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Notch signal reception is required in vascular smooth muscle cells for ductus arteriosus closure

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Summary

The ductus arteriosus is an arterial vessel that shunts blood flow away from the lungs during fetal life, but normally occludes after birth to establish the adult circulation pattern. Failure of the ductus arteriosus to close after birth is termed patent ductus arteriosus, and is one of the most common congenital heart defects. Our previous work demonstrated that vascular smooth muscle cell expression of the *Jag1* gene, which encodes a ligand for Notch family receptors, is essential for postnatal closure of the ductus arteriosus in mice. However, it was not known what cell population was responsible for receiving the *Jag1*-mediated signal. Here we show, using smooth muscle cell-specific deletion of the *Rbpj* gene, which encodes a transcription factor that mediates all canonical Notch signaling, that Notch signal reception in the vascular smooth muscle cell compartment is required for ductus arteriosus closure. These data indicate that homotypic vascular smooth muscle cell interactions are required for proper contractile smooth muscle cell differentiation and postnatal closure of the ductus arteriosus in mice.

Keywords

birth defects; vascular smooth muscle; lateral induction

INTRODUCTION

The ductus arteriosus is an arterial blood vessel that connects the pulmonary artery to the proximal descending aorta, directing blood flow away from the pulmonary circulation during fetal life. After birth the ductus arteriosus is first rapidly, and then permanently occluded, separating the pulmonary and systemic circulations to establish the normal adult circulatory pattern. Failure of the ductus arteriosus to close after birth is termed patent ductus arteriosus (PDA), and is one of the most common human congenital heart defects (Anilkumar, 2013; Coceani and Baragatti, 2012; Schneider and Moore, 2006). PDA patients are at increased risk for pulmonary and cardiac problems such as pulmonary hemorrhage, congestive heart failure, chronic lung disease, sepsis, and necrotizing enterocolitis.

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CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

We have shown previously that expression of the *Jag1* gene, which encodes a transmembrane ligand for Notch family receptors, is required in vascular smooth muscle cells for postnatal closure of the ductus arteriosus (Feng *et al.*, 2010). Mice with smooth muscle-specific deletion of the *Jag1* gene exhibited defects in contractile smooth muscle cell differentiation in the vascular wall of the ductus arteriosus and adjacent aorta, failed to close their ductus arteriosus postnatally, and died during the first few days after birth. It has not been demonstrated, however, what target cell population is required for reception of the *Jag1*-mediated signal. Here we show, by conditional deletion of the *Rbpj* gene (that encodes the transcriptional mediator of all canonical Notch signaling), that Notch signal reception is required in vascular smooth muscle cells, indicating that homotypic vascular smooth muscle cell interactions are required for postnatal closure of the ductus arteriosus in mice.

RESULTS AND DISCUSSION

Our previous work demonstrated that expression of the *Jag1* gene in vascular smooth muscle cells was essential for postnatal closure of the ductus arteriosus (Feng *et al.*, 2010). To assess whether Notch signal reception also is required in vascular smooth muscle cells, we performed smooth muscle-specific deletion using *SM22a-Cre* (also known as *Tagln-Cre*) mice (Holtwick *et al.*, 2002), the same Cre driver line we used in our previous study. We abrogated Notch signal reception in these cells by deleting the *Rbpj* gene, which encodes a transcription factor that mediates all canonical Notch signaling. *SM22a-Cre/+; Rbpj*$^{flox/-}$ (hereafter referred to as *Rbpj-SMcko*, for *Rbpj* smooth muscle conditional knockout) mice were generated by crossing *SM22a-Cre/+; Rbpj*$^{+/-}$ male mice to *Rbpj*$^{flox/flox}$ female mice. *Rbpj-SMcko* mice were found alive in expected Mendelian ratios when delivered by caesarean section at embryonic day (E) 18.5. However, approximately 50% of the *Rbpj-SMcko* neonates died by late on postnatal day (P) 0, the day of birth, and most had died by P2 (Table 1). However, one *Rbpj-SMcko* mouse survived until P6 (Table 1, Figure 1A).

All *Rbpj-SMcko* mice born exhibited patent ductus arteriosus (PDA), with the exception of one *Rbpj-SMcko* mouse in which closure of the ductus arteriosus could not be evaluated due to the presence of an interrupted aortic arch, a more severe defect of the great arteries in which part of the aortic arch is missing (Table 2). There was a low incidence (3%) of PDA in the control littermates, which consisted of all genotypes other than *SM22a-Cre/+; Rbpj*$^{flox/-}$. We had observed a similar low incidence of PDA in control littermate mice in our previous study (Feng *et al.*, 2010). To better visualize PDA, we injected the left ventricles of a subset of the control and *Rbpj-SMcko* neonates with Microfil injection compound (Figure 1B, C).

In our previous study of PDA in *Jag1* smooth muscle conditional knockout (*Jag1-SMcko*) mice, we observed defects in contractile vascular smooth muscle cell differentiation in the ductus arteriosus and adjacent aorta (Feng *et al.*, 2010). We assessed expression of smooth muscle actin protein, a marker for contractile smooth muscle cell differentiation, in sections from control littermate and *Rbpj-SMcko* embryos at E13.5 (Fig. 2). Similarly to the *Jag1-SMcko* mutants, *SM22a-Cre; Rbpj*$^{flox/-}$ mutant embryos (Fig. 2B) had greatly reduced expression of smooth muscle actin in the ductus arteriosus and adjacent aorta, but smooth muscle actin expression in the trachea and esophagus was unaffected in the mutants.

Prostaglandins are key regulators of ductus arteriosus patency and closure. The cyclooxygenases 1 and 2 (COX1 and 2; formally known as prostaglandin-endoperoxidase synthases 1 and 2) are rate-limiting enzymes involved in the conversion of arachidonic acid to prostaglandins. Non-steroidal anti-inflammatory drugs such as indomethacin and ibuprofen inhibit the formation of prostanoids by the COX enzymes (Mitchell and Warner, 2006). In premature infants exhibiting patent ductus arteriosus, COX inhibitors are used clinically as a first line treatment to induce ductus arteriosus closure by reducing prostaglandin levels, thereby inducing constriction of the ductus arteriosus and closure of its lumen. We found in our previous study that postnatal injection of indomethacin was able to rescue ductus arteriosus closure in a subset of the *Jag1-SMcko* mutants. When we injected indomethacin postnatally into control littermate and *Rbpj-SMcko* mutants, ductus arteriosus closure was rescued in only 1 of 9 *Rbpj-SMcko* mutants (Table 3, Figure 3). The enhanced resistance to ductus arteriosus closure after indomethacin administration exhibited by the *Rbpj-SMcko* mutants, compared to the *Jag1-SMcko* mutants, may indicate that more than one Notch ligand may be involved in regulating smooth muscle cell differentiation in the ductus arteriosus. Further work will be needed to assess that possibility.

The Notch signaling pathway plays multiple roles during vascular development in mammals and other vertebrates (Gridley, 2010; Kume, 2012), including vascular smooth muscle cell differentiation (Boucher *et al.*, 2012; Lin and Lilly, 2014). We showed previously that expression of the Notch ligand *Jag1* in vascular smooth muscle cells is essential for postnatal closure of the ductus arteriosus. However, the *Jag1* gene is expressed in both vascular smooth muscle cells and in endothelial cells (Villa *et al.*, 2001), making the recipient cell population for the *Jag1*-mediated signal unclear. In this study, we show that the *Jag1*-mediated signal must also be received by the vascular smooth muscle cell compartment.

While this manuscript was in preparation, Baeten and colleagues published an elegant study demonstrating that mutation of the Notch2 and Notch3 receptors caused patent ductus arteriosus in mice (Baeten *et al.*, 2015). In their study, they used smooth muscle-specific deletion of the *Notch2* gene (using a different Cre driver line, Myocardin-Cre) together with a constitutive *Notch3* deletion to produce mice with different combinations of mutant and wild type *Notch2/3* alleles in vascular smooth muscle cells. They found that all of the *Notch2*-smooth muscle null, *Notch3*-heterozygous mice exhibited PDA, and approximately 40% of *Notch2*-smooth muscle null mice with wild type alleles of the *Notch3* gene also had PDA.

Our previous results (Feng *et al.*, 2010) fit a model in which *Jag1* expression, first in endothelial cells and subsequently in vascular smooth muscle cells, initiated a process of lateral induction in which *Jag1* expression and contractile smooth muscle cell differentiation was induced in neighboring cells (Feng *et al.*, 2010). Manderfield and colleagues reached similar conclusions concerning the role of *Jag1* expression and Notch signal reception during assembly of arterial vessel walls (Manderfield *et al.*, 2012). Our current results, as well as those of Baeten and colleagues (Baeten *et al.*, 2015), confirm that *Jag1*-mediated homotypic intercellular interactions are essential for contractile smooth muscle cell differentiation and ductus arteriosus closure in mice. Interestingly, PDA has been identified in some case reports (Harris *et al.*, 2002; Sanchez-Angulo *et al.*, 1997) of Alagille syndrome,

which is most commonly caused by *JAG1* haploinsufficiency (Turnpenny and Ellard, 2012), suggesting that similar mechanisms likely occur in humans.

METHODS

Mice

Rbpj^{flox} and *Rbpj*^{null} (the deleted form of the *Rbpj*^{flox} allele) mice (Tanigaki *et al.*, 2002) were obtained from Dr. T. Honjo. The transgenic *SM22a-Cre* (official name Tg(Tagln-cre)1Her; MGI ID: 2446975) line (Holtwick *et al.*, 2002) was obtained from the Jackson Laboratory. All experimental procedures on mice were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and were approved by the Institutional Animal Care and Use Committee at Maine Medical Center.

Histology and Immunofluorescence

Embryos and neonatal mice were fixed in 4% paraformaldehyde. Chest cavities were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. For immunofluorescence, sections were dewaxed in a standard xylene and ethanol series, then rehydrated with phosphate buffered saline (PBS). An antigen retrieval step was performed in boiling 10 mM sodium citrate, pH 6.0, for 10 minutes. Slides were then blocked in 5% goat serum/2% BSA/PBST for 2 hours at room temperature before being covered with diluted primary antibodies at 4°C overnight. Antibodies used in this study were polyclonal anti-smooth muscle alpha actin primary antibody (Abcam, 1:200), and Alexafluor-labeled fluorescent secondary antibodies (Molecular Probes). All slides were mounted by using Vectashield mounting medium with DAPI (Vector Laboratories).

Intracardiac Injections

To better visualize the outflow tract and ductus arteriosus, hearts of *SM22a-Cre; Rbpj*$^{flox/-}$ mutant and control littermate mice were injected with Microfil silicone rubber injection compound (MV-122; Flow Tech Inc.). Pups were isolated at E18.5 by caesarean section, and were euthanized seven hours post surgery. The chest cavities were opened and fixed in 10% neutral buffered formalin. After fixing for one hour, neonates were rinsed and Microfil compound was injected into the left ventricle using a 27 gauge needle, as described previously (Feng *et al.*, 2010).

Indomethacin treatment

To determine whether postnatal indomethacin administration could rescue closure of the ductus arteriosus of *SM22a-Cre; Rbpj*$^{flox/-}$ mutant neonatal mice, pups from caesarean delivery at embryonic day (E)18.5, or naturally delivered pups at postnatal day (P)0, were injected subcutaneously with indomethacin (6 mg/kg). Pups were euthanized six hours after injection, the chest cavities were opened and closure of the ductus arteriosus was scored visually. In some treated mice, outflow tracts were visualized by Microfil injection.

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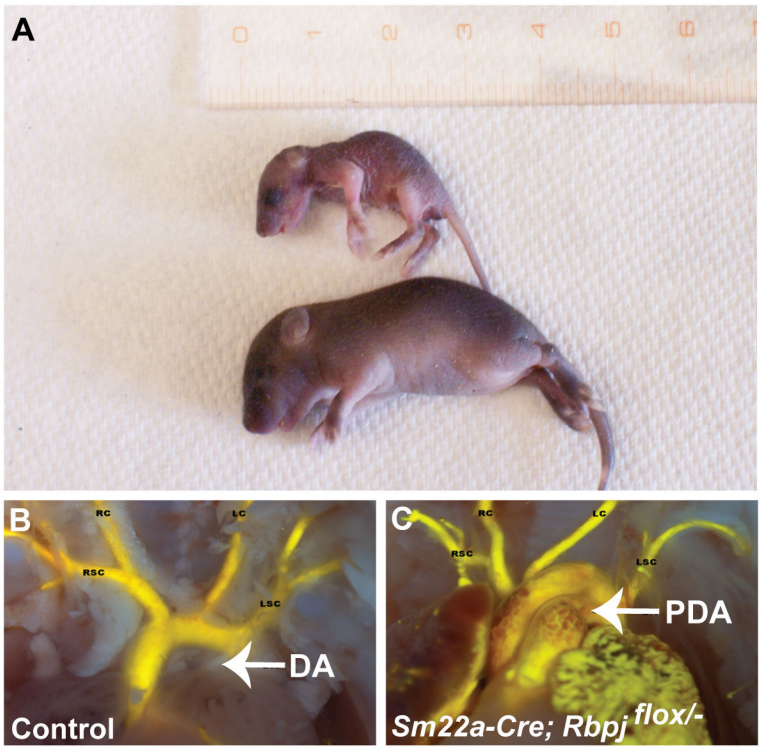

FIGURE 1.

(A) *SM22a-Cre; Rbpj*$^{flox/-}$ mutant (top) that survived until P6 and control littermate. (B, C) Intracardiac Microfil injections of a control littermate (B) and the *SM22a-Cre; Rbpj*$^{flox/-}$ mouse that survived until P6 (C). The mutant exhibits a large patent ductus arteriosus, while the ductus arteriosus of the littermate has closed (thin white band of tissue). Abbreviations: DA, ductus arteriosus; LC, left carotid artery; LSC, left subclavian artery; PDA, patent ductus arteriosus; RC, right carotid artery; RSC, right subclavian artery.

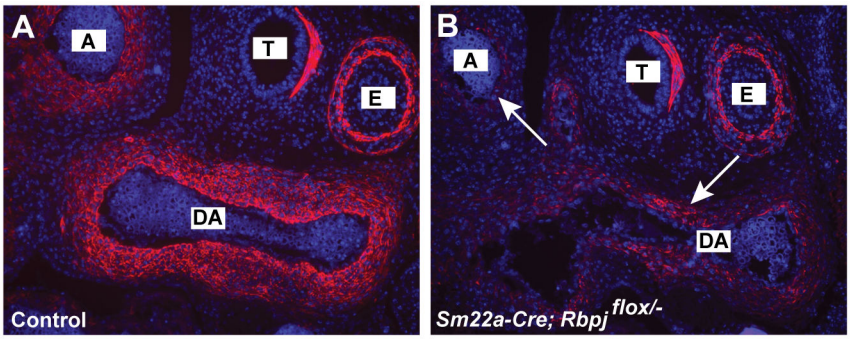

FIGURE 2.

Reduced contractile smooth muscle cell differentiation in the aorta and ductus arteriosus of *SM22a-Cre; Rbpj*$^{flox/-}$ mutants. Immunofluorescent staining of outflow tract vessels in E13.5 embryos stained with antibodies against smooth muscle actin. Sections were counterstained with DAPI. The *SM22a-Cre; Rbpj*$^{flox/-}$ mutant (B) has greatly reduced expression of smooth muscle actin in the ductus arteriosus (DA) and adjacent aorta (A). Smooth muscle actin expression in the trachea (T) and esophagus (E) is unaffected in the mutant.

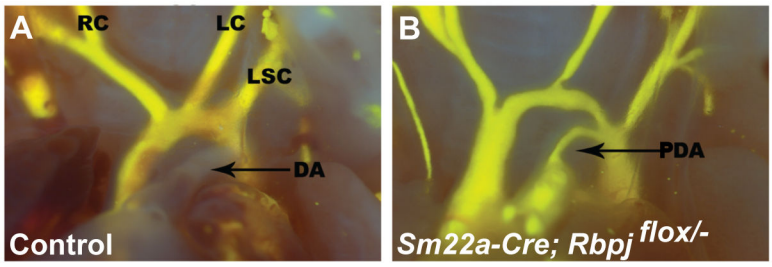

FIGURE 3.

Failure of indomethacin injection to rescue patent ductus arteriosus in a *SM22a-Cre; Rbpj*$^{flox/-}$ mutant (B). Abbreviations as in Figure 1.

Table 1

Survival of *SM22a-Cre; Rbpj*$^{flox/}$ neonatal mice.

Age	*SM22a-Cre; Rbpj*$^{flox/-}$ (number alive)	Control littermates
E18.5	18 (18)	73
P0	12 (6)	52
P1	6 (2)	29
P4	2 (1)	14
P6	1 (0)	7
Wean	0	89

Control: All littermate genotypes other than *SM22a-Cre; Rbpj*$^{flox/-}$.

Table 2

Penetrance of patent ductus arteriosus in *SM22a-Cre; Rbpj*$^{flox/-}$ and control littermate neonatal mice.

Phenotype	*SM22a-Cre; Rbpj*$^{flox/-}$	Control littermates
PDA	11/11 (100%) *	2/52 (3.4%)
OTPD	2/12 (17%)	1/52 (2%)

OTPD: outflow tract patterning defects; PDA: patent ductus arteriosus.

* One *SM22a-Cre; Rbpj*$^{flox/}$ mouse was excluded due to the presence of an interrupted aortic arch.

Table 3

Indomethacin injection in *SM22a-Cre; Rbpj*$^{flox/}$ mutant neonates.

Phenotype	*SM22a-Cre; Rbpj*$^{flox/}$	Control littermates
Closed DA	1/9 (11%)	29/29 (100%)
Patent DA	8/9 (89%)	0/29 (0%)

Indomethacin (6 mg/kg) was injected subcutaneously into pups within 12 hours of birth on day 0, or after caesarean delivery on day E18.5. After six hours, pups were euthanized and the ductus arteriosus scored for patency or closure. Mice from four litters are included in this table. DA: ductus arteriosus.