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BRIEF COMMUNICATION

Tissue-Specific Roles for the Slit–Robo Pathway During Heart, Caval Vein, and Diaphragm Development

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BACKGROUND: Binding of Slit ligands to their Robo receptors regulates signaling pathways that are important for heart development. Genetic variants in *ROBO1*and *ROBO4* have been linked to congenital heart defects in humans. These defects are recapitulated in mouse models with ubiquitous deletions of the Slit ligands or Robo receptors and include additional heart defects not currently linked to SLIT or ROBO mutations in humans. Given the broad expression patterns of these genes, the question remains open which tissue-specific ligand-receptor interactions are important for the correct development of different cardiac structures.

METHODS AND RESULTS: We used tissue-specific knockout mouse models of *Robo1*/*Robo2*, *Robo4*, *Slit2* and*Slit3* and scored cardiac developmental defects in perinatal mice. Knockout of *Robo2* in either the whole heart, endocardium and its derivatives, or the neural crest in ubiquitous *Robo1* knockout background resulted in ventricular septal defects. Neural crest-specific removal of *Robo2* in *Robo1* knockouts showed fully penetrant bicuspid aortic valves (BAV). Endocardial knock-out of either *Slit2*or *Robo4* caused low penetrant BAV. In contrast, endocardial knockout of *Slit3* using a newly generated line resulted in fully penetrant BAV, while removal from smooth muscle cells also resulted in BAV. Caval vein and diaphragm defects observed in ubiquitous *Slit3* mutants were recapitulated in the tissue-specific knockouts.

CONCLUSIONS: Our data will help understand defects observed in patients with variants in ROBO1 and ROBO4. The results strongly indicate interaction between endocardial Slit3 and neural crest Robo2 in the development of BAV, highlighting the need for further studies of this connection.

Key Words: bicuspid aortic valves ■ congenital heart defects ■ Robo ■ signaling pathway ■ Slit

Loss-of-function variants in Roundabout Guidance Receptor 1 (*ROBO1*) have recently been linked to tetralogy of Fallot, atrial septal and ventricular septal defects in patients,[1] whereas variants in *ROBO4* predispose to bicuspid aortic valves (BAVs).[2] These defects are recapitulated in ubiquitous mouse mutants for the Robo receptors and their Slit Guidance Ligands.[3,4] However, mouse mutants show a much broader range of congenital heart defects, including atrial septal and ventricular septal defects, BAVs, bicuspid pulmonary valves, and pericardial and caval vein defects. This correlates with the broad expression patterns of the different ligands and receptors in several cardiac cell types[3,4] from specific parts of the myocardium, the endocardium, the cushions/valves, and the neural crest to smooth muscle cells. As a result, it is still unknown which cell type–specific expression is important for the correct development of the different cardiac structures. Here, we have scored congenital heart defects in a broad range of tissue-specific knockouts to better understand the interaction between the source tissues of the ligands and the responsive tissues expressing the

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Table 1. Range of Congenital Defects Observed in the Conditional Slit and Robo Lines

		Robo1$^{-/-}$; *Robo2*$^{fl/fl}$ (Domyan et al, [5] *Dev Cell*. 2013)				*Robo4*$^{fl/fl}$ (Zheng et al,[8] 2012)	*Slit2*$^{fl/fl}$ (Gibson et al,[6] 2014; Rama et al,[7] 2015)		*Slit3*$^{fl/fl}$	
		E18.5				E18.5	E18.5		P0	E18.5
Congenital defect	Wild type	No cre Full *Robo1*$^{-/-}$	Nkx2.5-cre (Moses et al,[9] 2001) All cardiac tissues including second heart field and excluding neural crest	Tie2-cre (Koni et al,[11] 2001) Endocardium, endocardially derived tissues	Wnt1-cre (Danielian et al,[10] 1998) Neural crest	Tie2-cre (Koni et al,[11] 2001) Endocardium, endocardially derived tissues	Tie2-cre (Koni et al,[11] 2001) Endocardium, endocardially derived tissues	Wnt1-cre (Danielian et al,[10] 1998) Neural crest	Tie2-cre (Koni et al,[11] 2001) Endocardium, endocardially derived tissues	Myh11-cre^{ERT2} (Wirth et al,[12] 2008) Smooth muscle
Ventricular septal defect—membranous	0% (0/35)	11% (1/9)	33% (2/6)	20% (1/5)	17% (1/6)	0% (0/6)	0% (0/5)	0% (0/5)	17% (1/6)	17% (1/6)
Ventricular septal defect—muscular	6% (2/35)	11% (1/9)	17% (1/6)	20% (1/5)	0% (0/6)	0% (0/6)	20% (1/5)	0% (0/5)	0% (0/6)	0% (0/6)
Atrial septal defect	0% (0/33)	11% (1/9)	0% (0/6)	0% (0/5)	0% (0/6)	0% (0/6)	0% (0/5)	0% (0/5)	0% (0/5)	20% (1/5)
Bicuspid aortic valves	0% (0/36)	11% (1/9)*1/9	17% (1/6)*1/6	40% (2/5)‡1/5†1/5	100% (6/6)*4/6†1/6‡1/6§	17% (1/6)	20% (1/5)*1/5	0% (0/5)	100% (6/6)‡6/6§	50% (3/6)‡1/6†2/6§
Bicuspid pulmonary valves	0% (0/35)	0% (0/9)	0% (0/6)	20% (1/5)	33% (2/6)	0% (0/5)	0% (0/5)	0% (0/5)	17% (1/6)	17% (1/6)
Immature aortic valves¶	0% (0/36)	33% (3/9)	67% (4/6)	60% (3/5)	100% (5/5)	0% (0/6)	20% (1/5)	20% (1/5)	100% (5/5)	33% (2/6)
Immature pulmonary valves¶	0% (0/36)	22% (2/9)	33% (2/6)	40% (2/5)	40% (2/5)	0% (0/6)	0% (0/5)	20% (1/5)	60% (3/5)	67% (4/6)
Left persistent inferior caval vein	0% (0/36)	0% (0/9)	0% (0/6)	0% (0/5)	0% (0/6)	0% (0/6)	0% (0/5)	0% (0/5)	100% (6/6)	20% (1/5)
Absent left superior caval vein	0% (0/36)	0% (0/9)	0% (0/6)	0% (0/5)	0% (0/6)	0% (0/6)	0% (0/5)	0% (0/5)	0% (0/6)	50% (3/6)
Pericardial defect	0% (0/36)	11% (1/9)	0% (0/6)	0% (0/5)	0% (0/6)	0% (0/6)	0% (0/5)	0% (0/5)	0% (0/6)	0% (0/6)
Diaphragmatic hernia	3% (1/36)	0% (0/9)	33% (2/6)	0% (0/5)	0% (0/6)	0% (0/6)	0% (0/5)	0% (0/5)	100% (6/6)	83% (5/6)

E indicates embryonic day, P indicates postnatal day. Slashed data in parentheses indicate the number affected hearts of the total analyzed. Robo, Roundabout Guidance Receptor. Slit, Slit Guidance Ligand. Nkx2.5, NK2 Homeobox 5. Wnt1, Wnt Family Member 1. Tie2/Tek, TEK Receptor Tyrosine Kinase. Myh11, Myosin Heavy Chain 11.

*Bicuspid aortic valve subtype: bicuspid valves without visible raphe—noncoronary leaflet missing.

†Bicuspid aortic valve subtype: bicuspid vales with visible raphe—fusion of left and noncoronary leaflets.

‡Bicuspid aortic valve subtype: bicuspid vales with visible raphe—fusion of right and noncoronary leaflets.

§Indication of interaction between endocardial SLIT3 and neural crest ROBO2 for bicuspid aortic valves.

¶Defined as average valve size at least 25% larger than the average littermate wild-type valve size.

receptors during heart development. These data are important to fully understand the effects of disruption of this pathway in patients with variants in these genes.

METHODS

The data supporting the findings of this study are available from the corresponding author upon request. All experimental procedures were performed in accordance with the UK revised Animals (Scientific Procedures) Act 1986 and the European Directive 2010/63/EU, and approval has been obtained from Oxford University's central Committee on Animal Care and Ethical Review. *Robo1*^{tm1Matl}*; Robo2*^{tm1Rilm} (*Robo1*$^{-/-}$*; Robo2*^{flox}),[5] *Slit2*$^{tm1.1Ics}$ (*Slit2*^{flox}),[6,7] *Robo4*^{flox},[8] *Nkx2-5*$^{tm1(cre)Rjs}$ (NK2 Homeobox 5, *Nkx2.5-cre*),[9] *H2az2*$^{Tg(Wnt1-cre)11Rth}$ (Wnt Family Member 1, *Wnt1-cre*),[10] *Tg(Tek-cre)12Flv* (TEK Receptor Tyrosine Kinase,*Tie2-cre*),[11] and *Tg(Myh11-cre/ERT2)1Soff/J* (Myosin Heavy Chain 11, *Myh11-cre*^{ERT2})[12] were all maintained on a pure C57BL/6J background. A conditional *Slit3*$^{tm1c(EUCOMM)Hmgu}$ line was generated using an EUCOMM (European Conditional Mouse Mutagenesis Programme) embryonic stem cell line (http://www.informatics.jax.org/allele/key/615486). The day the vaginal plug was found was considered embryonic day (E) 0.5. For tamoxifen-dependent, tissue-specific gene activation, two 100 mg/kg doses of tamoxifen were administered by oral gavage to pregnant dams at E12.5 and E14.5. E18.5 embryos or postnatal day (P) 0 neonates were fixed overnight in 4% paraformaldehyde and embedded in paraffin. 10 µm paraffin sections were mounted and stained for 4′,6-diamidino-2-phenylindole and cardiac troponin I by immunohistochemistry.[4] Sections were scored for defects and volume measurements were carried out blinded as described previously using Amira 6.7.0 (Thermo Fisher Scientific).[4]

RESULTS

As a conditional *Robo1* line was unavailable and we failed to generate a floxed line using the *Robo1*$^{tm1a(KOMP)WTsi}$ vector from KOMP (The Knockout Mouse Programme) (https://www.komp.org/geneinfo.php?geneid=77367), we removed *Robo2* in a tissue-specific manner from ubiquitous *Robo1* knockouts. This *Robo1* gene trap is a different ubiquitous mutant from previous studies (full gene removal, *Robo1*^{tm1Wian}),[3,4,7] showing lower penetrance of pericardial defects as well as membranous ventricular septal defect (all phenotypes are summarized in the Table). Additional removal of *Robo2* specifically from either the whole heart, endocardium and its derivatives, or the neural crest increased the incidence of ventricular septal defect, but not to the level previously observed in ubiquitous *Robo1; Robo2* knockouts. This suggests that *Robo2* expression is important in all these tissues despite the absence of defects in ubiquitous *Robo2* knockouts.[4] BAV observed in all ubiquitous *Robo1; Robo2* knockouts showed full penetrance in the *Robo1*$^{-/-}$*; Robo2*$^{fl/fl}$*; Wnt1-cre* line, indicating that Robo2 is specifically important in the neural crest for semilunar valve development. Besides a contribution from neural crest cells, the cells in the valves derive from endocardial to mesenchymal transformation and the second heart field.[13] Accordingly, crosses with *Tie2-cre* and *Nkx2-5cre*, which target both the endocardial and second heart field contributions, showed a higher percentage of immature valves and BAV than observed when knocking out *Robo1* alone, indicating a role for *Robo2* in all 3 lineages. *Robo4* expression seems specific to the endocardium and,[3] correspondingly, removing *Robo4* from the endocardium resulted in a low but similar penetrance of BAV as has been observed in ubiquitous *Robo4* mutants.[2] Ubiquitous *Slit2* knockouts described before showed low penetrant BAV,[4] and this is fully recapitulated by endocardial-specific, but not neural crest-specific, knockout of *Slit2*. As a conditional *Slit3* line did not exist, we generated a *Slit3*^{flox} line. Intriguingly, although showing a similar range of defects as observed in ubiquitous *Slit3* knockouts,[3,4] the penetrance of these defects was higher in the different tissue-specific mutants. Specifically removing *Slit3* from the endocardium resulted in fully penetrant BAV, whereas removal from smooth muscle cells, in which *Slit3* is highly expressed, also resulted in BAV. In addition, although caval vein defects were observed in ubiquitous *Slit3* knockouts, these were more severe in the conditional lines. A persistent left inferior caval vein was observed in all the *Slit3*$^{fl/fl}$*; Tie2-cre* hearts analyzed, whereas smooth muscle–specific knockout of *Slit3* resulted in the complete absence of the left superior caval vein. Diaphragmatic hernias as described in the full *Slit3* knockout[3] were also seen when removing *Slit3* from either the endocardium or smooth muscle and, unexpectedly as the *Nkx2.5-cre* is not known to target the diaphragm, in the *Robo1*$^{-/-}$*; Robo2*$^{fl/fl}$*; Nkx2.5-cre*.

CONCLUSIONS

These data will help understand the defects observed in patients with variants in *ROBO1* and *ROBO4*. The similarity in phenotypes strongly indicates an interaction between endocardial SLIT3 and neural crest ROBO2 for BAV (see § in Table) and highlights the need for further studies of this connection. Clinically, *SLIT3* is an especially promising candidate for further screening in patients.

ARTICLE INFORMATION

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Disclosures

None.

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