

## *Ott1* (*Rbm15*) Is Essential for Placental Vascular Branching Morphogenesis and Embryonic Development of the Heart and Spleen<sup>∇</sup>

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**The infant leukemia-associated gene *Ott1* (*Rbm15*) has broad regulatory effects within murine hematopoiesis. However, germ line *Ott1* deletion results in fetal demise prior to embryonic day 10.5, indicating additional developmental requirements for *Ott1*. The *spen* gene family, to which *Ott1* belongs, has a transcriptional activation/repression domain and RNA recognition motifs and has a significant role in the development of the head and thorax in *Drosophila melanogaster*. Early *Ott1*-deficient embryos show growth retardation and incomplete closure of the notochord. Further analysis demonstrated placental defects in the spongiotrophoblast and syncytiotrophoblast layers, resulting in an arrest of vascular branching morphogenesis. The rescue of the placental defect using a conditional allele with a trophoblast-sparing *cre* transgene allowed embryos to form a normal placenta and survive gestation. This outcome showed that the process of vascular branching morphogenesis in *Ott1*-deficient animals was regulated by the trophoblast compartment rather than the fetal vasculature. Mice surviving to term manifested hyposplenia and abnormal cardiac development. Analysis of global gene expression of *Ott1*-deficient embryonic hearts showed an enrichment of hypoxia-related genes and a significant alteration of several candidate genes critical for cardiac development. Thus, *Ott1*-dependent pathways, in addition to being implicated in leukemogenesis, may also be important for the pathogenesis of placental insufficiency and cardiac malformations.**

*spen* (*split ends*) family proteins have a significant role in development. In *Drosophila*, *spen* was isolated from screens searching for modifiers of *egfr* (*Epidermal Growth Factor Receptor*)/*ras* (*Rat Sarcoma Virus*)/*mapk* (*Mitogen-Activated Protein Kinase*) pathways (32, 49). In addition, *spen* has been implicated in downstream *hox* (*homeobox*) gene pathways responsible for head and thorax development (66). The murine homolog, *Mint* (*Msx-Interacting Nuclear Target*), suppresses marginal zone B-cell development while promoting follicular B-cell differentiation in the spleen, purportedly through an inhibition of Notch2 signaling (33). The deletion of *Mint* is embryonic lethal at embryonic day 14.5 (E14.5) and has a phenotype including defects in cardiac, pancreatic, and skin cells as well as a quantitative loss of fetal liver cells (33).

The *spen* family member *OTT1* (*One-Twenty Two 1*) (*RBM15* [*RNA Binding Motif 15*]) was originally isolated as a fusion partner in t(1;22)(p13;q13), associated with infant acute megakaryocytic leukemia (39, 41), but its function is not well understood. In vitro experiments showed a cell context-dependent stimulation or inhibition of Notch-dependent transcription in myeloid cell lines and nonhematopoietic lines, respectively (38). An additional function in RNA transport and splicing has been described; however, thus far, only viral transcripts rather than physiological mRNAs have been identified (27, 37).

We previously reported that the germ line deletion of *Ott1* is embryonic lethal beyond E9.5, whereas conditional deletion within the hematopoietic compartment results in a block in B-cell development at the pre-B stage and increases in hematopoietic progenitor, myeloid, and megakaryocytic populations (48). Because *Ott1* has a significant role in multiple aspects of hematopoiesis and is widely expressed in multiple tissue types, additional roles of *Ott1* in development were investigated. Analysis of *Ott1* homozygous null embryos revealed a specific defect in placental trophoblast development and placental vascular branching morphogenesis that is responsible for the demise of the early embryos. Using a conditional deletion of *Ott1* that spared trophoblastic lineages, the early placental phenotype was rescued, allowing for the delineation of additional

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requirements of *Ott1* during development, including roles in cardiac and splenic organogenesis.

#### MATERIALS AND METHODS

**Mice.** Mice containing the *Ott1<sup>fllox</sup>* and *Ott1<sup>null</sup>* alleles were generated as previously reported (48). Transgenic *Sox2-cre* animals were obtained from Jackson Labs (Bar Harbor, ME). Genotyping was performed by PCR analysis of murine or embryonic tissue (48). Timed matings were determined by the presence of a vaginal plug at E0.5. Animals were housed in microisolator cages, and all experiments were performed with the approval of the Institutional Animal Care and Use Committee.

**Histology.** Mouse litters were sacrificed at the appropriate gestational age. For routine histology and light microscopy, tissues were fixed overnight in 10% formalin or Bouin's solution, processed, paraffin embedded, sectioned (5- $\mu$ m thickness), and stained with hematoxylin and eosin (H&E). E8.5 and E9.5 embryos were kept within deciduas and sectioned along the embryonic/abembryonic axis. For detailed analysis of cardiac phenotype, thoraces from E18.5 embryos were embedded and serially sectioned transversely at a 10- $\mu$ m thickness. Immunohistochemical staining for CD34 was performed with a rat monoclonal antibody, MEC14.7 (Abcam, Cambridge, MA).

**In situ hybridization.** Plasmids to generate in situ probes for *Tp53*, *Gcm1*, and *Vegf* were generously provided by J. Rossant (Toronto, Ontario, Canada). The *Ott1* probe was generated by cloning the first 1,000 bp of exon 1 of *Ott1* from murine genomic DNA using PCR amplification with primers 5'-ATGAGGTCTGCGGGGCGGGAG-3' and 5'-ACGGATAGTCTCGTTCTCTTTC CA-3' into pCR4-TOPO (Invitrogen, Carlsbad, CA). A sense *Ott1* probe was used as a control for the antisense probe. Linearized plasmids were transcribed using T7 polymerase. <sup>33</sup>P-radiolabeled in situ hybridization of E9.5 sections was performed. After deparaffinization, the slides were fixed in 4% paraformaldehyde for 10 min and then treated with proteinase K for 10 min at 37°C. The slides were hybridized overnight at 60°C with labeled riboprobe diluted in hybridization buffer. The slides were washed at 65°C for 2 h in 0.1 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate), immersed in Kodak NTB emulsion, and exposed for 1 to 4 weeks at 4°C. Following exposure, the slides were developed and counterstained with hematoxylin.

**Gene expression analysis.** RNA was extracted from three hearts each from either *Ott1<sup>null/null</sup> Sox2-cre* or *Ott1<sup>null/wt</sup> Sox2-cre* E18.5 embryos using the RNeasy microkit (Qiagen, Valencia, CA) and treated with RNase-free DNase (Qiagen, Valencia, CA). Fifty nanograms of purified total RNA was linearly amplified using the Ovation RNA amplification system, V2 (Nugen, San Carlos, CA), and biotinylated with the FL-Ovation Biotin Module, V2 (Nugen, San Carlos, CA), according to the supplied protocol. cDNA was then hybridized to Affymetrix mouse expression array 430A2.0 chips by the Dana Farber Microarray Core Facility (Boston, MA). The raw gene expression values were preprocessed with a robust multiarray analysis algorithm (28) using BioConductor software (21). To find differentially expressed genes, the preprocessed gene expression values were then filtered to remove genes with relatively constant expression. The filter kept genes that had at least a threefold change and an absolute change of 100 gene expression units. On this filtered set, differential expression was measured with the Comparative Marker Selection algorithm (50) using GenePattern software (50). The signal-to-noise ratio was used as the test statistic to rank order the genes for permutation testing. In addition to finding differentially expressed genes, we also considered gene sets. Gene sets were tested for enrichment with gene set enrichment analysis (GSEA) (61). We tested all 1,837 gene sets in the Molecular Signatures Database (<http://www.broad.mit.edu/gsea/msigdb/index.jsp>).

**Microarray data accession number.** Array data are listed in the Gene Expression Omnibus repository under accession number GSE12628.

#### RESULTS

**Deletion of *Ott1* results in numerous developmental defects prior to fetal demise at E10.5.** Analysis of embryos resulting from timed matings of *Ott1<sup>null/+</sup>* mice shows that *Ott1<sup>null/null</sup>* embryos are dead or undergoing degeneration by E10.5 (48). Further comparison with wild-type or heterozygous littermates showed severe growth retardation, delayed turning, and incomplete closure of the neural tube in *Ott1* homozygous null embryos (Fig. 1A and B). Blood was present and the heart was



FIG. 1. Gross histopathology of E9.5 *Ott1<sup>null/null</sup>* and wild-type embryos. (A and B) Micrographs of whole E9.5 embryos (A) and embryos fixed in Bouin's solution (B). (Left) Wild-type embryo; (right) *Ott1<sup>null/null</sup>* embryo. (C) H&E-stained cross section. Exencephaly and spina bifida are indicated by large and small arrows, respectively. (Top) Wild-type embryo; (bottom) *Ott1<sup>null/null</sup>* embryo. Magnification bars represent 200  $\mu$ m.

visibly pumping in the *Ott1<sup>null/null</sup>* embryos (data not shown). Histological cross sections of E9.5 *Ott1<sup>null/null</sup>* embryos showed that the neural tube defect was manifested by exencephaly and spina bifida (Fig. 1C). Somitogenesis did not appear disorganized but reflected an otherwise delayed state. No major structures were missing, and no specific tissues were observed to undergo degeneration.

**Placentas of *Ott1<sup>null/null</sup>* embryos have defective vascularization.** Numerous gene deletions in mice with similar growth delay phenotypes due to inadequate placentation, thus limiting fetal oxygen exchange and resulting in fetal death, have been described (53). Because the embryonic defects in the mutant embryos were similar to one of global growth retardation and occurred at a point requiring placentation function, we studied

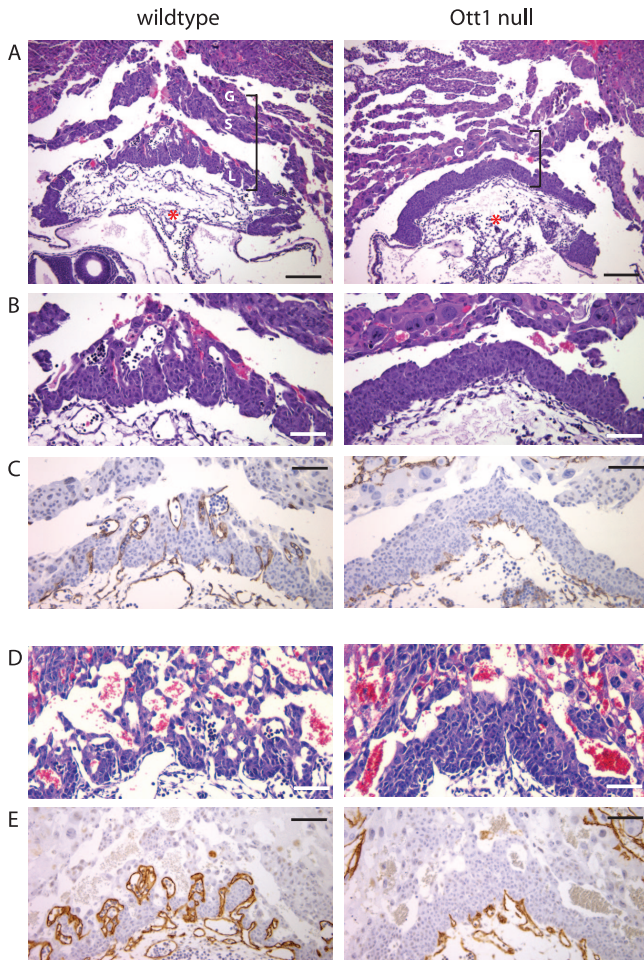


FIG. 2. *Ott1*<sup>null/null</sup> placentas have impaired fetal vascularization. Shown are cross sections through E8.5 (A to C) and E9.5 (D and E) placenta and decidual maternal tissues. Littermate control placentas are depicted on the left, and *Ott1*<sup>null/null</sup> placentas are depicted on the right. (A and B) H&E stain. Brackets indicate the layers comprising the placenta. G, giant cell trophoblast layer; S, spongiotrophoblast layer; L, labyrinthine syncytiotrophoblast layer; asterisk, allantoic stalk. (B) Higher magnification of placenta. (C) Immunostaining of the same embryo with endothelial marker CD34 to highlight fetal vasculature. (D and E) E9.5 placentas showing a highly vascularized labyrinth in controls but not mutants. (E) CD34 stain. Magnification bars represent 200  $\mu$ m (A) and 100  $\mu$ m (B to E).

placenta formation in *Ott1*<sup>null/null</sup> deciduas. At E7.5, the allantois was seen in both wild-type and mutant embryos (not shown), and by E8.5, chorioallantoic fusion had occurred in both genotypes (Fig. 2A). Histologically, beginning at E8.5 and progressing at E9.5, *Ott1*-null embryos exhibited a smaller, thinner placenta. The outermost placental layer consisting of trophoblast giant cells appeared unchanged in size and number (Fig. 2A). However, the region consisting of the spongiotrophoblast and the labyrinthine syncytiotrophoblast layers was markedly reduced and did not show a vascular network that was present in the control littermate embryos (Fig. 2B). To examine this vascular phenotype further, immunoperoxidase staining with the endothelial marker CD34 was performed. CD34 staining showed a nearly complete lack of vascularization of the syncytiotrophoblast layer by fetal vessels (Fig. 2C).

Penetration of the placenta by fetal vessels was observed; however, there was no expansion, branching, or development of adjacent maternal sinuses. While an increase in vascular branching was seen in control E9.5 embryos, no developmental progression was seen in mutants (Fig. 2D and E). Collectively, these findings are consistent with an arrest of fetal vascular branching morphogenesis in *Ott1*<sup>null/null</sup> animals.

**Loss of *Ott1* causes a decrease in the syncytiotrophoblast and spongiotrophoblast layers.** To ascertain the involvement and tissue specificity of *Ott1* in the embryo and placenta, in situ hybridization of *Ott1* RNA expression was performed with *Ott1*<sup>null/null</sup> mice and their wild-type littermates. *Ott1* was widely expressed throughout the embryo, similar to the nearly ubiquitous expression in adult tissues (Fig. 3A) (39, 41). The neural tube, however, did not show a significant expression of *Ott1*, providing additional evidence that the observed defects were likely to be secondary rather than primary effects of the loss of *Ott1* within the neural tube. Within the placenta, *Ott1* was observed to have a diffuse expression pattern and was present in all three trophoblast layers (Fig. 3B). As expected, *Ott1* null homozygous embryos lacked expression of *Ott1* within both the embryo proper and placenta (Fig. 3A and B).

To further define the involvement of the syncytiotrophoblast and spongiotrophoblast layers in *Ott1*<sup>null/null</sup> animals, in situ hybridization for *Gcm1* (*Glial Cell Missing 1*) and *Tpbpa* (*Trophoblast-Specific Protein  $\alpha$ 4311*) expression was performed. The lineage-specific *Gcm1* is expressed on syncytiotrophoblast cells, specifically on cells adjacent to fetal vessels (2). In situ analysis of the expression of *Gcm1* in *Ott1*-deleted E9.5 placentas showed markedly decreased cell numbers (Fig. 3C). Punctate clusters of *Gcm1*-positive cells were observed abutting narrow, nonbranching fetal vessels. In situ hybridization for *Tpbpa* that identified the spongiotrophoblast cells (7) demonstrated a dramatic reduction in the spongiotrophoblast layer in the *Ott1*<sup>null/null</sup> placentas.

The *Vegf* (*Vascular Endothelial Growth Factor*) pathway plays a pivotal role in fetal vascular branching morphogenesis (51). To determine whether an *Ott1* deficiency causes a loss of *Vegf* induction, in situ hybridization for *Vegf* was performed. *Vegf* expression was observed within the syncytiotrophoblast layer in wild-type littermates, as previously noted (17). The *Ott1*-deleted placentas still demonstrated expression of *Vegf*; however, and similar to *Gcm1* expression, it was confined to punctuate areas adjacent to fetal vessels. These data indicate that *Ott1*-mediated fetal vascular branching morphogenesis occurs either downstream or independent of *Vegf* expression. In summary, the deletion of *Ott1* results in the hypoplasia of the spongiotrophoblast and syncytiotrophoblast layers and an arrest of fetal vascular branching morphogenesis within the placenta independent of *Vegf* expression.

***Ott1* is required solely in extraembryonic tissue for normal placental development and vascular branching morphogenesis.** The formation of a vascular network within the placenta is thought to be driven primarily by trophoblast cells rather than the penetrating fetal vessels (16). To determine whether placental development and vascular branching morphogenesis required *Ott1* expression within the trophoblast compartment and/or the fetal vasculature, we utilized the *Sox2* (*SRY-containing gene 2*)-*cre* transgene. *Sox2-cre* expression is confined to the epiblast and has a high excision efficiency for alleles

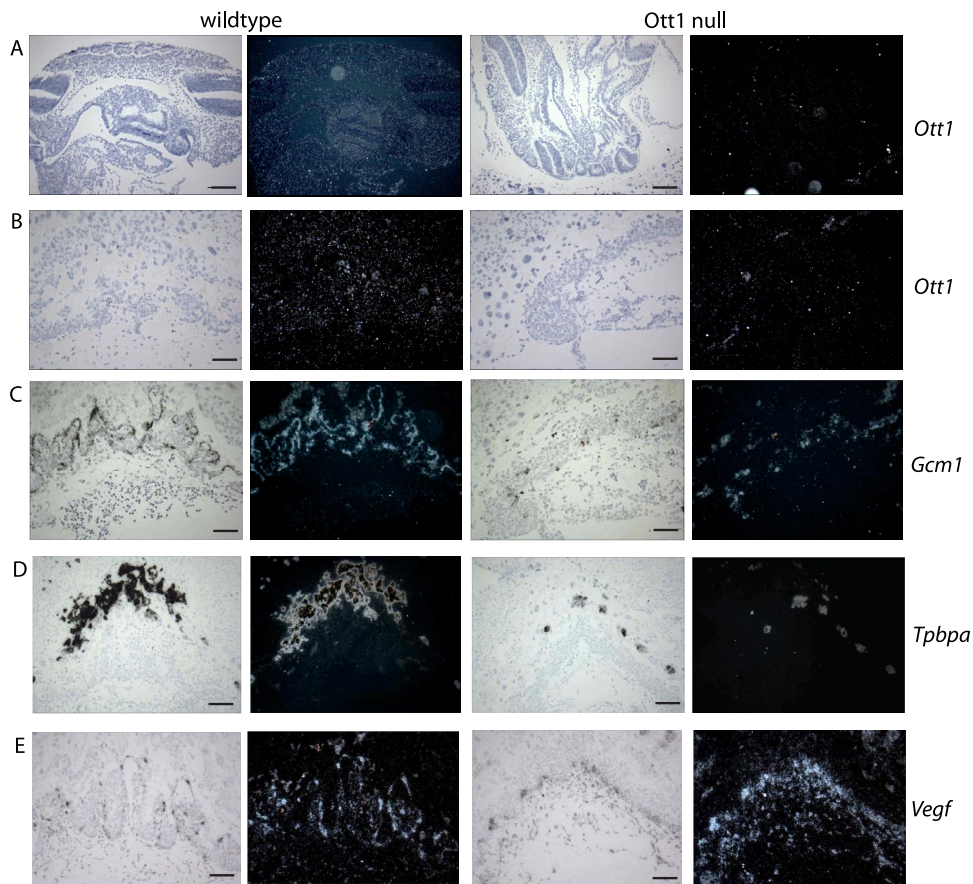


FIG. 3. In situ gene expression in E9.5 *Ott1*<sup>null/null</sup> embryos and placentas. (A) In situ hybridization of E9.5 embryos with the *Ott1* probe. (B to E) In situ hybridization of E9.5 placentas with *Ott1* (B), *Gcm1* (C), *Tpbpa* (4311) (D), and *Vegf* (E) probes. (Left panes) bright field; (right panes) dark field. Magnification bars, 200  $\mu\text{m}$  (A and D) and 100  $\mu\text{m}$  (B, C, and E).

flanked by loxP (floxed) sites (24). This results in a complete *cre*-mediated excision of floxed loci within the embryo proper including the yolk sac but leaves the trophoectoderm, which gives rise to the placental trophoblast lineages, intact. Male *Ott1*<sup>null/wt</sup> *Sox2-cre* mice were bred with female *Ott1*<sup>flox/flox</sup> mice in timed matings (Table 1). *Ott1*<sup>null/null</sup> embryos, the result of the excision of the original *Ott1*<sup>null/flox</sup> *Sox2-cre* genotype, were

identified with near the expected Mendelian ratio of 1:3 throughout gestation, in contrast to the nonconditional *Ott1*<sup>null/null</sup> embryos, which died beyond E9.5. Genotyping of *Ott1*<sup>null/flox</sup> *Sox2-cre* embryos by PCR demonstrated a complete conversion of the floxed *Ott1* alleles to the null allele, and residual floxed alleles were undetectable (data not shown).

The histology of *Ott1*<sup>null/flox</sup> *Sox2-cre* E9.5 placentas was indistinguishable from that of littermate placentas with an intact *Ott1* allele (Fig. 4A and B), with the preservation of the spongiotrophoblast and syncytiotrophoblast layers. Therefore, defects in spongiotrophoblast and syncytiotrophoblast development caused by a loss of *Ott1* are intrinsic to *Ott1* function within the trophoblast cell compartment alone. Placental vascular branching morphogenesis was present, and *Ott1*<sup>null/flox</sup> *Sox2-cre* placentas had a normal juxtaposition of fetal vasculature and maternal sinuses (Fig. 4A). Therefore, in the context of *Ott1*-dependent mechanisms, placental vascular branching morphogenesis is mediated via the trophoblasts rather than by the invading embryonic fetal vessels.

**Rescue of the placental defect in *Ott1*-deleted embryos enables peripartum survival and reveals defects in cardiac and splenic organogenesis.** Timed matings of *Ott1*<sup>null/wt</sup> *Sox2-cre* and *Ott1*<sup>flox/flox</sup> mice demonstrated viable embryos at near-

TABLE 1. Genotypes of embryos from *Ott1*<sup>null/wt</sup> transgenic *Sox2-cre*  $\times$  *Ott1*<sup>flox/flox</sup> mice

DPC <sup>a</sup>	No. of litters	No. of embryos	No. of flox/wt or flox/null <i>Sox2-cre</i> embryos (%)	No. of null/wt <i>Sox2-cre</i> embryos (%)	No. of null/null <i>Sox2-cre</i> embryos (%)
E9.5	2	21	12 (57)	6 (29)	3 (14)
E10.5	1	12	7 (58)	3 (25)	2 (17)
E14.5	7	55	26 (47)	15 (27)	14 (26)
E15.5	13	74	38 (51)	16 (22)	20 (27)
E16.5	3	21	14 (67)	1 (5)	6 (29)
E17.5	1	7	1 (14)	4 (57)	2 (29)
E18.5	7	44	25 (58)	12 (27)	7 (16)
E19.5/P0	5	34	17 (50)	11 (32)	6 (18) <sup>b</sup>

<sup>a</sup> Days postconception. The day of the vaginal plug is counted as E0.5. P0 is postpartum day 0.

<sup>b</sup> All pups were found dead or dying <5 h after birth.

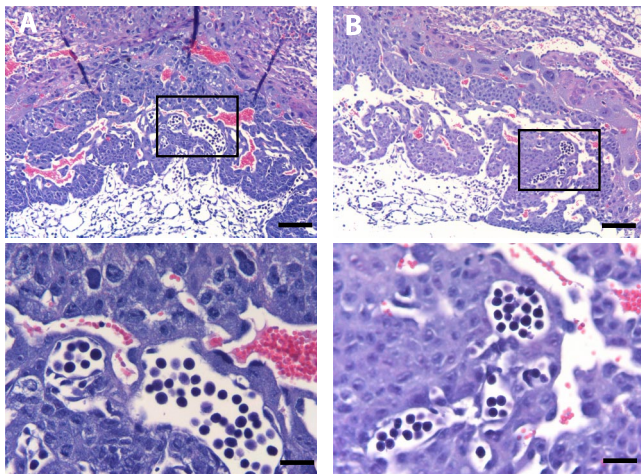


FIG. 4. Normal E9.5 placental vascularization after *Sox2-cre*-mediated *Ott1* deletion, sparing extraembryonic tissue. Shown are H&E-stained cross sections of *Ott1*<sup>null/null</sup> (A) and wild-type E9.5 (B) placentas. Bottom panels represent enlargements of the indicated areas. Magnification bars, 200  $\mu$ m (top) and 25  $\mu$ m (bottom).

Mendelian ratios throughout gestation (Table 1). However, *Ott1*<sup>null/null</sup> pups, the product of the *Sox2-cre Ott1*<sup>fllox/null</sup> genotype, were often found dead after birth. Occasional live mutant pups were observed, but they were runted and unable to feed and died within a few hours peripartum (Fig. 5A, left). Necropsies at E18.5 or postpartum day 0 were performed to assess the peripartum lethal phenotype. Examination of the lungs showed no histologic abnormalities, with the exception of a lack of lung inflation in mutant pups. However, pericardial effusion was often observed, and histological evaluation of the heart revealed a membranous ventricular septal defect in the majority of mutant embryos (Fig. 5D). No atrial defects or defects in the endocardial cushions, valves, or great vessels were seen. *Ott1*<sup>null/null</sup> embryos harvested during late gestation (E18.5) were often edematous, consistent with heart failure. In the absence of other major organ defects, this was a likely cause of peripartum demise (Fig. 5A, right). Interestingly, gross dissection also revealed hyposplenism with variable penetrance (Fig. 5B). Histological analysis showed a small remnant of splenic tissue attached to the splenic ligament (Fig. 5C).

**Cardiac gene expression in E18.5 embryos.** To gain insight into the pathways affected by the loss of *Ott1*, total RNA was isolated from E18.5 *Ott1*<sup>null/null</sup> *Sox2-cre* and littermate hearts to analyze global changes in gene expression. Total RNA was linearly amplified and then hybridized to Affymetrix 430 chips. Cardiac development in the embryo has overlapping gene expression requirements with other organs, including spleen and placenta. A hierarchy of gene expression in the developing spleen was previously determined and involves *Pbx1* (*Pre-B-cell leukemia transcription factor 1*), *Tlx1* (*T-cell leukemia homeobox 1*), *Nkx2.5* (*NK2 transcription factor related, locus 5*), and *Wt1* (*Wilms' Tumor 1*), each of which has also been implicated in cardiogenesis (6, 10, 13). *Sox11* (*SRY-box-containing gene 11*), a high-mobility-group protein, and *Acvr2b* (*Activin Receptor type IIB*) were also shown to be required for both cardiac and splenic development (47, 59). Of the above-mentioned genes,

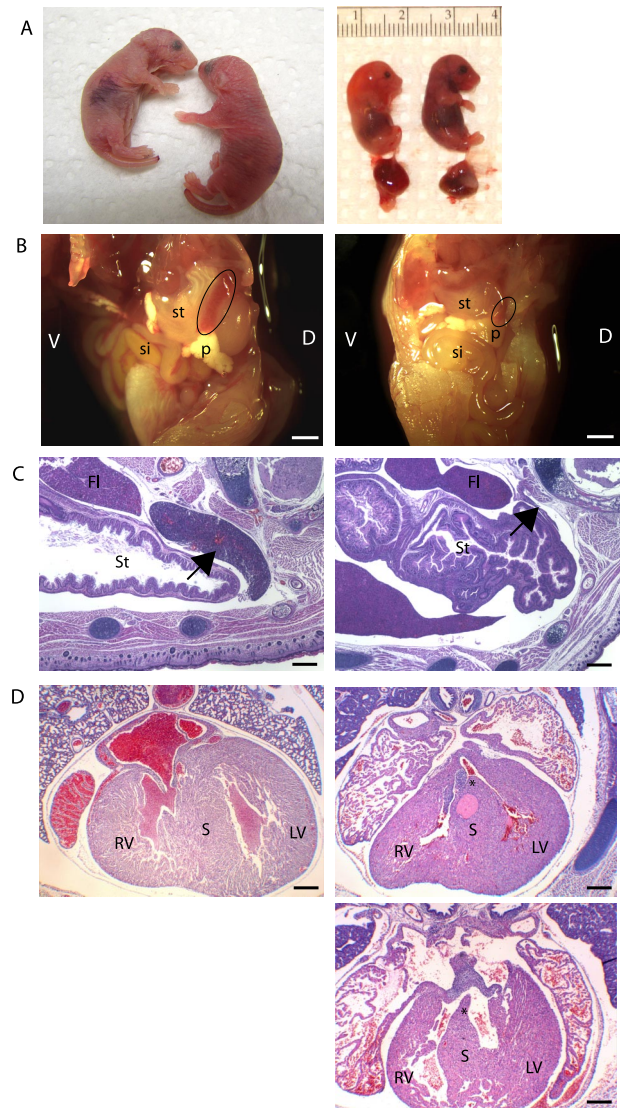


FIG. 5. *Ott1*<sup>null/null</sup> *Sox2-cre* mice are runted and hyposplenic and have a cardiac ventricular septal defect. (A, left) Photograph of newborn pups. (Right) Photograph of E18.5 embryos. *Ott1*<sup>null/null</sup> embryos are shown at left, and wild-type embryos are shown at right. (B) Gross photograph of dissected left flank/abdomen of E18.5 embryos. The black circle overlays the spleen. The fetal liver has been removed. V, ventral; D, dorsal; si, small intestine; st, stomach; p, pancreas. (C) H&E-stained cross section of E18.5 embryos. The arrow points to the spleen. St, stomach; Fl, fetal liver. (D) H&E-stained cross section of E18.5 embryo through the cardiac ventricles. RV, right ventricle; S, ventricular septum; LV, left ventricle. Asterisks indicate the location of the septal defect. (Left) Wild-type embryo; (right) two representative *Ott1* null embryos. Magnification bars represent 1,000  $\mu$ m (B) and 200  $\mu$ m (C and D).

*Acvr2b* was significantly altered, with a threefold upregulation ( $P = 0.001$ ) (Table 2). Other genes implicated in cardiac development that were found to have significantly upregulated expression in *Ott1*<sup>null/null</sup> hearts included *Gnb1l* (*guanine nucleotide binding protein, beta-1-like*) (twofold;  $P < 0.001$ ), a gene deleted in DiGeorge syndrome (23), and *E2f3* (*E2F transcription factor 3*) (twofold;  $P = 0.05$ ). Significantly downregu-

TABLE 2. Changes in gene expression among selected genes in *Ott1*-deleted E18.5 hearts<sup>c</sup>

Gene	Fold difference <sup>a</sup>	Signal-to-noise ratio <sup>b</sup>	P value
<i>Pbx1</i>	1.16	0.59	0.23
<i>Tlx1</i>	-1.12	-0.50	0.39
<i>Nkx2.5</i>	-1.07	-0.29	0.53
<i>Wt1</i>	1.06	0.21	0.63
<i>Sox11</i>	1.06	0.27	0.62
<i>Acvr2b</i>	<b>3.02</b>	<b>7.35</b>	<b>0.001</b>
<i>Hif1<math>\alpha</math></i>	1.26	0.68	0.17
<i>Cited2</i>	1.11	0.39	0.41
<i>Gnb11</i>	<b>1.95</b>	<b>4.43</b>	<b>&lt;0.001</b>
<i>E2f3</i>	<b>2.03</b>	<b>2.05</b>	<b>0.05</b>
<i>Il18r1</i>	<b>-1.91</b>	<b>-2.94</b>	<b>0.004</b>
<i>Hes1</i>	-1.06	-0.28	0.53
<i>EphrinB2</i>	1.15	0.37	0.52
<i>Hey1</i>	1.11	-0.35	0.48
<i>Hey2</i>	1.06	0.79	0.21

<sup>a</sup> Mean change of *Ott1*<sup>null/null</sup> compared to *Ott1*<sup>null/wt</sup> genotypes ( $n = 3$ ).

<sup>b</sup> Signal-to-noise ratio of gene expression values.

<sup>c</sup> Values in boldface type indicate significant changes.

lated cardiac-associated genes in *Ott1*<sup>null/null</sup> embryo hearts included *Il18r1* (*Interleukin 18 receptor 1*) (twofold;  $P < 0.004$ ).

*Ott1* and the family member *Mint* have been implicated in Notch signaling. In vitro studies of *Ott1* showed a cell context-dependent inhibition or stimulation of Notch-activated pathways (38). Notch signaling is also known to play a crucial role in cardiac and trophoblast development (26, 35, 45). The expression levels of downstream Notch targets, including *Hes1* (*hairly and enhancer of split 1*), *EphrinB2* (*eph-related receptor tyrosine kinase ligand 5*), *Hey1* (*hairly/enhancer-of-split related with YRPW motif 1*), and *Hey2* (*hairly/enhancer-of-split related with YRPW motif 2*) were not significantly altered between the *Ott1*-deleted and wild-type embryos, arguing against a significant change in Notch pathway activation (Table 2).

The hypoxia-inducible genes *Hif1 $\alpha$*  (*hypoxia-inducible factor 1  $\alpha$* ) and *Cited2* (*Cbp/p300-interacting transactivator, with Glu/Asp-rich C-terminal domain, 2*) have essential roles in cardiac development and in placental vascular branching morphogenesis (1, 4, 14, 58, 67). We examined expression levels of these genes between *Ott1*<sup>null/null</sup> embryos and littermates, but no significant differences were observed. To further understand global changes resulting from the deletion of *Ott1* in embryonic hearts, the expression array data were used for GSEA to compare to 1,837 curated gene sets in the Molecular Signature Database (61). Interestingly, the *Ott1*<sup>null/null</sup> set was significantly enriched (normalized enrichment score of 1.98;  $P < 0.001$ ) in comparison to a gene set composed of genes upregulated in response to hypoxia (Fig. 6) (34). However, a gene set specifically composed of HIF-1 $\alpha$  targets was not enriched (normalized enrichment score of 1.12;  $P = 0.12$ ) (57). In addition, Notch pathway gene sets were also not enriched (3, 44).

## DISCUSSION

The *Ott1* gene is widely expressed in adult and fetal tissues including the placenta. Early embryonic lethality of *Ott1*<sup>null/null</sup> embryos at E9.5 to E10.5 can be avoided through the use of the epiblast-specific *Sox2-cre* transgene, which spares the extraem-

brionic tissue that develops into the placenta. Therefore, the early stages of embryogenesis such as gastrulation are independent of *Ott1*. Although germ line *Ott1*<sup>null/null</sup> embryos have incomplete closure of the neural tube, the *Sox2-cre* deletion of *Ott1* confined to the embryo proper lacks this defect. Taken together with the dearth of *Ott1* expression seen in wild-type neural tube tissue, the neural tube defect in germ line *Ott1*<sup>null/null</sup> embryos must be a consequence of placental insufficiency rather than a primary consequence of the loss of *Ott1*.

*Ott1* is required for the vascular branching morphogenesis of fetal vessels into the placental labyrinth. The disruption of this process by the deletion of *Ott1* results in fetal death at E9.5 to 10.5, as diffusion alone is unable to supply the oxygen requirements of the embryo beyond this stage (52). Both the spongiotrophoblast and syncytiotrophoblast layers are required for vascular branching morphogenesis (53). Identification of the individual trophoblast layers showed a significant reduction in the sizes of both the spongiotrophoblast and syncytiotrophoblast layers but a preservation of the giant cell trophoblast layer. The formation of the syncytiotrophoblast layer into the labyrinth is in part dependent on the process of vascular branching morphogenesis; therefore, the defect in *Ott1*<sup>null/null</sup> placentas could be either intrinsic to syncytiotrophoblasts or a non-cell-autonomous effect resulting from the failure of vascular branching morphogenesis (16). In contrast, the development of the spongiotrophoblast layer was previously observed in the absence of an intact labyrinth in murine knockouts of the *Hai-1* (*hepatocyte growth factor activator inhibitor 1*) and *Fosl1* (*fos-like antigen 1*) genes, suggesting that the loss of *Ott1* causes a primary defect in spongiotrophoblasts (55, 62). The role of the spongiotrophoblast layer is not completely understood; however, it is thought to be required for the proper establishment of the fetal and maternal portions of the labyrinth through the production of vasoactive factors (15, 63).

The expansion and branching of the fetal vessels are presumed to be guided through growth factors and contact originating from the trophoblast tissues rather than directed by the fetal vasculature itself (15). Evidence for this include the observations that (i) there is expression of vascular remodeling genes such as *Vegf* in the trophoblast cells but not the fetal vessels and (ii) *Vegf* expression is essential for vascular branching morphogenesis (8, 19, 64). The deletion of *Tfeb* (*transcription factor EB*) causes a loss of vascular branching morphogen-

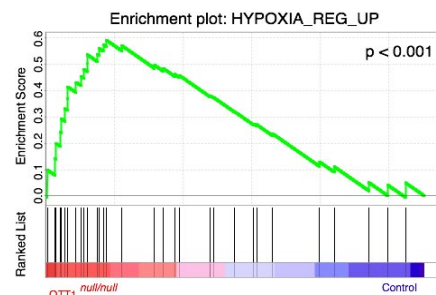


FIG. 6. Enrichment plot for genes upregulated in hypoxia. Shown are a profile of the running enrichment score and positions of the gene set members on the rank order list. *Ott1*<sup>null/null</sup> embryonic heart gene expression signatures are compared to those of *Ott1*<sup>null/wt</sup> embryonic hearts at E18.5 ( $n = 3$ ).

esis as well and specifically abolishes *Vegf* expression within the syncytiotrophoblast cells (60). *Vegf* expression can still be induced in the *Ott1*<sup>null/null</sup> placenta; therefore, *Ott1*-mediated vascular branching morphogenesis is either independent or downstream of *Vegf* signaling. In addition, the excision of *Ott1* by *Sox2-cre* in the embryo, including the fetal vasculature, demonstrates that vascular branching morphogenesis, at least in the context of *Ott1*, is a trophoblast-driven process.

The extraembryonic-sparing *Sox2-cre* excision demonstrated that *Ott1* was required for spleen and cardiac development in the later stages of embryonic development. Interestingly, splenic, cardiac, and hematopoietic tissues are all derived from mesoderm, suggesting that despite the nearly ubiquitous expression of *Ott1*, mesodermal tissue has specifically dependent or nonredundant requirements. Asplenia or hyposplenia has been associated with the deletion of a relatively small set of genes including *Tlx1*, *Pbx1*, *Nkx2.3* (*NK2 transcription factor related, locus 3*), *Nkx3.2* (*NK3 homeobox 2*), *Tcf21* (*transcription factor 21*), *Sox11*, *Wt-1*, *Acvr2b*, and *Cited2* (4, 6, 47). A transcriptional hierarchy among several of these genes has been established, placing *Wt-1* downstream of the homeobox genes *Tlx1*, *Pbx1*, and *Nkx3.2* (6). *Nkx2.5* marks the spleen primordium and is downstream of *Tcf21* and *Pbx1* (6). *Spen/Mint* was previously implicated in *Hox* gene regulation but not specifically with any of the above-mentioned genes or their homologs (43, 66).

*Sox11*, *Pbx1*, *Wt-1*, *Cited2*, and *Acvr2b* are known to be involved in both cardiac and splenic development (25, 47, 56, 59, 65). Of these genes, evaluation of cardiac gene expression in *Ott1* knockout embryos revealed a threefold increase in levels of *Acvr2b*. Deletion mutants of *Acvr2b* in mice demonstrate a complex phenotype including cardiac atrial and ventricular septal defects, hyposplenism, right isomerization of the lung, and vertebral defects (46, 47). Mutations of *ACVR2B* in humans have been observed in relation to cardiac defects including ventricular septal defects, left-right axis abnormalities, and splenic malposition (31). Although no placental abnormalities were noted in the knockout model, activin and activin receptors are believed to be important regulators of human trophoblast differentiation (29). No studies have yet examined the effects of an overexpression of *Acvr2b*; however, gene dosage appears to be an important factor in phenotype and penetrance in mice (46).

Twofold overexpression was also observed for *E2f3* and *Gnb11*. An *E2f3* deletion results in congestive heart failure in adult mice and causes apoptosis in cardiomyocytes when overexpressed (11, 18). *GNB11* is among the deleted genes in human DiGeorge syndrome (del22q11), which manifests cardiac abnormalities including ventricular septal defects; however, it is believed that another gene, *Tbx1* (*T-box 1*), is responsible for the cardiac phenotype (36, 42). A threefold underexpression of *Il18r1* was seen in the *Ott1* knockout hearts, and *Il-18* has been shown to be important for cardiac remodeling and the hypertrophic response (12, 40).

The expression levels of *Hif1 $\alpha$* , a regulator of the hypoxia response, and its downstream target *Cited2* have been shown to contribute to cardiac, splenic, and placental development. (4, 5). Therefore, it was important to exclude a perturbation of gene expression within this set as the basis for the *Ott1* knockout phenotype. GSEA showed a significant enrichment of hy-

poxia-related genes without a similar enrichment of a specific *Hif1 $\alpha$*  target set. It is unclear whether changes in hypoxia-related gene expression may be primary or secondary to inadequate cardiac function in the *Ott1*<sup>null/null</sup> embryos. Likewise, alterations in the expressions of *Acvr2b*, *E2f3*, *Gnb11*, and *Il18r1* will need further investigation to determine whether *Ott1* directly affects expression.

The Notch pathway is also critical for embryonic heart development. *Ott1* can inhibit or activate the Notch-responsive gene *Hes1* in vitro depending on cell context (38). The family member *Mint* has already been implicated in the suppression of Notch2 signaling via RbpJ $\kappa$  (recombination signal binding protein for immunoglobulin kappa J region) binding. Although *Ott1* lacks the domain identified for this function, an alternative RbpJ $\kappa$  binding site in *Ott1* has been identified (38). In murine gene knockout experiments, the Notch target genes *Hes1*, *EphrinB2*, *Hey1*, and *Hey2* have each been shown to be required for cardiac development and manifest phenotypes including ventricular septal defects (20, 22, 30). Surprisingly, the expression of these Notch targets did not change in *Ott1*<sup>null/null</sup> embryonic hearts compared to littermate controls, suggesting that the generation of the *Ott1*<sup>null/null</sup> cardiac phenotype is not mediated via these Notch pathway signaling intermediates and that an alternative pathway exists.

In summary, *Ott1* has essential roles in hematopoietic, trophoblast, cardiac, and splenic development. These findings implicate the *spen* family and homologs as significant global regulators of embryonic and adult hematopoietic development. It is possible that *Ott1* has a more expansive role than *Mint* by virtue of its wider expression pattern. The deletion of the family member *Mint* in mice results in a cardiac septal defect as well; however, *Ott1* must fulfill a nonredundant role. The deletion of *Ott1* causes a defect within the syncytiotrophoblast and spongiosotrophoblast cell layers responsible for placental vascular branching morphogenesis, as confirmed by embryo-specific *cre*. The diverse affected tissues in *Ott1*-deleted mice suggest that *Ott1* or *Ott1*-dependent pathways may play an important part in human pathophysiology, specifically, placental insufficiency and cardiac malformation. Of note, certain leukemia translocation-associated genes such as *Cdx2* (*caudal-type homeobox 2*) and *Rxr- $\alpha$*  (*retinoid X receptor alpha*) have significant roles in development and particularly in trophoblast development (9, 54). Further studies into the *Ott1*-dependent mechanisms essential for development, including the putative targets *Acvr2b*, *E2f3*, *Gnb11*, and *Il18r1*, may also yield significant insight into pathways coopted by OTT1-MAL in t(1;22)-associated leukemogenesis.

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