Edifying the role of genes that are important for the development of the vascular endothelium and their time course of expression throughout ontogeny is an important and exciting area of ongoing investigation. In addition to advances that can be made to better understand the vasculature from a developmental perspective, there are obvious clinical implications from determining how gene expression in the endothelium participates in blood vessel formation and function. For example, since tumors require an adequate supply of blood to proliferate, there is a tremendous interest in uncovering mechanisms controlling vascularization and angiogenesis. Moreover, enhancing our understanding of the regulation of gene expression in the blood vessel wall has clear clinical implications for improving collateral blood vessel formation in tissues after an ischemic insult and in peripheral vascular disease.

One of the most common methods to experimentally examine how a gene is regulated is by “promoter bashing.” Promoter bashing is usually performed by transient transfection of recombinant DNA constructs consisting of the 5′ flanking region of the gene of interest fused to easily assayable reporter genes such as luciferase or LacZ. The presumption is that the 5′ flanking sequence contains regulatory elements vital to the control of transcriptional activity that are required for targeting cell-specific expression. Systematic deletions or mutations created in the construct with subsequent evaluation of their effects on reporter gene activity, coupled with molecular assays designed to identify cognate transcription factors, has been a powerful approach to identify regulatory elements and proteins regulating gene expression. However, it is critical to recognize that for these sequences and factors to have true physiological relevance, the results obtained in vitro must be translated in a meaningful way in vivo.

The Tie-2 gene, a receptor tyrosine kinase that binds members of the angiopoietin family, is exclusively expressed in the vascular endothelium and is involved in angiogenesis and the remodeling and repair of the vascular wall. Its importance in the endothelium is evident from its evolutionary conservation, a homolog of Tie-2 is specifically expressed in the endothelium of Zebrafish, and from the lethality observed in knockout mice. Tie-2, like other endothelial-specific genes Flt-1, Flk-1, Tie-1, ICAM-2, eNOS, E-selectin and VE-cadherin, has been studied extensively to uncover mechanisms of endothelial specificity. Although each of these gene has been shown to specifically target endothelial cells in transgenic assays, current evidence suggests that they each target distinct subsets of endothelial cells. This has led Minami et al. to hypothesize that genes expressed in the vascular endothelium contain distinct “DNA modules” providing regional specificity to the vasculature.

In this issue of Arteriosclerosis, Thrombosis, and Vascular Biology, Minami et al. extend on previous studies that evaluated endothelial-specific expression of Tie-2, by identifying specific motifs in the 5′ flanking sequence and first intron that are required for endothelial-specific expression in vivo. Like numerous other experiments of this kind, they first evaluated variations of the Tie-2 promoter by transfection in cultured cells. Their experiments demonstrate that a short segment (~105 to +318) of the Tie-2 gene is sufficient to trigger robust expression of LacZ in cultured progenitor and mature endothelial cells. Transcriptional activity was significantly increased by the addition of a 300-base pair intronic segment; this increase occurred when the intronic DNA was placed in either the forward or reverse orientation, suggesting it may act as a transcriptional enhancer. DNase1 footprinting and mutational analysis revealed the presence of several Ets and GATA motifs. Extensive mutagenesis suggested that the Ets motifs in the first exon are crucial for both the magnitude and endothelial-specificity of expression. The Ets motifs in the intronic enhancer may cooperate with those in the first exon because their mutation in both locations markedly diminished transcriptional activity of the Tie-2 promoter. Ets motifs have also been identified in the regulatory sequences controlling some of the other endothelial-specific genes mentioned above.

What sets this study apart from so many others is the manner in which they extended their in vitro findings to the whole animal. Instead of simply deriving transgenic mouse lines, they used homologous recombination in embryonic stem cells to target several of their Tie-2-LacZ reporter constructs in a single copy to a known site in the genome. They chose the HPRT locus because recombination events in that locus are easily selectable in embryonic stem cells. This is a particularly elegant and novel approach because by targeting each transgene in a single copy and to the identical insertion site, the investigators have effectively circumvented the problems typically encountered when analyzing trans-
genic mice generated by pronuclear microinjection, that is, position artifacts caused by the insertion of the transgene in the proximity of other regulatory elements, and copy number effects caused by the integration of multiple units of the transgene at the site of insertion. Both these problems limit an investigator’s ability to make direct comparisons among transgenic lines and constructs. Instead, this targeting approach allowed the investigators to make direct comparisons between constructs either containing or lacking the intronic enhancer or the Ets motif.

Minami and colleagues demonstrate that mice containing the short Tie-2 promoter containing the intronic enhancer (T-short-LacZ) exhibited widespread endothelial-specific expression in the vasculature of E10.5 gestation-day embryos. The level of expression of the T-short-LacZ construct was significantly higher than a construct extending further upstream (~3.8 Kb) but lacking the intronic enhancer (T5-LacZ). Expression of the enhancer-containing construct was observed in sprouting blood vessels in the brain and in both large and small vessels in the yolk sac. In the adult heart, LacZ staining was observed in a subset of arteries, veins, and capillaries, at a level much higher than the longer “enhancerless” construct. Most significantly, a complete loss of reporter gene expression resulted when the Ets sites in the first exon and intronic enhancer were mutated (T-ShortmEts), thus demonstrating the importance of these transcription factor binding sites for endothelial expression in vivo.

Gene targeting to the HPRT locus was first reported by Doetschman et al who were able to correct, in a mouse embryonic stem cell line, a mutant HPRT gene that was rendered inactive due to a spontaneous deletion of the 5’ end. More recently, Bronson et al reported an easy-to-use targeting vector and embryonic stem cell line which could be used to generate transgenic mice with chosen integration at the HPRT locus. The HPRT locus is attractive because it is easy to target in embryonic stem cells (see the Figure and explanation in the legend) and is permissive for the expression of many genes. This method has now been used to target the expression of other endothelial-specific genes including, from the same investigative group as the Tie-2 report in this issue, Von Willebrand factor, FLT-1, and the eNOS promoter.

It is worth mentioning that there are other important uses for this methodology. For example, we recently used HPRT targeting to determine the physiological relevance of genetic variation in the human angiotensinogen gene. Direct comparisons of blood pressure were made between two lines of mice which were identical in all respects except they carried different haplotypic combinations of single nucleotide polymorphisms (SNPs) of the angiotensinogen gene. Mice carrying one of the variants had higher blood pressure than control mice whereas the blood pressure of mice carrying the other variant was identical to control nontransgenic mice. The comparison of mice carrying different constructs was possible because the influence of transgene insertion and copy number could be abolished.

In conclusion, Minami et al have set a higher standard for translating in vitro data of promoter analysis to its physiological relevance in a whole animal. In the process of elucidating mechanisms for the regulation of Tie-2 transcription, the authors have also described a potentially useful tool (Tie-short-promoter) that can be used to drive uniform expression of other interesting genes in the vascular endothelium in vivo.

References


HPRT Targeting: "Ets" A Powerful Tool For Investigating Endothelial-Cell Specific Gene Expression
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