Chapter 4

Future Directions

Investigation of ephrin-B2 function in arterial smooth muscle cells

Ephrin-B2 is expressed in arterial smooth muscle cells (SMCs) as well as in arterial endothelial cells (Gale et al., 2001; Shin et al., 2001). The essential role of ephrin-B2 in arterial endothelial cells was revealed by the analysis of *ephrin-B2* conventional knockout and endothelial-specific conditional knockout mice (Adams et al., 1999; Gerety and Anderson, 2002; Wang et al., 1998); however, the mutant mice die around E10 when *ephrin-B2* is not yet expressed in arterial SMCs, preventing the investigation of ephrin-B2 function in the SMCs, using the mutant mice. The essential function of ephrin-B2 in endothelial cells during cardiovascular development suggests that ephrin-B2 may play an important role in SMCs as well. However, the late onset of *ephrin-B2* expression in arterial SMCs suggest ephrin-B2 function in SMCs might be different from that in endothelial cells where *ephrin-B2* is expressed during early vascular development.

This remaining question can be investigated by utilizing *ephrin-B2* conditional knockout mice and smooth muscle-specific Cre deleter mice. *Ephrin-B2* conditional knockout mice were generated by Dr. Gerety in my lab (Gerety and Anderson, 2002); two transgenic smooth muscle-specific Cre deleter lines, SMMHC-Cre and SM22α-Cre, are available (Holtwick et al., 2002; Regan et al., 2000). SMMHC-Cre mediated deletion of *ephrin-B2* in arterial SMCs did not reveal any defects in the mutant mice. The careful analysis of the Cre activity of the transgenic SMMHC-Cre line revealed that the SMMHC-Cre mediated recombination occurred in a very small number of arterial smooth muscle cells at E15.5 (Fig. 1A-C), indicating that this Cre line is not useful. However, SM22α-Cre mediated recombination extensively occurs in arterial SMCs but not in endothelial cells at E13.5 (Fig. 1D-F), suggesting that it is worth while to use a

SM22α-Cre deleter line to specifically remove *ephrin-B2* in SMCs but not in endothelial cells.

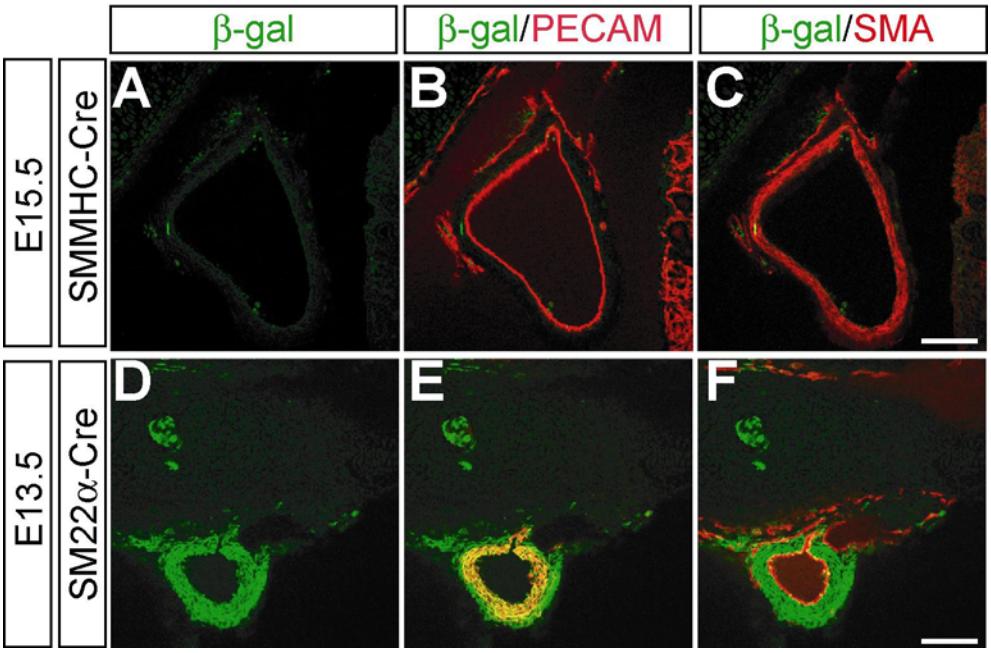

Fig. 1. SMMHC-Cre and SM22α-Cre mediated recombination in the dorsal aorta

What controls the initiation of *ephrin-B2* expression in arterial SMCs?

VEGF and Notch signaling are involved in *ephrin-B2* induction in arterial endothelial cells (Weinstein and Lawson, 2002), while the signal to induce the expression of *ephrin-B2* in arterial SMCs remains elusive. Several Notch receptors and ligands are expressed in arterial SMCs as well as in arterial endothelial cells (Villa et al., 2001), suggesting that Notch signaling might be involved in *ephrin-B2* induction in arterial SMCs like it induces *ephrin-B2* expression in endothelial cells. Since conventional knockouts of these genes result in early embryonic lethality due to their essential roles in endothelial cells, such knockout mice cannot be used to address this question. Instead, the conditional knockout approach is applicable to the question, by specifically deleting the genes in SMCs but not

in endothelial cells using SM22α-Cre deleter line. *Notch1* conditional knockout mice are available (Limbourg et al., 2005), but the conditional knockout mice of other Notch receptors and ligands have to be generated to address the question.

Neuropilin-1 (NP-1) is expressed in SMCs as well as in endothelial cells (Mukouyama et al., 2002; Yuan et al., 2002); therefore, the same approach suggested for Notch signaling could be applied to address whether NP-1-mediated VEGF signaling is involved in *ephrin-B2* induction in SMCs. In addition, conventional *NP-1* knockout mice die around E13.5, allowing me to assess whether *ephrin-B2* induction in arterial SMCs at E12.5-E13.5 is affected in the knockout mice.

Ephrin-B2 functions in adult stable vasculature and neovasculature

Although ephrin-B2 is essential for angiogenesis during embryonic development, it is not clear whether ephrin-B2 still plays an important role in adult vasculature. The observation that *ephrin-B2* is maintained in adult arterial endothelial cells, and expressed in a subset of endothelial cells in tumors, during wound healing, and in the corneal micropocket assay suggests that ephrin-B2 may play an important role in adult vasculature (Gale et al., 2001; Shin et al., 2001).

This issue can be addressed by conditional knockout of *ephrin-B2* in adults. The endothelial-specific, temporal control of Cre activity can be achieved by the use of inducible Cre-ER (cre recombinase-estrogen receptor fusion), which is only active in the presence of estrogen analog tamoxifen (Danielian et al., 1998; Kellendonk et al., 1996), under an endothelial specific promoter such as *Flk1*, *Tie2*, or *Scl*. Tie2-Cre-ER and Scl-Cre-ER mice are available (Forde et al., 2002; Gothert et al., 2004), and Scl-Cre-ER

mediated recombination occurs in the endothelial cells of most adult tissues including tumors (Gothert et al., 2004). This temporal, spatial ablation of *ephrin-B2* mediated by Tie2-Cre-ER or Scl-Cre-ER will reveal the function of ephrin-B2 in endothelial cells in adult tissues. In addition, using inducible Cre-ER under the promoter of pan-neovascular markers, such as D1.1, the function of ephrin-B2 in adult neovasculature could be investigated. However, the Cre-ER mice still need to be generated in order to perform these experiments.

If these studies demonstrate that ephrin-B2 is essential for adult vasculature and/or neovasculature, ephrin-B2 mediated signaling may serve as a useful target for therapeutic intervention.

Further application of the differential screening method

The differential screening method, which I used to isolate arterial specific genes from a small number of endothelial cells has several advantages over other differential screening methods. First, a small number of cells (about 10-100 cells) are sufficient for this screen; therefore, this screen is applicable to the case where it is impossible to isolate a large number of cells (more than 500 cells). Second, low abundant genes can be isolated by the subsequent subtraction of high abundant genes, identified in the previous subtraction, from cDNA libraries containing both high and low abundant genes. This subsequent subtraction followed by PCR amplification can further increase the number of cDNAs of low abundant genes in the cDNA libraries. Low abundant genes are not usually detected in a differential screen without subsequent enhancement of their signals; therefore, such enhancement is essential for detecting low abundant genes in a differential screen. If

these two points are crucial for studies involving a differential screen, this method may be a good choice.

This method can be used to isolate more markers to distinguish arterial SMCs from venous SMCs. Although no SMC-specific antibodies are available for fluorescence activated-cell sorter (FACS), arterial and venous SMCs can be isolated by sorting PECAM-1 negative populations from umbilical arteries and veins, respectively. The isolation and characterization of novel arterial or venous SMC-specific genes will improve the understanding of cardiovascular developments as well as the functions of vascular SMCs like those of endothelial-specific genes greatly improve the understanding of cardiovascular development.

Significance of the molecular heterogeneity of arterial endothelial cells revealed by *Depp*

Although the detailed analysis of *Depp* expression at E9.5 dorsal aorta reveals the molecular heterogeneity of arterial endothelial cells in a single vessel, the significance of this heterogeneity remains elusive. Whether the heterogeneity of *Depp* expression is essential for proper vascular development can be tested by ectopically expressing DEPP in arterial endothelial cells under *ephrin-B2* promoter. This genetic approach will result in the homogeneous expression of DEPP in arterial endothelial cells, permitting us to investigate any significance of the heterogeneous expression of *Depp*. In addition, I can investigate the significance of arterial-restricted expression of *Depp* by ectopically expressing DEPP in all endothelial cells under a pan-endothelial promoter such as *Tie2* or *PECAM-1*.

To get more clues about the significance of the heterogeneity, gene expression profiles of *Depp*$^{+}$ and *Depp*$^{-}$ arterial endothelial cells could be compared with each other. I purified *Depp*$^{+}$ and *Depp*$^{-}$ endothelial cells from the whole E10 embryos, and compared their gene expression profiles. Several known arterial specific genes were enriched in *Depp*$^{+}$ endothelial population; a known lymphatic marker, LYVE-1, was highly enriched in *Depp*$^{-}$ endothelial population. However, these experiments did not provide any clues about the significance. *Depp*$^{-}$ endothelial population contains a subset of arterial endothelial cells, all venous endothelial cells, all lymphatic endothelial cells, and maybe progenitor cells negative for *Depp*. The complexity of *Depp*$^{-}$ endothelial population might result in various differences in gene expression profiles, preventing me from deducing any clues. Furthermore, the use of the whole embryos, containing various types of vessels such as large, medium, and small vessels, could mask any differences that are specifically associated with one of these populations. Therefore, the use of *Depp*$^{+}$ and *Depp*$^{-}$ arterial endothelial cells from E9.5-E10 dorsal aorta of *Depp*$^{EGFP/+}$*;ephrin-B2*$^{lacZ/+}$ double heterozygous embryos will reduce the complexity in each population and increase the specific differences related to *Depp* expression. Any clues deduced from this approach will serve as a starting point to investigate the functional significance of the heterogeneity in detail.

Distinction between primary and secondary defects in cardiovascular development

Most homozygous mutant mice, showing severe angiogenesis defects in embryos and yolk sacs, display severe heart defects. The genes responsible for these phenotypes are typically expressed in both peripheral endothelial cells and endocardial cells, suggesting

their essential roles in endothelial and 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. If any mutant mice display both peripheral angiogenesis defects and heart defects, it should be carefully considered whether these defects are primary or secondary defects.

Depp is expressed in peripheral endothelial cells, but not in the endocardial cells of the heart; furthermore, *Depp-Cre* knockin mice reveal that Depp-Cre mediated recombination does not occur in atrial or ventricular endocardial cells of the heart (as described in Chapter 2). This unique expression pattern allows Depp-Cre mice to be used as a Cre deleter line to specifically remove genes in peripheral endothelial cells but not in heart endocardial cells. By deleting genes, expressed in both peripheral endothelial and heart endocardial cells, only in peripheral endothelial cells, the function of the genes in peripheral endothelial cells can be investigated without being affected by heart defects.

Ephrin-B2 conventional knockout and endothelial specific knockout mice show the same angiogenesis and heart defects; *ephrin-B2* is expressed in both peripheral endothelial cells and heart endocardial cells. Therefore, it is not clear whether the peripheral angiogenesis defects in the mutant reflect a local requirement for ephrin-B2 signaling, or rather are secondary to the heart defects. I used the Depp-Cre line and a conditional *ephrin-B2* allele, in order to distinguish whether the peripheral angiogenesis

defects is primary or secondary to the heart defects. The detailed analysis and data are described in the Appendix.

Although the Depp-Cre deleter line is useful in terms of peripheral endothelial specificity, the Cre-mediated recombination occurs in a subset of arterial endothelial cells, and the number of recombination-occurring cells varies among different embryos. I recently found that connexin40 (Cx40), a gap junction protein, is expressed in arterial endothelial cells but not in endocardial cells like *Depp*. In addition, Cx40 is expressed in most arterial endothelial cells, while *Depp* is expressed in a subset of arterial endothelial cells, suggesting that Cx40-Cre-mediated recombination might occur in most arterial endothelial cells, and the number of recombination-occurring cells might be similar among different embryos. Thus, it is worthwhile to generate Cx40-Cre mice, serving as a useful arterial-specific Cre deleter line.

D1.1 monoclonal antibody production

Since *D1.1* may be a useful marker of adult neovasculature, the generation of monoclonal antibodies would be valuable for diagnosis of neovascularization and for drug targeting to neovasculature.

Mouse monoclonal antibodies to mouse D1.1 can be generated using *D1.1* homozygous mutant mice lack of any phenotypic defects. The mutant mice do not produce D1.1 proteins; thus, they recognize mouse D1.1 proteins as a foreign antigen, initiating antibody production. By injecting mouse D1.1-Fc proteins, that I used for in vivo and in vitro angiogenesis assays, into the homozygous mutant mice, and by subsequently testing specific binding of antibodies in hybridoma supernatants on cells

transfected with mouse *D1.1* expression plasmids, mouse monoclonal antibodies to mouse D1.1 might be generated and selected.

In addition, mouse monoclonal anti-human D1.1 antibodies could be generated in a similar way to anti-mouse D1.1 antibodies, by injecting human D1.1-Fc proteins into wild-type or the homozygous mutant mice and the subsequent testing. If the expression pattern of human D1.1 is similar to that of mouse D1.1, anti-human D1.1 antibodies could be useful to detect neovessels in human samples. Furthermore, the antibodies could be used to deliver conjugated drugs into the endothelial cells of neovasculature.

Overexpression studies of D1.1

Despite the absence of any phenotypic defects in *D1.1* homozygous mutant mice, the overexpression of D1.1 in all endothelial cells might reveal the function of D1.1 in cardiovasculature. *D1.1* is barely expressed in the veins of embryonic yolk sacs, and is barely or weakly expressed in the microvessels of most adult tissues. The mis-expression of D1.1 under a pan-endothelial promoter, such as *Flk1*, *Tie-2*, or *PECAM-1*, would yield homogeneous D1.1 expression in all endothelial cells during development and in adulthood. If the absence and down-regulation of *D1.1* expression in the veins of yolk sacs and in the microvessels of adult tissues, respectively, are essential for proper vessel development or maintenance of vessel integrity, the gain-of-function studies might reveal the function of D1.1, which can not be observed by loss-of-function studies.

Acute loss-of-function studies of D1.1 in vitro

Although D1.1-Fc acts as an angiogenic inhibitor in several angiogenic assays, it is not clear whether it is an agonist or antagonist of D1.1, making it hard to conclude an actual function of D1.1 based on D1.1-Fc activity. Any phenotypic defects in *D1.1* homozygous mutant mice will clarify the issue, but there are no such defects in the mutant mice.

From the beginning of development, *D1.1* is absent in *D1.1* homozygous mutant mice; therefore, it is possible that the homozygous mutant mice might have built up mechanisms to compensate for lack of *D1.1*. If this is the reason that the homozygous mutants appear phenotypically normal, the acute deletion of *D1.1* might reveal the function of D1.1, by depriving endothelial cells from sufficient time to compensate for lack of D1.1. The acute deletion of *D1.1* can be achieved through RNAi approach using HUVEC cells, whose general and VEGF-induced migration is significantly reduced in the presence of D1.1-Fc. If the acute deletion reduces the migration of HUVEC cells, D1.1-Fc acts as an antagonist, and the actual function of D1.1 is pro-angiogenic. If the acute deletion enhances the migration of HUVEC cells, D1.1-Fc acts as an agonist, and the actual function of D1.1 is anti-angiogenic.

REFERENCES

Adams, R. H., Wilkinson, G. A., Weiss, C., Diella, F., Gale, N. W., Deutsch, U., Risau, W., and Klein, R. (1999). Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. *Genes & Development* **13,** 295-306.

Danielian, P. S., Muccino, D., Rowitch, D. H., Michael, S. K., and McMahon, A. P. (1998). Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. *Current Biology* **8,** 1323-1326.

Forde, A., Constien, R., Grone, H. J., Hammerling, G., and Arnold, B. (2002). Temporal Cre-mediated recombination exclusively in endothelial cells using Tie2 regulatory elements. *Genesis* **33,** 191-197.

Gale, N. W., Baluk, P., Pan, L., Kwan, M., Holash, J., DeChiara, T. M., McDonald, D. M., and Yancopoulos, G. D. (2001). Ephrin-B2 selectively marks arterial vessels and neovascularization sites in the adult, with expression in both endothelial and smooth-muscle cells. *Developmental Biology* **230,** 151-160.

Gerety, S. S., and Anderson, D. J. (2002). Cardiovascular ephrinB2 function is essential for embryonic angiogenesis. *Development* **129,** 1397-1410.

Gothert, J. R., Gustin, S. E., van Eekelen, J. A. M., Schmidt, U., Hall, M. A., Jane, S. M., Green, A. R., Gottgens, B., Izon, D. J., and Begley, C. G. (2004). Genetically tagging endothelial cells in vivo: bone marrow-derived cells do not contribute to tumor endothelium. *Blood* **104,** 1769-1777.

Holtwick, R., Gotthardt, M., Skryabin, B., Steinmetz, M., Potthast, R., Zetsche, B., Hammer, R. E., Herz, J., and Kuhn, M. (2002). Smooth muscle-selective deletion

of guanylyl cyclase-A prevents the acute but not chronic effects of ANP on blood pressure. *Proceedings of the National Academy of Sciences of the United States of America* **99,** 7142-7147.

Huang, C. Q., Sheikh, F., Hollander, M., Cai, C. L., Becker, D., Chu, P. H., Evans, S., and Chen, J. (2003). Embryonic atrial function is essential for mouse embryogenesis, cardiac morphogenesis and angiogenesis. *Development* **130,** 6111-6119.

Kellendonk, C., Tronche, F., Monaghan, A. P., Angrand, P. O., Stewart, F., and Schutz, G. (1996). Regulation of cre recombinase activity by the synthetic steroid RU 486. *Nucleic Acids Research* **24,** 1404-1411.

Limbourg, F. P., Takeshita, K., Radtke, F., Bronson, R. T., Chin, M. T., and Liao, J. K. (2005). Essential role of endothelial Notch1 in angiogenesis. *Circulation* **111,** 1826-1832.

Mukouyama, Y., Shin, D., Britsch, S., Taniguchi, M., and Anderson, D. J. (2002). Sensory nerves determine the pattern of arterial differentiation and blood vessel branching in the skin. *Cell* **109,** 693-705.

Regan, C. P., Manabe, I., and Owens, G. K. (2000). Development of a smooth muscle-targeted cre recombinase mouse reveals novel insights regarding smooth muscle myosin heavy chain promoter regulation. *Circulation Research* **87,** 363-369.

Shin, D., Garcia-Cardena, G., Hayashi, S. I., Gerety, S., Asahara, T., Stavrakis, G., Isner, J., Folkman, J., Gimbrone, M. A., and Anderson, D. J. (2001). Expression of ephrinB2 identifies a stable genetic difference between arterial and venous vascular smooth muscle as well as endothelial cells, and marks subsets of

microvessels at sites of adult neovascularization. *Developmental Biology* **230,** 139-150.

Tanaka, M., Chen, Z., Bartunkova, S., Yamasaki, N., and Izumo, S. (1999). The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development. *Development* **126,** 1269-1280.

Villa, N., Walker, L., Lindsell, C. E., Gasson, J., Iruela-Arispe, M. L., and Weinmaster, G. (2001). Vascular expression of Notch pathway receptors and ligands is restricted to arterial vessels. *Mechanisms of Development* **108,** 161-164.

Wang, H. U., Chen, Z. F., and Anderson, D. J. (1998). Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell* **93,** 741-753.

Weinstein, B. M., and Lawson, N. D. (2002). Arteries, veins, notch, and VEGF. *Cold Spring Harbor Symposia on Quantitative Biology* **67,** 155-162.

Yuan, L., Moyon, D., Pardanaud, L., Breant, C., Karkkainen, M. J., Alitalo, K., and Eichmann, A. (2002). Abnormal lymphatic vessel development in neuropilin 2 mutant mice. *Development* **129,** 4797-4806.