Appendix

Specific Deletion of *Ephrin-B2* in Peripheral Endothelial Cells, but not in Heart Endocardial Cells Reveals an Essential Role of Ephrin-B2 in Peripheral Endothelial Cells

Ephrin-B2 conventional knockout and endothelial specific knockout mice show the same angiogenesis defects in head, trunk and yolk sac as well as the same heart defects during embryonic development (Gerety and Anderson, 2002; Wang et al., 1998); *ephrin-B2* is expressed in both peripheral endothelial cells and heart endocardial cells (Gerety and Anderson, 2002; Wang et al., 1998), suggesting that the defects in peripheral vasculature and the heart result from the absence of *ephrin-B2* in peripheral endothelial and heart endocardial cells, respectively. However, the fact that targeted deletion of heart-specific genes such as *Nkx2.5* and *MLC2a* (atrial myosin light chain 2) (Huang et al., 2003; Tanaka et al., 1999), which are not expressed in peripheral endothelial cells, results in peripheral angiogenesis defects as well as heart defects indicates that the peripheral angiogenesis defects in these mutant mice are secondary to the heart defects. Therefore, it is not clear whether the peripheral angiogenesis defects in *ephrin-B2* conventional knockout and endothelial specific knockout mice reflect a local requirement for ephrin-B2 signaling, or rather may be secondary to the heart defects.

To address this issue, I generated a novel Cre line by inserting an *EGFP-Cre* fusion construct into the locus of *Depp* that is expressed in peripheral arterial endothelial cells, but not in endocardial cells, as described in Chapter 2. Using this Cre line and a conditional *ephrin-B2* allele, I have deleted *ephrin-B2* in peripheral endothelial cells but not in atrial or ventricular endocardial cells of the heart. Apparently 45% of the conditional *ephrin-B2* mutants show severe angiogenesis defects in head, trunk, and yolk sac as well as heart defects at E9.5 (Fig. 1). About 45% penetrance of the phenotypes may stem from the variable Cre activity which I observed in *Depp-Cre;Rosa26R* double heterozygous embryos and yolk sacs by β-gal expression pattern (Fig. 2). The

angiogenesis defects in the conditional mutants are similar to those in *ephrin-B2* conventional mutants. However, endocardial cells in the conditional mutants display normal trabeculation in the right ventricle (Fig. 1J, arrows), whereas endocardial trabeculation is greatly reduced in the conventional mutants (Gerety and Anderson, 2002; Wang et al., 1998), suggesting that ephrin-B2 in ventricular endocardial cells is required for the endocardial trabeculation.

The detailed analysis of Depp-Cre activity and *ephrin-B2* expression in the heart reveals that Depp-Cre mediated recombination occurs in a subset of endocardial and myocardial cells of the outflow tract (Fig. 3D-F), and *ephrin-B2* is expressed in the endocardial and myocardial cells of the outflow tract but barely expressed in myocardial cells of the atria and ventricles (Fig. 3A-C, and data not shown). Therefore, it is not clear whether the severe heart defects and the angiogenesis defects witnessed in the half of the conditional mutants at E9.5 are caused by *ephrin-B2* deletion in peripheral endothelial cells or by the deletion in the endocardial and/or myocardial cells of the outflow tract.

To clarify this issue, several other Cre lines, which are active in the heart but not in peripheral endothelial cells, were used to delete *ephrin-B2* specifically in the heart. Isl1-Cre is active in a subset of endocardial and myocardial cells of the outflow tract and in a subset of myocardial cells in the rest of the heart (Cai et al., 2003); SM22α-Cre (Holtwick et al., 2002) and Nkx2.5-Cre (Moses et al., 2001) are active in most of the myocardial cells throughout the heart; and αMHC-Cre (Gaussin et al., 2002) is active in most of the myocardial cells of the atria and ventricles, but not in the outflow tract (Fig. 3G-R). None of these Cre-mediated *ephrin-B2* conditional knockout mice display any angiogenesis or heart defects at E9.5, suggesting that ephrin-B2 in the endocardial cells

of the outflow tract and in the myocardial cells of the entire heart may be not essential for proper cardiovascular development at least until E9.5. However, the fact that the number of Isl1-Cre positive endocardial cells in the outflow tract is lower than that of Depp-Cre positive endocardial cells (Fig. 3G, H vs D, E) makes it unclear whether *ephrin-B2* deletion in the endocardial cells of the outflow tract contributes to the peripheral angiogenesis defects. To clarify this point, another Cre line, active in most endocardial cells of the outflow tract but not in the endocardial cells of the atria or the ventricles, should be used. I am using a novel Cre line, NFATc1-Cre (Zhou et al., 2005), which may be active in the endocardial cells of the outflow tract and the atrioventricular canal but not in the atrial or ventricular endocardial cells, to clarify this issue.

At this point, I can not exclude the possibility that *ephrin-B2* deletion in the endocardial cells of the outflow tract causes peripheral angiogenesis defects; however, these data suggest that the angiogenesis defects in *ephrin-B2* conventional knockout mice are not due to the deletion in atrial or ventricular endocardial cells, or in the myocardial cells of the entire heart including the outflow tract.

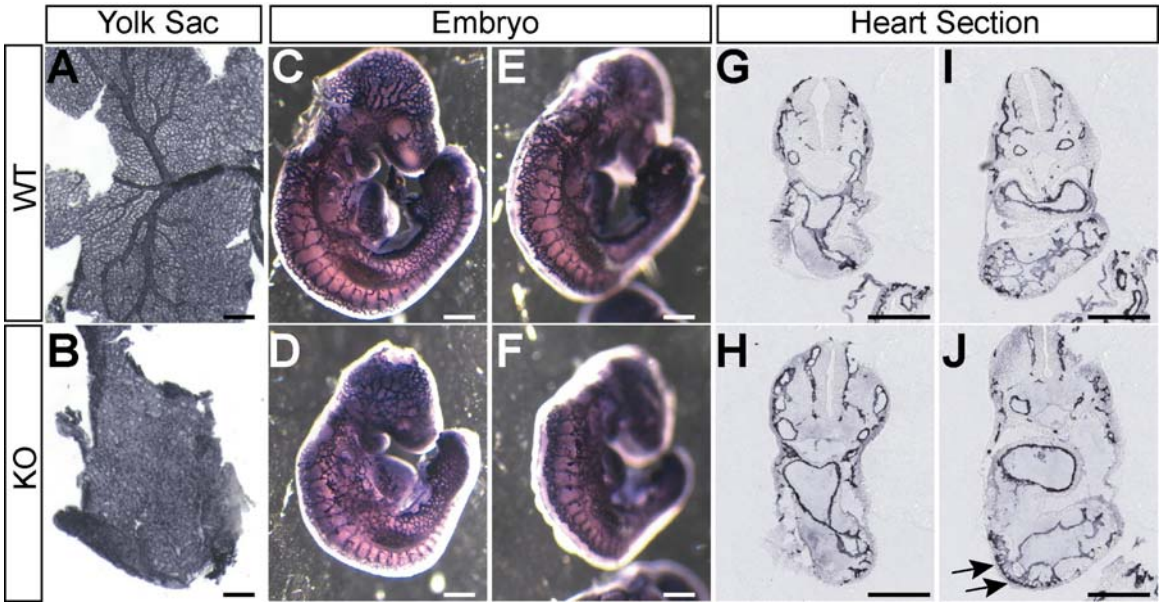

Fig. 1 Depp-Cre mediated *ephrin-B2* conditional knockout mice display peripheral angiogenesis defects as well as heart defects at E9.5

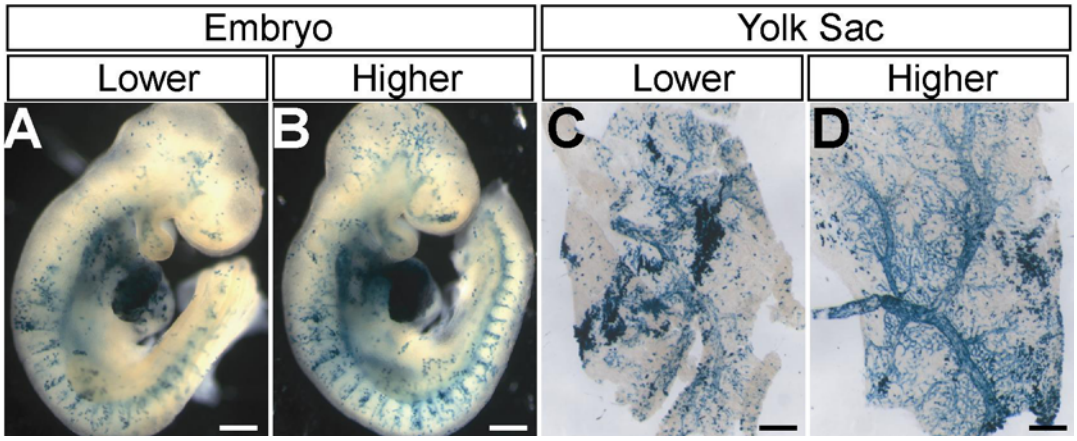

Fig. 2 Variegation of Depp-Cre mediated recombination

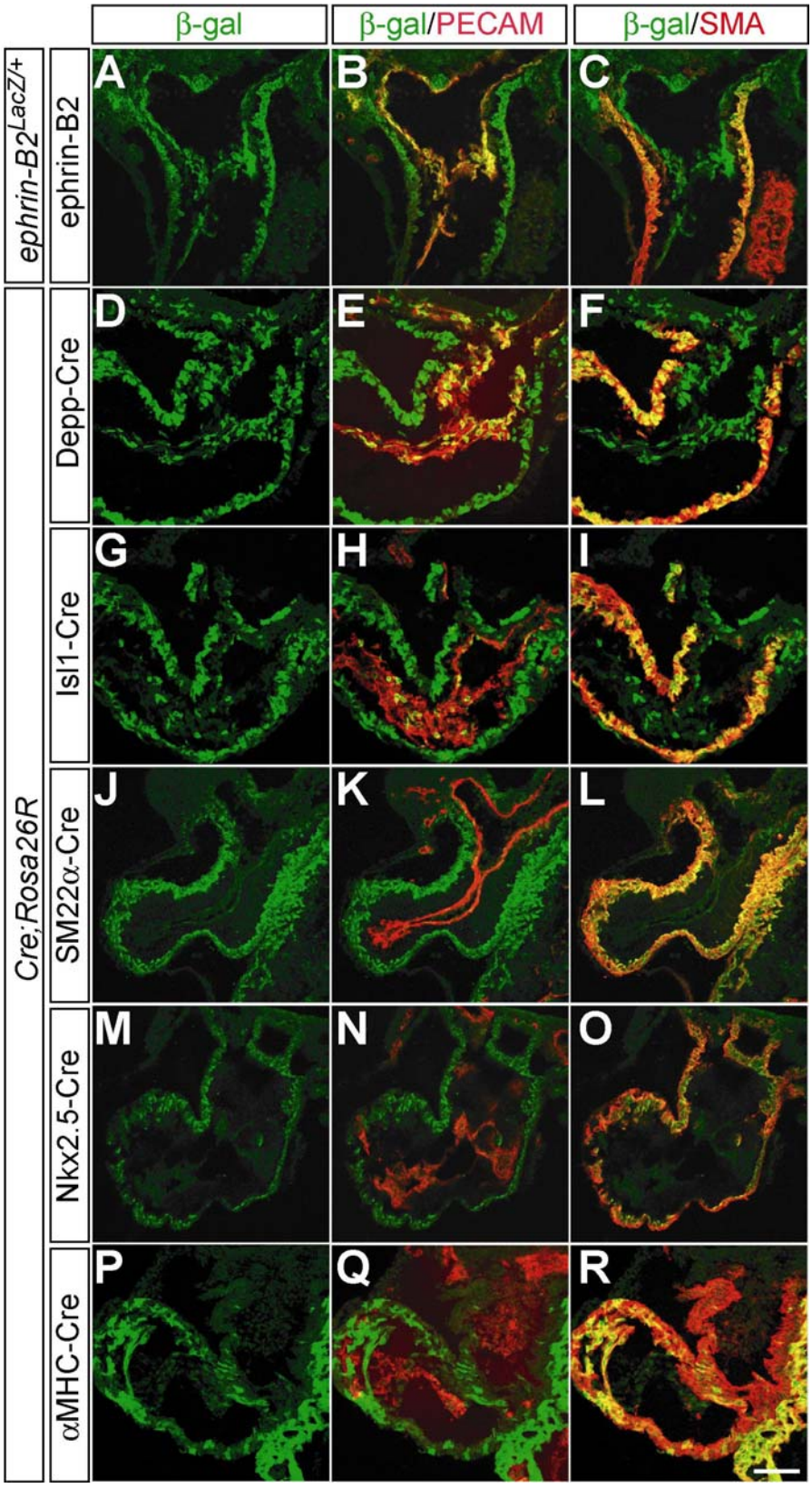

Fig. 3 *Ephrin-B2* expression and several Cre activities in the outflow tract at E9.5

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