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

Juanjuan Zhao, PhD*; Susann Bruche, PhD*; Helen G. Potts, MS; Benjamin Davies, PhD; Mathilda T. M. Mommersteeg, PhD

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 knockout 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
| Congenital defect                  | Wild type | Full Robo1<sup>−/−</sup> | Robo<sup>1−/−</sup>; Robo2<sup>−/−</sup> (Domyan et al, 2013) | Robo4<sup>−/−</sup> (Zheng et al, 2012) | Slit2<sup>−/−</sup> (Gibson et al, 2014; Rama et al, 2015) | Slit3<sup>−/−</sup> |
|-----------------------------------|-----------|--------------------------|---------------------------------------------------------------|-----------------------------------------|---------------------------------------------------------------|----------------|
| Ventricular septal defect—membranous | 0% (0/35) | 11% (1/9)                | Nkx2.5-cre (Moses et al, 2001)                                | E18.5                                   | Wnt1-cre (Danielian et al, 1998)                              | E18.5 |
| Ventricular septal defect—muscular | 6% (2/35) | 11% (1/9)                | Tie2-cre (Koni et al, 2001)                                   | E18.5                                   | Tie2-cre (Koni et al, 2001)                                   | E18.5 |
| Atrial septal defect              | 0% (0/33) | 11% (1/9)                | Endocardium, endocardially derived tissues                    | E18.5                                   | Endocardium, endocardially derived tissues                    | E18.5 |
| Bicuspid aortic valves            | 0% (0/36) | 11% (1/9)<sup>†</sup>    | E18.5                                                         | E18.5                                   | Wnt1-cre (Danielian et al, 1998)                              | E18.5 |
| Bicuspid pulmonary valves         | 0% (0/35) | 0% (0/9)                 | E18.5                                                         | E18.5                                   | Tie2-cre (Koni et al, 2001)                                   | E18.5 |
| Immature aortic valves            | 0% (0/36) | 33% (3/9)                | Endocardium, endocardially derived tissues                    | E18.5                                   | Endocardium, endocardially derived tissues                    | E18.5 |
| Immature pulmonary valves         | 0% (0/36) | 22% (2/9)                | Endocardium, endocardially derived tissues                    | E18.5                                   | Endocardium, endocardially derived tissues                    | E18.5 |
| Left persistent inferior caval vein| 0% (0/36) | 0% (0/9)                 | Endocardium, endocardially derived tissues                    | E18.5                                   | Endocardium, endocardially derived tissues                    | E18.5 |
| Absent left superior caval vein    | 0% (0/36) | 0% (0/9)                 | Endocardium, endocardially derived tissues                    | E18.5                                   | Endocardium, endocardially derived tissues                    | E18.5 |
| Pericardial defect                | 0% (0/36) | 11% (1/9)                | Endocardium, endocardially derived tissues                    | E18.5                                   | Endocardium, endocardially derived tissues                    | E18.5 |
| Diaphragmatic hernia              | 3% (1/36) | 0% (0/9)                 | Endocardium, endocardially derived tissues                    | E18.5                                   | Endocardium, endocardially derived tissues                    | E18.5 |

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 valves with visible raphe—fusion of left and noncoronary leaflets.
‡Bicuspid aortic valve subtype: bicuspid valves 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. Robo1tm1Matl; Robo2tm1Rilm (Robo1−/−; Robo2flox), 5Slit2tm1.1cs (Slit2flox), 6,7Robo4flox 8Nkx2.5tm1crePrf2 (NK2 Homeobox 5, Nkx2.5-cre), 9H2az2tg(Wnt1-cre)11Rth (Wnt Family Member 1, Wnt1-cre),10Tg(Tek-cre)12Flv (TEK Receptor Tyrosine Kinase,Tie2-cre),11 and Tg(Myh11-cre/ERT2)1Soff/J (Myosin Heavy Chain 11, Myh11-creERT2)12 were all maintained on a pure C57BL/6J background. A conditional Slit3tm1c(EUCOMM) Hmgu line was generated using an EUCOMM (European Conditional Mouse Mutagenesis Programme) embryonic stem cell line (http://www.informatics.jax.org/alleles/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. 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 Robo1tm1a(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, Robo1tm1Wian),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−/−; Robo2flox; 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 Nkx2.5-cre, 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, correspondingly, removing Robo4 from the endocardium resulted in a low but substantial 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 Slit3flox 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 Slit3flox; 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 knockout4 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−/−; Robo2flox; 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

Received July 24, 2021; accepted January 11, 2022.

Affiliations

Department of Physiology, Anatomy & Genetics, Burdon Sanderson Cardiac Science Centre, University of Oxford, United Kingdom (J.Z., S.B., H.G.P.,
Zhao et al  Slit- Robo Signaling in Congenital Heart Disease

M.T.M.); and Nuffield Department of Medicine, Wellcome Centre for Human Genetics, University of Oxford United Kingdom, (B.D.).

Sources of Funding
This work was supported by the British Heart Foundation (PG/15/50/31594; FS/17/68/33478; RE/18/3/34214) and the Wellcome Trust (203141/Z/16/Z).

Disclosures
None.

REFERENCES

1. Kruszka P, Tanpaiboon P, Neas K, Crosby K, Berger SL, Martinez AF, Addissie YA, Pongprot Y, Sittiwangkul R, Silivilairat S, et al. Loss of function in ROBO1 is associated with tetralogy of Fallot and septal defects. J Med Genet. 2017;54:825–829.
2. Gould RA, Aziz H, Woods CE, Seman-Senderos MA, Sparks E, Preuss C, Wünnemann F, Bedja D, Moats CR, McClymont SA, et al. ROBO4 variants predispose individuals to bicuspid aortic valve and thoracic aortic aneurysm. Nat Genet. 2019;51:42–50. doi: 10.1038/s41588-018-0265-y
3. Mommersteeg MTM, Andrews WD, Ypsilanti AR, Zelina P, Yeh ML, Norden J, Kispert A, Chédotal A, Christoffels VM, Parnavelas JG. Slit-roundabout signaling regulates the development of the cardiac systemic venous return and pericardium. Circ Res. 2013;112:465–475. doi: 10.1161/CIRCRESAHA.112.277426
4. Mommersteeg MTM, Yeh ML, Parnavelas JG, Andrews WD. Disrupted Slit-Robo signaling results in membranous ventricular septum defects and bicuspid aortic valves. Cardiovasc Res. 2015;106:55–66. doi: 10.1093/cvr/crv040
5. Domyan ET, Branchfield K, Gibson DA, Tessier-Lavigne M, Ma L. Sun X Roundabout Receptors Are Critical for Foregut Separation from the Body Wall. Dev Cell. 2013;24:52–63. doi: 10.1016/j.devcel.2012.11.018
6. Gibson DA, Tymanskyj S, Yuan RC, Leung HC, Lefebvre JL, Sanes JR, Chédotal A, Ma L. Dendrite self-avoidance requires cell-autonomous slit/robo signaling in cerebellar Purkinje cells. Neuron. 2014;81:1040–1056. doi: 10.1016/j.neuron.2014.01.009
7. Rama N, Dubrac A, Mathivet T, Ni Chárthaigh R-A, Genet G, Cristofaro B, Pibouin-Fragner L, Ma L, Eichmann A, Chédotal A. Slit2 signaling through Robo1 and Robo2 is required for retinal neovascularization. Nat Med. 2015;21:483–491. doi: 10.1038/nm.3849
8. Zheng W, Geng AQ, Li PF, Wang Y, Yuan XB. Robo4 regulates the radial migration of newborn neurons in developing neocortex. Cereb Cortex. 2012;22:2587–2601. doi: 10.1093/cercor/bhr330
9. Moses KA, Demayo F, Braun RM, Reecy JL, Schwartz PJ. Embryonic expression of an Nkx2-5/Cre gene using ROSA26 reporter mice. Genesis. 2001;31:176–180. doi: 10.1002/gene.10022
10. Danielian PS, Muccino D, Rowitch DH, Michael SK, McMahon AP. Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. Curr Biol. 1998;8:1323–1326. doi: 10.1016/S0960-9822(07)00562-3
11. Koni PA, Joshi SK, Temann UA, Olson D, Burky L, Flavell RA. Conditional vascular cell adhesion molecule 1 deletion in mice: impaired lymphocyte migration to bone marrow. J Exp Med. 2001;193:741–753. doi: 10.1084/jem.193.6.741
12. Wirth A, Benyó Z, Lukasova M, Leutgeb B, Wettchureck N, Gorbe y S, Órsy P, Horváth B, Maser-Gluth C, Greiner E, et al. G12–G13-LARG-mediated signaling in vascular smooth muscle is required for salt-induced hypertension. Nat Med. 2008;14:64–68. doi: 10.1038/nm1666
13. Henderson DJ, Eley L, Chaudhry B. New concepts in the development and malformation of the arterial valves. J Cardiovasc Dev Dis. 2020;7:1–27. doi: 10.3390/jcdd7040038