuperscript{2}. Since Rbpj was expressed in both the allantois and chorion, to ascertain the respective contribution of trophoblast versus allantoic Rbpj to chorioallantoic fusion and branching, we employed the trophoblast-specific \textit{Cyp19}\textsuperscript{+} mouse model. In order to achieve efficient Rbpj depletion, one allele of Rbpj was first deleted systemically and then the other was deleted via \textit{Cyp19}\textsuperscript{+}. As shown in Fig. S2a–c, Rbpj expression was deleted specifically and efficiently in trophoblast cells, not in fetal cells (\textit{Rbpj}\textsuperscript{−/−}/\textit{Cyp19}\textsuperscript{+}).
placenta). Upon trophoblast-specific Rbpj deletion, chorioallantoic attachment and fetal vascular development occurred normally in Rbpjf/f and Rbpjf−/− placenta, comparable with that of the controls (Rbpjf/f and Rbpjf−/−) (Fig. 2a, b). These observations suggest that Rbpj located in the chorionic trophoblast is not required for chorioallantoic fusion and branching morphogenesis.

After chorioallantoic fusion at E8.5, chorionic trophoblast cells coordinate with the allantoic mesoderm and the underlying fetal vessels to initiate primary branching in the chorioallantoic placenta. We then analyzed the expression of marker genes involved in chorionic trophoblast differentiation in Rbpjf/f and Rbpjf−/− placentas with chorioallantoic fusion. At E9.0, Gcm1, which defines branching points and promotes chorionic trophoblast differentiation into syncytiotrophoblast layer II (SynT-II)19,20, was expressed normally at branching points in both Rbpjf/f and Rbpjf−/− placentas. Accordingly, the expressions of Synb and Cebpα, downstream targets of Gcm1 and markers for SynT-II, were comparable in both Rbpjf/f and Rbpjf−/− placentas (Fig. 2c and S2g). Moreover, the expression of Syna localized to SynT-I21, was not disturbed by Rbpj deletion (Fig. 2c and S2g). These results reinforce the notion that trophoblast-expressed Rbpj is dispensable for chorioallantoic trophoblast differentiation and branching morphogenesis.

Based on the aforementioned observations, we surmised that allantois-expressed Rbpj could be a vital player directing normal chorioallantoic fusion. To test this hypothesis, we first performed tetraploid aggregation assay, via which the Rbpj−/− embryos were aggregated with wild-type tetraploid embryos with EGFP expression (Fig. 3a).

Tetraploid cells contribute exclusively to trophoblast cells of the placenta and endoderm of the yolk sac, rather than to allantois. As illustrated in Fig. 3b, c, while the EGFP-expressing cells derived from wild-type tetraploid embryos contributed exclusively to Rbpjf/f placentas, as well as Rbpjf−/− placenta at E10.5, impaired chorioallantoic fusion and branching were not rescued in the chimera conceptus. These findings clearly demonstrate that allantois-expressed Rbpj is essential for normal chorioallantoic fusion and branching morphogenesis. To verify this finding, we employed Sox2cre/+ mouse model to delete Rbpj selectively in the epiblast, and therefore in extraembryonic mesoderm-derived allantois, as well as fetal vessels in the placenta22 (Fig. S3a, b). While growth arrest and defective chorioallantoic morphogenesis were observed in Rbpjf/f/Sox2 cre/+ placenta (Fig. 3d and S3c), the expression of markers involved in chorionic trophoblast differentiation was unaffected (Fig. 3e). These data further reinforce the notion that allantois-expressed Rbpj is essential for normal chorioallantoic fusion and branching morphogenesis.
Rbpj directs allantoic Vcam1 expression in a Notch-dependent manner

Chorion–allantois fusion is based on cell–cell contact that is mediated by a variety of cell adhesions and signaling molecules. The vascular cell adhesion molecule-1 (Vcam1), expressed on the distal two-thirds of allantois, and α4 integrin (Itga4) localized to the chorionic basal side, lie at a pivotal step during chorioallantoic attachment and fusion, since deletion of each led to defective chorioallantoic fusion. To search for the underlying molecular basis intrinsic to allantoic defects of Rbpj mutants, we examined the expression of Itga4 and Vcam1 in Rbpjf/f and Rbpj−/− placentas. While Itga4 expression was detectable in both the Rbpjf/f and Rbpj−/− basal chorions, the expression of Vcam1 almost disappeared in Rbpj−/− placentas at E8.5 and E9.5 (Fig. 4a, b). These findings suggest a potentially functional association between Rbpj and Vcam1 during chorioallantoic attachment and branching. However, it remained unclear how allantoic Rbpj ensures normal Vcam1 expression during early placentation.

Since Rbpj is a transcriptional factor, we speculated that Vcam1 could be a direct target gene of Rbpj. In order to test this hypothesis, we searched the promoter region of Vcam1 and found two prospective binding sites of Rbpj (Fig. 4c), located at −1006 to −1000 bp (VP-BS1) and
−1824 to −1818 bp (VP-BS2) upstream of the transcription start site (TSS), respectively. Chromatin immunoprecipitation (ChIP) assay using antibodies against Rbpj revealed an enriched accumulation of Rbpj at the two binding sites in E8.5 placenta (Fig. 4d). Luciferase assay further revealed that VP-BS1 was responsive to Rbpj overexpression, while its mutant form abrogating the conserved Rbpj-binding sequence (mVP-BS1) failed to respond to Rbpj signaling (Fig. 4e). Since Notch1 and Notch2 were expressed in allantois (Fig. S4) and the phenotype of Notch1-deficient mice was similar to that of Rbpj-deficient mice, we thus surmised that Notch1 and Notch2 could be the prospective receptors driving Rbpj signaling to regulate Vcam1 expression during chorioallantoic fusion. Indeed, luciferase activity driven by Vcam1 promoter was increased prominently when N1ICD (Notch1) and N2ICD (Notch2) were cotransfected with Rbpj, respectively (Fig. 4e). The results suggest that
Rbpj signaling ensures normal chorioallantoic fusion and branching morphogenesis via directly inducing Vcam1 expression in a Notch-dependent manner.

To further confirm that the participation of Rbpj signaling in chorioallantoic morphogenesis is driven by canonical Notch activation, we employed DNMAML$^{+/+}$/Prtm$^{m+/+}$ and DNMAML$^{+/+}$/Cyp19$^{pro+/+}$ mouse models with haploid overexpression of dominant negative MAML (DNMAML), which blocks canonical Notch signaling. As shown in Fig. 5a, defective chorioallantoic development was observed in

![Image](image_url)
DNMAML$f^{+/+}$/Prmcre$^{+/+}$ placentas, similar to that of Rbpj$^{-/-}$ placentas. Most importantly, Vcam1 expression was almost diminished in DNMAML$f^{+/+}$/Prmcre$^{+/+}$ allantois at E8.5, while Itga4 was expressed normally (Fig. 5b). Moreover, as expected, normal labyrinth formation was observed in DNMAML$f^{+/+}$/Cyp19cre$^{+/+}$ placenta, in which DNMAML functions specifically in trophoblast cells (Fig. 5c), consistent with the recent findings, further demonstrating that Rbpj-mediated canonical Notch signaling in chorionic trophoblast cells is dispensable for chorioallantoic attachment and branching. These data provided a new line of genetic evidence that allantoic Rbpj directs Vcam1 expression in a Notch-dependent manner.

Rbpj deficiency attenuates specification of Tpbpa-positive trophoblast in EPC

Regardless of the dispensability of Rbpj signaling in chronic trophoblast differentiation, we eventually looked into the significance of Rbpj during ektoplacental cone development, since its expression was persistent in EPC and TGCs at E8.0–10.5 (Fig. 1a). We analyzed mRNA expression of marker genes important for EPC development. The expression of heart and neural crest derivatives expressed transcript 1 (Hand1), an essential regulator of TGC specification, was comparable between Rbpj$f^{+/−}$ and Rbpj$^{-/-}$ placentas (Fig. 6a). This observation well correlated with the normal differentiation of Pl1-expressing TGCs in Rbpj$^{-/-}$ placentas (Fig. 6a). By contrast, while the expression of achaete–scute complex homolog-like 2 (Ascl2, also Mash2), known to stimulate specification of the spongiotrophoblast lineage, was not disturbed, the population of trophoblast-specific protein α (Tpbpa)-positive cells was decreased significantly by Rbpj deletion (Fig. 6a). These findings suggest that Rbpj deletion attenuates specification of Tpbpa-positive trophoblast in EPC.

Since Tpbpa-positive trophoblast progenitors could differentiate into spongiotrophoblast cells, glycogen...
trophoblast cells, as well as endovascular TGCs that invade into the maternal decidua and SPA, respectively, beyond mid-gestation\textsuperscript{31,32}, we wonder whether attenuated specification of Tpbp\textsubscript{α}-positive trophoblast would hamper the functions of mature placenta, particularly trophoblast invasion into the maternal decidua and SPA. Indeed, the E13.5 fetuses with Rbpjf\textsuperscript{−/−}/Cyp19\textsuperscript{cre/+} placenta were a little pale, compared with those of control placentas (Fig. S5a). Moreover, the weight of the fetuses with Rbpjf\textsuperscript{−/−}/Cyp19\textsuperscript{cre/+} placenta was decreased significantly, while the placental weight was unaffected (Fig. S5b). We next analyzed the differentiation of Tpbp\textsubscript{α}-positive trophoblast in E13.5 placentas. As illustrated in Fig. 6b, Tpbp\textsubscript{α}-positive spongio trophoblast population was reduced significantly upon Rbpj deficiency (Rbpjf\textsuperscript{−/−}/Cyp19\textsuperscript{cre/+}). More importantly, the population and invasion of glycogen trophoblasts expressing Prl7b1 and Pcdh12 were reduced substantially in Rbpjf\textsuperscript{−/−}/Cyp19\textsuperscript{cre/+} placentas (Fig. 6b). Moreover, the differentiation and invasion of Prl7b1-positive and Plf-positive TGCs were disturbed by Rbpj deletion (Fig. 6b). These results indicate that intact Rbpj signaling is essential for normal specification of Tpbp\textsubscript{α}-positive trophoblast cells.

**Rbpj facilitates Mash2 to promote specification of Tpbp\textsubscript{α}-positive trophoblast**

Previous evidence disclosed that Notch signaling regulated Mash2 through hairy/Enhancer of split (Hes) genes and Groucho genes (Tle) in Drosophila\textsuperscript{33,34}, implying a potential coupling of Notch signaling with Mash2 during the specification of Tpbp\textsubscript{α}-positive cells. To reveal the underlying molecular mechanism governing normal specification of Tpbp\textsubscript{α}-positive trophoblast driven by Rbpj signaling, we further employed cultured trophoblast stem (TS) cell lines (Fig. S6a). Rbpj deletion did not perturb the stemness of trophoblast cells, since Rbpj\textsuperscript{−/−} trophoblast stem cells have been established successfully. We found that while the expression of Mash2 was not disturbed, Tpbp expression was decreased significantly, but not disappeared in Rbpj-deficient TS cells undergoing default differentiation in culture (Fig. 7a), consistent with in vivo observations (Fig. 6a). In addition, Mash2 knockdown by
siRNA almost diminished Tpbpa expression (Fig. 7b), similar to the findings in Mash2 knockout mice. These findings suggest that Rbpj might facilitate the functions of Mash2 during specification of Tpbpa-positive trophoblast. Co-immunoprecipitation analysis further revealed physical interaction between Mash2 and Rbpj in trophoblast cells (Fig. 7c). Since Rbpj and Mash2 are both transcription factors, we performed ChIP assay using antibodies against Mash2 and Rbpj, respectively. We found an enriched accumulation of both Mash2 and Rbpj in a trophoblast-specific regulatory region of Tpbpa, a 340-bp region that is 3.7 kb upstream of the TSS of Tpbpa, in both E8.5 placenta (Fig. 7d) and TSC (Fig. 7e). These data demonstrate a functional association of Rbpj and Mash2 during the specification of Tpbpa-positive trophoblast cells.

Regarding the physiological significance of Mash2-Rbpj interaction, we employed luciferase assay to assess the activity of Tpbpa promoter in both HEK 293 T cells and TS cells. While Mash2 increased luciferase activity significantly, Rbpj alone exhibited no influence on luciferase activity in HEK 293 T cells (Fig. 7f), which have extremely low expression of endogenous Mash2. Importantly, co-transfection of Mash2 and Rbpj increased the activity of Tpbpa promoter (Fig. 7f). In consistence, a similar phenomenon was observed in cultured TS cells undergoing differentiation; besides that, Rbpj alone was able to promote the activity of Tpbpa promoter (Fig. 7g), since TS cells expressed endogenous Mash2. Moreover, employing in silico analysis of the trophoblast-specific regulatory region of Tpbpa, we found the binding site of Mash2, but not that of Rbpj. When the binding site of
Mash2 was deleted, the activity of Tpbra promoter increased by Rbpj and Mash2 was decreased significantly (Fig. 7f, g). In addition, while endogenous Tpbra expression was promoted by Rbpj alone in the presence of endogenous Mash2, its expression was further increased synergistically by Mash2 and Rbpj overexpression in differentiated trophoblast cells (Fig. S6b, c). These data demonstrate that Rbpj facilitates Mash2 to promote the specification of Tpbra-positive trophoblast.

Discussion

Placenta acts as the interface between the mother and fetus for the exchanges of nutrients, gases, and wastes. During placental development, fetal vessels grow into the labyrinth via chorioallantoic branching morphogenesis. Simultaneously, the maternal blood enters the placenta through SPA remodelled by TGCs and facilitated by invasive glycogen trophoblast cells, and forms blood sinus in the placental labyrinth layer. We provided herein genetic and molecular evidence that allantoic Rbpj guides normal chorioallantoic fusion and branching morphogenesis by targeting Vcam1, while trophoblast Rbpj facilitates the functions of Mash2 during specification of Tpbra-positive trophoblast, which are important for the differentiation and invasion of SPA-TGCs and glycogen trophoblast cells during placental development (Fig. S7). These findings reinforce the content that allantoic Notch-Rbpj-Vcam1 signaling is also essential for remodeling of placental fetal vessels beyond the initial fusion7,24,37. In addition, Dll4 was reported to be expressed in fetal vascular endothelial cells of allantois and subsequent umbilical arteries. Haploid insufficiency of Dll4 exhibits similar phenotypes to that of Rbpj-null mice38–40, suggesting that Dll4 is a potential upstream driver of Rbpj-Vcam1 signaling governing normal vascular remodeling and the maintenance of fetal vessels during chorioallantoic morphogenesis.

During pregnancy, maternal blood flow through the placenta increases dramatically, largely due to significant vasodilation of blood vessels10. In mice, TGCs invading utero-placental SPA and glycogen trophoblast cells invading interstitially into the decidua, are associated with

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**Fig. 8** Diagram illustrating the functions of RBPJ-mediated signaling during chorioallantoic development and trophoblast differentiation in EPC (Sp). In the presence of Rbpj (with Rbpj), Rbpj regulates the expression of Vcam1 in the allantois through a Notch-dependent manner to ensure normal chorioallantoic branching morphogenesis, and facilitated Mash2 in EPC (Sp) to promote the specification of Tpbra-positive trophoblast cells simultaneously. However, failed chorioallantoic branching and defective trophoblast differentiation in EPC (Sp) were observed in the absence of Rbpj (without Rbpj). Dec decidual basalis, Sp spongiotrophoblast layer, SPA spiral arteries.
the remodeling of maternal SPA into dilated, inelastic tubes to increase maternal blood flow to the placenta. It has been shown that TGCs and glycogen trophoblast cells are differentiated from progenitor cells localized within the ectoplacental cone, as well as the spongiotrophoblast layer later, and expressing spongiotrophoblast-specific gene Tpbpa. In our work, we found that decreased Tpbpa expression by Rbpj deletion led to defective invasion of SPA-TGCs and glycogen trophoblast cells. This may help explain why fetal weight was not perturbed at E10.5, but was decreased at E13.5, in the presence of trophoblast-specific Rbpj deletion. Fetal weight at E10.5 was not disturbed, maybe due to normal development of placental labyrinth after Rbpj deletion. However, with rapid fetal growth since E10.5, the fetuses failed to get enough nutrients to meet their increasing needs, might attribute to impaired remodeling of maternal SPA and insufficient placental perfusion upon Rbpj deletion, leading to decreased fetal weight at E13.5.

Although it has been reported that Mash2 is a key determinant for specification of Tpbpa-positive trophoblast cells, the mechanism controlling specialization of Tpbpa-positive cells remains largely unknown. In our study, we demonstrated that Rbpj facilitates Mash2 to promote Tpbpa expression and specialization of Tpbpa-positive cells. These findings provide a new line of evidence regarding the regulation of Tpbpa-positive trophoblast cells specification. Moreover, the findings that decreased Tpbpa expression upon Rbpj deletion led to defective invasion of SPA-TGCs and glycogen trophoblast cells are similar to the phenotypes of mice with conditional deletion of Notch2 via Tpbpa-Cre. Although Notch2 is involved in the process during which the positive cells differentiate into invasive SPA-TGCs and glycogen trophoblast cells, it seems that Notch2 does not participate in Rbpj-mediated signaling responsible for specification of Tpbpa-positive trophoblast cells in EPC, since the population of Tpbpa-positive cells was not disturbed in Notch2-deficient placentas. Moreover, it has been shown that Rbpj could also function in a Notch-independent manner, and trophoblast-specific inhibition of canonical Notch signaling did not disturb placental development. We also found that Rbpj had no binding sites in the trophoblast-specific regulatory region of Tpbpa. Therefore, Rbpj-mediated signaling might regulate specification of Tpbpa-positive trophoblast cells in a noncanonical manner. In addition, it has been reported that trophoblast cells expressing Prdm/Blimp1 gave rise to SPA/endoovascular TGCs, canal TGCs, and glycogen trophoblast cells, and Tpbpa expression was unaffected in the absence of Blimp1, suggesting that Blimp1 might function after the specification of Tpbpa-positive trophoblast cells, and Rbpj signaling may also regulate specification of Blimp1-positive cells.

In summary, our study further shed light on the molecular network governing placental development and functions, and the findings might have clinical relevance, since abnormal Notch signaling contributes to pathogenesis of gestational diseases with aberrant placentation and trophoblast function in women.

**Materials and methods**

**Animals**

RbpjloxP/loxP (Rbpjfl/fl) mice, DNMAMLf/f mice, and Cyp19fcre/+ transgenic mice were generated as previously described. Enhanced green fluorescent protein (Egfp)Tgg/+, Rosa26-Laczfl/fl, Prm3cre/+Zp3cre/+, and Sox2cre/+ transgenic mice were obtained from Jackson Laboratory. Rbpjfl/fl mice were mated with Zp3Cre/+, and Prm3cre/+ to achieve systemic Rbpj deletion. Rbpjfl/fl mice were mated with Sox2Cre/+ and Cyp19fcre/+ transgenic mice to get conditional deletion of Rbpj in the epiblast and trophoblast derivates, respectively. DNMAMLf/f mice were mated with Prm3Cre/+ transgenic mice and Cyp19Cre/+ transgenic mice to silence canonical Notch signaling in the global and trophoblast-derived cells, respectively. Females were mated with fertile males of the same strain to induce pregnancy, and mice with virginal plus were considered as embryonic day 0.5 (E0.5). Mice were housed in the animal care facility of the Xiamen University according to the guidelines for the care and use of laboratory animals.

**Histological analysis and immunostaining**

For hematoxylin and eosin staining, isolated implantation sites or whole dissected placental tissues were collected at various stages of gestation and fixed in 10% neutral buffer formalin. Then tissues were dehydrated and embedded in paraffin wax before being cut into 5-μm sections. For immunohistochemistry analysis, the rehydrated sections were incubated with RBPJ (1:200, Cell Signaling Technology) and Laminin (1:200, Sigma) antibodies, respectively. A Histostain-plus Kit (Zhongshan Golden Bridge Biotechnology) was used to visualize the antigen. For immunofluorescence, antibodies specific to Laminin (1:500, Sigma) and Vcam1 (1:500, Abcam) and secondary antibodies conjugated with Cy3 dyes (Jackson ImmunoResearch Laboratories) were used to stain rehydrated sections. Immunofluorescence images were captured in a Zeiss LSM 780 confocal scanning laser microscope.

**In situ hybridization**

In situ hybridization was performed on cryosections as previously described. Briefly, frozen sections (10 μm) were mounted onto poly-L-lysine-coated slides and fixed in 4% paraformaldehyde solution in PBS at 4 °C. After prehybridization, sections were hybridized at 45 °C for 4 h.
in 50% formamide buffer containing \textsuperscript{35}S or digoxigenin (DIG)-labeled sense or antisense RNA probes. The primers for probe production are listed in Table S1. After hybridization, sections were incubated with RNase A (10 mg/ml) at 37 °C for 20 min, and RNase A-resistant hybrids were detected by autoradiography, using liquid emulsion or NBT/BCIP chromogenic agent. Sections hybridized with the sense probes served as negative controls.

**Tetraploid aggregation assay**

Tetraploid aggregation chimeras were generated as described previously with some modifications\textsuperscript{51}. In brief, wild-type tetraploid embryos were generated by electrofusion of two-cell embryos derived from EGFP intercrosses. Fused embryos were cultured overnight in KSOM medium and developed to form four-cell embryos before the removal of zona pellucida with acidic Tyrode solution. Diploid \textit{Rbpj}-null embryos at eight-cell or morula stage were collected at E2.5. Each diploid embryo with zona pellucida removed was aggregated with two tetraploid embryos. Aggregated chimeric embryos were allowed to develop into the blastocyst stage and then transferred into the pseudopregnant uteri of wild-type females. Chimeric developing embryos were dissected at E10.5. Both the placenta and embryo proper were visualized for EGFP under a dissecting microscope and subsequently fixed for staining with hematoxylin–eosin. The placental and embryo tissue was used as a DNA source for genotyping of diploid embryos.

**Quantitative real-time PCR**

Total RNA was extracted from embryonic tissues or trophoblast cells using TRIzol reagent (Invitrogen), and 1–3 μg of total RNA after DNase treatment was used to synthesize cDNA. Quantitative RT-PCR analysis of different genes was performed using ABI QuantStudio 5 Real-Time PCR system, according to the manufacturer’s instructions. Assays were performed at least three times with each in duplicate. All the primers for real-time PCR are listed in Table S1.

**Plasmid construction, transfection, and luciferase assay**

The promoter region of \textit{Vcam1} gene (2 kb upstream relative to the transcription start site) with VP-BS1 and VP-BS2 wild-type versus mutant vectors (TGGGAA to TCTCAA) was generated using mouse genomic DNA as template and cloned into pGL3-basic vectors (Promega). Similarly, pGL3-basic vectors containing a trophoblast-specific regulatory region of \textit{Tpbpa}, a 340-bp region that has been reported and is 3.7 kb upstream of the TSS of \textit{Tpbpa}\textsuperscript{35}, were constructed from genomic DNA similarly. Mutations of the Mash2 binding site were achieved by Fast Mutagenesis System (Stratagene). The coding sequence region for the intracellular domain of Notch1 and Notch2 were amplified from the mouse placenta and cloned into the pCDH expression vector. In addition, the coding sequence region of \textit{Rbpj} and \textit{Mash2} was constructed with pEGFP-N1 and pcmv-myc expression vectors, respectively. Primers used are listed in Table S1.

HEK293T cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Hyclone) containing 10% fetal calf serum, 1 mM sodium pyruvate, 2 mM glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. All constructs were transiently transfected into HEK293T cells in 96-well plates, using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer’s instructions. 

**Allantois culture and immunostaining**

Briefly, \textit{Rbpj}\textsuperscript{fl/fl} and \textit{Rbpj}\textsuperscript{−/−} pregnant females were killed by cervical dislocation at E8.0. Allantois could be clearly identified at the end of PS in the exocoelom after wiping off the Reichert’s membrane. The floating allantoises were excised at the basal part, rinsed in cold CZB and culture medium three times, respectively, transferred to the 24-well Petri dish that has been coated with fibronectin, and then cultured for 24 h. The allantois explants were fixed in 4% PFA, permeabilized in 0.2% Triton X-100, and incubated with rat anti-PECAM1 (1:500, BD) overnight at 4 °C. Then they were washed in 0.1% BSA–PBS, incubated with the Cy3-labeled secondary antibody.

**Co-immunoprecipitation (Co-IP) and chromatin immunoprecipitation (ChIP)**

Protein samples (1 mg) from E8.5 placentas or trophoblast cells lysated by mild buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 5% glycerol; pH 7.4) were used for Co-IP experiments. Antibodies to \textit{Rbpj} (Santa Cruz Biotechnology), \textit{Mash2} (Merck) were used. Protein A agarose beads (Thermo-Fisher Scientific) were washed and incubated with protein lysates overnight at 4 °C. Immunoprecipitated proteins were separated by SDS/PAGE and detected by immunoblotting using the respective antibodies. ChIP analysis was performed according to the instructions of the ChIP Assay Kit (Millipore). In brief, E8.5 placentas or trophoblast cells were collected and suspended in 1% formaldehyde (Sigma)–PBS solution for cross-linking. After the cross-linking was terminated, cell pellets were lysed and sheared by sonication, until the average length of DNA was
500 bp as evaluated by agarose gel electrophoresis. The sheared chromatin fragments were incubated with the RBPJ (Santa Cruz Biotechnology) or Mash2 (Millipore) antibodies overnight at 4 °C. Normal goat IgG (Santa Cruz Biotechnology) was used as a negative control for non-specific immunoprecipitation. After washing, the beads were suspended in elution buffer and the precipitated protein/DNA complexes were eluted from the antibodies/beads. Then the chromatin was subjected to cross-link reversal and DNA was purified by phenol/chloroform extraction and ethanol precipitation. Specific primers listed in Table S1 were used to detect immunoprecipitated chromatin fragments and input chromatin.

Statistical analysis
Statistical analysis was performed with SPSS11.5 program. Comparison of means was performed using the independent-samples t test. Each experiment was performed at least three times with each in duplicate. Data were shown as means ± SEM. *p < 0.05; **0.01 < p < 0.05.

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Author details
1 Reproductive Medical Center, The First Affiliated Hospital of Xianning University, 361033 Xianning, Hubei, People’s Republic of China. 2 Fujian Provincial Key Laboratory of Reproductive Health Research, School of Medicine, Xiamen University, 361102 Xiamen, Fujian, People’s Republic of China. 3 National Engineering Laboratory for Animal Breeding, Key Laboratory of Animal Genetics, Breeding and Reproduction of the Ministry of Agriculture, College of Animal Science and Technology, China Agricultural University, 100193 Beijing, People’s Republic of China. 4 National Engineering Laboratory for Animal Breeding, Key Laboratory of Animal Genetics, Breeding and Reproduction of the Ministry of Agriculture, College of Animal Science and Technology, China Agricultural University, 100193 Beijing, People’s Republic of China

Author contributions
WH, WC, and WS designed research; LJ, WW, XQ, ZC, WJ, and NZ performed research; WH, WC, WS, LJ, WW, XQ, HJ, and KS analyzed the data; LJ, WW, and WH wrote the paper.

Conflict of interest
The authors declare that they have no conflict of interest.

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