Repressors NFI and NFY Participate in Organ-Specific Regulation of von Willebrand Factor Promoter Activity in Transgenic Mice

Marjan Nassiri, Ju Liu, Stephen Kulak, Richard R.E. Uwiera, William C. Aird, Barbara J. Ballermann, Nadia Jahroudi

Objective—To determine the role of repressors in cell type and organ-specific activation of von Willebrand factor (VWF) promoter sequences –487 to 247 in vivo.

Methods and Results—Activation patterns of wild-type and mutant VWF promoters (sequences –487 to 247) containing mutations in repressors NFI- and NFY-binding sites were analyzed in transgenic mice. Mutation of the NFI-binding site activated the promoter in heart and lung endothelial cells, whereas mutation of the NFY-binding site activated the promoter in kidney vasculature. Immunofluorescence analyses showed that NFIB was predominant in heart and lung endothelial cells, whereas NRFIX was predominantly detected in kidney endothelial cell nuclei. By using chromatin immunoprecipitation, we demonstrated that the distal lung-specific enhancer (containing a YY1 site) of the VWF gene is brought in proximity to the NFI binding site.

Conclusion—The NFI and NFY repressors contribute differentially to organ-specific regulation of the VWF promoter, and the organ-specific action of NFI may reflect its organ-specific isoform distribution. In addition, the lung-specific enhancer region of the endogenous VWF gene may inhibit NFI repressor function through chromatin looping, which can approximate the 2 regions. (Arterioscler Thromb Vasc Biol. 2010;30:00-00.)

Key Words: endothelium ■ gene expression ■ molecular biology ■ vascular biology ■ promoter ■ transcription ■ von Willebrand factor

Although they share a common lineage, endothelial cells of different organs exhibit substantial tissue-to-tissue variation in vivo, reflected by their functions and patterns of gene expression.1,2 The activation patterns of endothelial-specific gene promoters in vivo are also manifested in the heterogeneity of the endothelial cell phenotype.3–5 Despite well-established endothelial subset–restricted activity of a number of endothelial-specific promoters (ie, distinct regions that target expression to specific endothelial subsets), the mechanisms and regulators of this differential expression are not known.

It was previously demonstrated that nucleotides –487 to 247 of the von Willebrand factor (VWF) gene function as an endothelial-specific promoter in cultured cells and transgenic mice.4,6 However, the activity of this promoter fragment in adult transgenic mice was restricted to a subset of brain vascular endothelial cells,4,7 suggesting that other sequences of the VWF gene in addition to the –487 to 247 region are required for the pattern of transcription observed with endogenous genes. Aird et al8 have demonstrated that an extended fragment of the VWF promoter (designated VWF2, spanning –2182 to the end of the first intron) directs expression in microvascular endothelial cells of the heart, skeletal muscle, and brain. Recently, it was demonstrated that a region in intron 51 of the VWF gene confers lung-specific activation to the VWF promoter.9

Analyses of the VWF promoter sequence –487 to 247 in vitro demonstrated that several transacting factors function as activators and repressors of the promoter. GATA6, histone H1-like protein, NFY (interacting with a consensus CCAAT element), and Ets activate the VWF promoter; NFI, Oct1, and NFY (interacting with a novel nonconsensus DNA element) are repressors of the promoter.6,10–15 Thus, we hypothesized that, along with a requirement for other VWF DNA sequences, some of these repressor elements may also participate in the organ-restricted activation pattern of the VWF promoter. Herein, we explored the roles of NFI- and NFY-binding sites (novel nonconsensus NFY-binding DNA element) in the regulation of the VWF promoter sequence –487 to 247 in vivo.
we generated transgenic mice harboring the LacZ gene under the regulation of the VWF promoter that was either wild type (LacZK) or contained a mutation of the NFI-binding site (LacZKNFI), the NFY-binding site (LacZKNFY), or both (LacZKNFI-NFY). The VWF promoter in all transgenes constituted sequence −487 to 247, and the mutations contained 3 base substitutions that were used previously to analyze NFI- and NFY-binding sites in vitro (Figure 1A).10,12,14 We analyzed 2 to 3 independent lines of mice carrying each transgene, with only 1 line for LacZK (previously analyzed extensively). Organs were harvested from adult transgenic mice LacZK (1 line), LacZKNFY (3 lines), and LacZKNFI-NFY (2 lines each) and processed for quantitative RT-PCR for the detection of LacZ mRNA and for immunohistochemistry analysis for the detection of LacZ protein.

RT-PCR of the LacZK (the wild-type promoter) transgenic mouse detected LacZ mRNA only in the brain and kidney (Figure 1B). In contrast, in 3 lines of LacZKNFY, LacZ mRNA was detected most significantly in the lung, but also in the heart and brain and to a lesser degree in the liver. Although the level of expression was variable among the lines (potentially reflecting transgene variation in integration site and copy number), the pattern of expression was similar (Figure 1C: the scales on the y-axis reflect significantly higher levels in lines 92 and 108 [C and E, respectively] versus line 109 [D]).

RT-PCR of the 2 lines of LacZKNFY detected LacZ mRNA in the brain, kidney, and heart of 1 line; in the other line, expression was also detected in the lung and liver (Figure 2A). RT-PCR of organs from 2 lines of double-mutant LacZKNFI-NFY detected LacZ mRNA in all 5 organs of both lines (Figure 2B).

To determine LacZ protein expression, tissue arrays from formaldehyde-treated organs were processed for immunohistochemistry with specific anti–β-galactosidase antibody. In these analyses, sections from multiple tissues were processed on the same slide to minimize interexperimental staining variability. Consistent with previous reports,4,7 immunohistochemistry showed that LacZ protein was detected only in the brain vasculature of the transgenic mouse LacZK (Figure 3, panel LacZK).

In contrast, analyses of LacZKNFY transgenic mice detected significant LacZ protein expression in the brain, lung, and heart vasculature. Similar results were obtained for all 3 LacZKNFI lines. Results for 1 line are shown (Figure 3, LacZKNFI). In LacZKNFY transgenic mice, the expression of LacZ was detected in the brain and kidney vasculature in both lines (results for 1 line are shown in Figure 3, LacZKNFY). However, in 1 of the lines that demonstrated RNA expression in all organs, barely detectable expression in heart and lung vasculature was also observed (data not shown). In LacZKNFI-NFY transgenic mice, LacZ expression was detected in the vasculature of the brain, heart, lung, kidney, and liver in both lines (results for 1 line are shown in Figure 3, LacZKNFI-NFY).

The previously described results demonstrate that mutation of the NFI-binding site (LacZKNFI) activates the VWF promoter, resulting in LacZ transgene expression detectable at both RNA and protein levels in heart and lung vasculature in addition to brain vasculature. A double mutation in both NFI- and NFY-binding sites (LacZKNFI-NFY) activates the VWF promoter in the vasculature of all 5 major organs. The analyses of LacZ

**Methods**

A more detailed description is provided in the supplemental Methods (available online at http://atvb.ahajournals.org).

**Generation and Analyses of Transgenic Mice**

LacZK plasmid, containing the wild-type VWF promoter sequence −487 to 247 fused to the LacZ gene and SV40 poly-A signal, was generated as previously described.4 Plasmids LacZKNFY, LacZKNFY, and LacZKNFI-NFY contain sequences similar to LacZK, except for 3-bp substitution mutations in the repressor binding sites of NFI, NFY, or both. C57BL/6 strain mice were used to generate transgenic mice by Ozgene. A minimum of 2 independent lines for each newly generated transgenic mouse were analyzed. Because the LacZK transgenic mouse was previously described,4 only 1 line was analyzed.

**RNA Preparation and RT-PCR Analyses**

RNA was prepared from heart, lung, liver, kidney, and brain samples using a minikit (RNeasy), according to the manufacturer’s instructions, and used as a template for RT-PCR analyses, as previously described.10 The primers used for the detection of GAPDH and transgene LacZ are shown in the Table.

**Immunohistochemical Detection of β-Galactosidase**

Tissue macroarrays of heart, lung, liver, kidney, and brain were prepared by Histobest Inc and subjected to immunohistochemistry using primary anti–LacZ antibodies (Abcam ab116100) and LacZK as previously described.3 Immunofluorescent detection of NFI, PECAM, and LacZ was performed as described (supplemental Methods).

**Cell Culture and Chromatin Immunoprecipitation**

Human embryonic kidney (HEK) 293 cells were maintained as previously described.10 Human microvascular lung endothelial cells were purchased from Lonza. Chromatin immunoprecipitation (ChIP) and RT-PCR were performed with primers detecting NFI- and YY1-binding sequences of the VWF gene, as previously described (Table).10 Data are given as mean (SD), unless otherwise indicated. Statistical analyses used the paired t test.

**Results**

**Mutation of NFI-Binding Sites Result in VWF Promoter Activation Specifically in Lung and Heart Vasculature**

To determine whether 2 repressors (NFI and NFY) participate in the organ-restricted activity of the VWF promoter in vivo, we generated transgenic mice harboring the LacZ gene under the regulation of the VWF promoter that was either wild type (LacZK) or contained a mutation of the NFI-binding site (LacZK/NFI), the NFY-binding site (LacZK/NFY), or both (LacZK/NFI-NFY). The VWF promoter in all transgenes constituted sequence −487 to 247, and the mutations contained 3 base substitutions that were used previously to analyze NFI- and NFY-binding sites in vitro (Figure 1A).10,12,14 We analyzed 2 to 3 independent lines of mice carrying each transgene, with only 1 line for LacZK (previously analyzed extensively). Organs were harvested from adult transgenic mice LacZK (1 line), LacZK/NFY (3 lines), and LacZK/NFI-NFY (2 lines each) and processed for quantitative RT-PCR for the detection of LacZ mRNA and for immunohistochemistry analysis for the detection of LacZ protein.

RT-PCR of the LacZK (the wild-type promoter) transgenic mouse detected LacZ mRNA only in the brain and kidney (Figure 1B). In contrast, in 3 lines of LacZK/NFY, LacZ mRNA was detected most significantly in the lung, but also in the heart and brain and to a lesser degree in the liver. Although the level of expression was variable among the lines (potentially reflecting transgene variation in integration site and copy number), the pattern of expression was similar (Figure 1C: the scales on the y-axis reflect significantly higher levels in lines 92 and 108 [C and E, respectively] versus line 109 [D]).

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In contrast, analyses of LacZK/NFY transgenic mice detected significant LacZ protein expression in the brain, lung, and heart vasculature. Similar results were obtained for all 3 LacZK/NFI lines. Results for 1 line are shown (Figure 3, LacZK/NFI). In LacZK/NFY transgenic mice, the expression of LacZ was detected in the brain and kidney vasculature in both lines (results for 1 line are shown in Figure 3, LacZK/NFY). However, in 1 of the lines that demonstrated RNA expression in all organs, barely detectable expression in heart and lung vasculature was also observed (data not shown). In LacZK/NFI-NFY transgenic mice, LacZ expression was detected in the vasculature of the brain, heart, lung, kidney, and liver in both lines (results for 1 line are shown in Figure 3, LacZK/NFI-NFY).

The previously described results demonstrate that mutation of the NFI-binding site (LacZK/NFI) activates the VWF promoter, resulting in LacZ transgene expression detectable at both RNA and protein levels in heart and lung vasculature in addition to brain vasculature. A double mutation in both NFI- and NFY-binding sites (LacZK/NFI-NFY) activates the VWF promoter in the vasculature of all 5 major organs. The analyses of LacZK/NFY
transgenic mice demonstrated variation in the LacZ expression pattern between the 2 lines, most significantly at the RNA level. However, consistent RNA and protein expression in the kidney vasculature of both lines of this transgenic mouse suggests that mutation of the NFY-binding site is necessary for activation of the promoter in the kidney.

Based on the consistent results from 3 lines of LacZKNFI transgenic mice, attention was focused on these lines and confocal immunofluorescence was used to determine the localization of LacZ expression in OCT frozen sections of the brain, heart, and lung of these mice. These analyses showed that LacZ (Alexa488-conjugated secondary antibodies [green]) colocalizes specifically with PECAM, a marker of endothelial cells (Alexa594-conjugated secondary antibodies [red]) in the brain, lung, and heart, thus confirming the endothelial-specific activation of the NFI mutant promoter in the vasculature of these organs (Figure 3B).

**NFIB and NFIX Are Differentially Expressed in the Vascular Endothelial Cells of the Lung, Heart, and Kidney**

Based on the transgenic mouse analyses, we hypothesized that NFI-transacting factor specifically represses the VWF promoter...
in lung and heart, but not kidney, vasculature. There are 4 known NFI isoforms, designated as A, B, C, and X.17 Thus, we explored whether endothelial cells of the lung, heart, kidney, and brain have different expression patterns of these isoforms. Because cultured endothelial cells may not accurately reflect organ-specific endothelial cell characteristics, we performed immunofluorescence/confocal microscopy analyses of mouse organ sections using specific antibodies to detect A, B, C, and X isoforms of NFI (Alexa 594-conjugated secondary antibodies [red]) and PECAM (Alexa488-conjugated secondary antibodies [green]) as a general marker for endothelial cells. Nuclei were stained with 4′,6-diamidino-2-phenylindole to determine nuclear localization of NFI. There were significant variations in the expression of NFI isoforms that were detected in the nuclei of endothelial cells of the organs studied (Figure 4). The expression of NFIC and NFIX was most significantly detected in brain endothelial cell nuclei, whereas barely detectable levels of NFIA and no NFIB were detected. NFIB, followed by NFIA, was most significantly detected in heart endothelial cell nuclei, with no detection of NFIX or NFIC. NFIX was predominantly detected in kidney endothelial cell nuclei, with significantly lower levels of NFIC and no NFIA or NFIB. In lung endothelial cell nuclei, NFIB was detected to the greatest extent, whereas NFIA and C and X isoforms were not detected.

These results demonstrate that distinct nonoverlapping isoforms of NFI are present specifically in lung, heart, and kidney endothelial cell nuclei. NFIX is the major isoform detected in the kidney, with no detectable NFIB; in the lung and heart, NFIB is the major isoform detected, with no detectable NFIX.

Because mutation of the NFI-binding site activates the VWF promoter in the lung and heart, but not the kidney, we hypothesize that NFIB, but not NFIX, may function as a repressor of the VWF promoter. Our experiments do not exclude the possibility that NFIC and NFIA (when present) may also function as repressors of the VWF promoter.

DNA Looping Mediates Proximity of the Lung-Specific Enhancer Located in VWF Intron 51 to the Upstream NFI-Binding Site

Recently, it was reported that a region of intron 51 of the VWF gene that interacts with transacting factor YY1 functions as a lung-specific enhancer.9 In vitro, this enhancer activity required YY1 interaction with this region. When the intron 51 region was inserted upstream or downstream of the VWF promoter, the promoter was activated specifically in lung and brain endothelial cells of transgenic mice.9 Because NFI functions as a repressor of the VWF promoter in lung (and heart) endothelium, we hypothesized that (without mutation of the NFI-binding site) intron 51 sequences potentially overcome the inhibitory effect of NFI in lung endothelial cells through YY1. Because the YY1-binding site of intron 51 lies more than 100 kb downstream of the NFI-binding site, we also hypothesized that YY1 (interacting with a region of intron 51) may be brought in proximity to NFI, potentially through chromatin looping.

To test this hypothesis, we reasoned that if the intron 51 YY1-binding site and the NFI-binding site are in close proximity, the cross-linking reagents in the ChIP assay may result in a complex containing NFI-binding sequences, NFI factor, YY1 factor, and YY1-binding sequences (Figure 5A). Thus, we would expect the detection of both NFI- and YY1-binding sequences using either NFI or YY1 antibodies. Analyses of intron 51 did not reveal an NFI-binding site, and upstream VWF sequences (within 1 kb) did not contain a YY1-binding site. In addition, immunoprecipitation assays did not demonstrate coprecipitation of free (not bound to DNA) nuclear NFI and YY1 proteins (data not shown).
Therefore, using NFI-specific antibodies for ChIP, if we detect both amplified YY1-binding intron 51 and NFI-binding sequences and vice versa, this strongly suggests the close proximity of these elements, potentially through chromatin looping (Figure 5A).

Human lung microvascular endothelial cells (LMECs) and nonendothelial HEK 293 cells were used for ChIP analyses. ChIP from LMECs using YY1 antibody or all 4 isoform-specific NFI antibodies demonstrated that the NFI-binding sequences of the VWF promoter were amplified. Fold increases over results from ChIP with control IgG were indicative of specific immunoprecipitation with the target antibodies (Figure 5B, NFI primers). The reciprocal experiment also demonstrated that intron 51 sequences were amplified in ChIP with NFI antibodies and YY1 antibody (Figure 5B, I51 primers). These analyses also demonstrated that the amplification of NFI-binding sequences was highest when NFIB-specific antibody was used, suggesting that NFIB preferentially interacts with the VWF promoter in LMECs. This result is consistent with the organ analyses demonstrating preferential expression of NFIB in lung endothelial cells (Figure 4, lung panel); however, in cultured cells, other NFI isoforms can interact with the VWF promoter, suggesting the presence of multiple isoforms. In ChIP of LMECs, the intron 51 sequences were highly amplified when YY1-specific antibodies were used, suggesting that NFIB preferentially interacts with the VWF promoter in LMECs. This result is consistent with the organ analyses demonstrating preferential expression of NFIB in lung endothelial cells (Figure 4, lung panel); however, in cultured cells, other NFI isoforms can interact with the VWF promoter, suggesting the presence of multiple isoforms. In ChIP of LMECs, the intron 51 sequences were highly amplified when YY1-specific antibodies were used, demonstrating significant interaction of YY1 with intron 51 sequences. Together, these results show significant interaction of NFIB with the VWF promoter and YY1 with intron 51 sequences, and are consistent with the hypothesis that chromatin looping brings the YY1-binding intron 51 sequences in close proximity to the NFI-binding site in LMECs. This arrangement may promote association of YY1 and NFI and/or allow YY1 to somehow influence the inhibitory function of NFI.

However, in HEK 293 cells, ChIP showed that the NFI-binding site sequence was not amplified when YY1 antibodies were used (<1-fold versus IgG), whereas it was amplified when NFIC-specific antibodies (almost 4-fold) or to a lesser extent when NFIA-specific antibodies (2-fold) were used. Furthermore, the YY1-binding site of intron 51 was not amplified (<1-fold) when either NFIC- or YY1-specific antibodies were used (Figure 5C). These results demonstrate that interaction of YY1 with VWF intron 51 in the context of chromatin does not occur in HEK 293 cells. These results also demonstrate that, in HEK 293 cells, NFIC predominantly interacts with the NFI-binding site, whereas NFIB or NFIX does not interact with this site.

Discussion

The results of in vivo analyses of the VWF promoter with mutations in NFI- and NFY-binding sites support the hypothesis that the activity of the VWF promoter sequence 487 to 247 is repressed by at least these 2 repressors. RNA and immunohistochemical analyses demonstrated the expression of the LacZ transgene activated by mutant VWF promoters in distinct sets of organs, consistent with previous observations,4 the wild-type promoter activated LacZ protein expression only in brain vasculature, although expression of mRNA in the kidney was also detected. The transgenic mice were generated using standard approaches and were subject to transgene variation in integration sites and copy numbers, which could influence expression level, lead to aberrant transcription, and/or cause ectopic expression. Therefore, the detection of both protein and mRNA LacZ transgene expression in any organ and, similarly, in 2 or more lines for each transgene was used as the criterion for promoter activation. Accordingly, detection of LacZ mRNA in the kidney of LacZ transgenic mice without detectable LacZ protein suggests low-level ectopic expression and/or aberrant transcripts (untranslatable) in this line. Previously, multiple lines of transgenic mice harboring this VWF promoter sequence fused to LacZ or amyloid precursor protein genes demonstrated transgene activation in the brain, but not the heart, lung, liver, or kidney.4,7

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Based on analyses of 3 lines of LacZKNFI demonstrating LacZ expression at both mRNA and protein levels in heart and lung (and brain), we conclude that mutation of the NFI-binding site results in promoter activation specifically in heart and lung vasculature, but not in kidney or liver (where only mRNA was detected). On the other hand, mutation of the NFY-binding site (LacZKNFY) was required for activation of the VWF promoter in kidney vasculature. However, because in 1 of the 2 transgenic mice with double mutations in both NFI- and NFY-binding sites (LacZKNFINFY) demonstrated promoter activity in the heart, lung, kidney, and liver vasculature. This supports the results of individual mutations by demonstrating that double-mutant transgenic mice exhibit a pattern of expression that overlaps the patterns observed with individual mutations. In addition, the results also suggest that the simultaneous mutation of both repressor elements has a synergistic effect on the VWF promoter activity because it results in promoter activation in liver vasculature that was not detected at the protein level in either of the single mutants. Together, the results of transgenic mouse analyses demonstrate that repressors NFI and NFY function differentially to inhibit the VWF promoter in distinct organs. Although NFI inhibits VWF promoter activity in the heart and lung, but not the kidney, NFY inhibits promoter activity primarily in kidney vasculature.

To date, a repressive function for NFY specifically in endothelial cells has been reported only for the VWF promoter.20,21 However, an organ-specific repressive function for NFI in the lung has been reported.18 In addition, analyses in vitro have demonstrated differential activation/repression functions for various NFI isoforms, depending on the target promoter.17,19 Consistent with these in vitro observations, our in vivo analyses of mouse organ sections showed that NFIB was predominantly detected in lung and heart endothelial cell nuclei and that NFIX was predominantly detected in kidney endothelial cell nuclei. Based on these observations and on the fact that the NFI mutant VWF promoter (LacZKNFI) is activated in heart and lung, but not kidney, endothelial cells, we hypothesize that NFIB predominantly functions as a repressor of VWF promoter activity. Analyses of knockout mice specific for each NFI isoform have demonstrated that, although all NFI isoforms contribute to development of the central nervous system, NFIB is required specifically for lung development.20,21 Also, a report19 on the role of NFI in regulating the p21 promoter in cultured cells demonstrated that while NFIB functions as repressor, NFIX and isoform A appear to have opposing roles. Taken together, we hypothesize that NFIB predominantly functions as a repressor of VWF promoter activity.

Analyses of additional transgenic lines are necessary to unambiguously determine the LacZKNFY activation pattern. Nevertheless, our results unambiguously demonstrate that mutation of the NFY- but not NFI-binding site activates the VWF promoter in kidney vasculature. Analyses of transgenic mice with double mutations in both NFI- and NFY-binding sites (LacZKNFIMFY) demonstrated promoter activity in the heart, lung, kidney, and liver vasculature. This suggests that the simultaneous mutation of both repressor elements has a synergistic effect on the VWF promoter activity because it results in promoter activation in liver vasculature that was not detected at the protein level in either of the single mutants. Together, the results of transgenic mouse analyses demonstrate that repressors NFI and NFY function differentially to inhibit the VWF promoter in distinct organs. Although NFI inhibits VWF promoter activity in the heart and lung, but not the kidney, NFY inhibits promoter activity primarily in kidney vasculature.

Several NFI isoforms (A, B, C, and X) and various alternatively spliced mRNAs of these isoforms have been reported.17 An overlapping, but distinct, expression pattern for each isoform during embryonic development has been shown.17 In addition, analyses in vitro have demonstrated differential activation/repression functions for various NFI isoforms, depending on the target promoter.17,19 Consistent with these in vitro observations, our in vivo analyses of mouse organ sections showed that NFIB was predominantly detected in lung and heart endothelial cell nuclei and that NFIX was predominantly detected in kidney endothelial cell nuclei. Based on these observations and on the fact that the NFI mutant VWF promoter (LacZKNFI) is activated in heart and lung, but not kidney, endothelial cells, we hypothesize that NFIB predominantly functions as a repressor of VWF promoter activity. Analyses of knockout mice specific for each NFI isoform have demonstrated that, although all isoforms contribute to development of the central nervous system, NFIB is required specifically for lung development.20,21 Also, a report19 on the role of NFI in regulating the p21 promoter in cultured cells demonstrated that while NFIB functions as repressor, NFIX and isoform A appear to have opposing roles. Taken together, we hypothesize that NFIB appears to have specific functions in the lung, including repression of the VWF promoter in lung endothelial cells.

It was previously reported that intron 51 sequences confer lung-specific activity on the VWF promoter.9 In regard to the
endogenous gene, we hypothesize that activators that interact with intron 51 sequences, including YY1, overcome the repressive function of NFI in lung endothelial cells. We reasoned that a prerequisite for this function of intron 51 sequences may be a chromatin structure of the VWF gene that brings 2 distant regulatory elements (NFI-binding site and intron 51 sequences) into close proximity. We tested this hypothesis by ChIP and demonstrated that anti–NFI antibodies can amplify intron 51 YY1- and NFI-binding sequences (and vice versa) in human LMECs, but not in HEK 293 cells. These results support the hypothesis that the NFI and intron 51 YY1-binding regions are in close proximity in endothelial cells.

By using ChIP and in contrast to LMECs, YY1 in HEK 293 cells does not interact with its cognate intron 51-binding site in the context of VWF chromatin. However, we cannot eliminate the possibility that chromatin looping also occurs in HEK 293 cells, but cannot be detected using ChIP, which is based on the potential association of both YY1 and NFI with their cognate binding sites. Recently, we reported that YY1 forms a specific complex with actin in HEK 293 cell nuclei, which was not observed in human umbilical vein endothelial cells. This was demonstrated by gel mobility, DNA pull-down, and immunoprecipitation/Western blot analyses. Based on ChIP failing to detect YY1 interaction with intron 51 sequences in HEK 293 cells, we hypothesize that nuclear actin association with YY1 may function, as a regulatory switch to prevent YY1 interaction with intron 51 sequences, thus rendering it ineffective as an enhancer of the VWF gene in nonendothelial cells.

In summary, we propose the following hypothesis for lung-specific activation of the VWF promoter, NFI, predominantly NFIB, functions as a repressor in the lung, whereas YY1 binding to intron 51, which is in close proximity to the NFI-binding site, overcomes the repression by NFI, thereby promoting transcription. In nonendothelial cells, even if chromatin looping is similar, interaction of nuclear actin with YY1 inhibits YY1 association with intron 51 sequences, thus rendering it ineffective as an activator.

Differential levels of VWF expression in endothelial cells of different organs and vessel types are reflective of the heterogeneity of endothelial cells. Furthermore, vascularatures of different organs were reported to modulate VWF expression differentially in response to stimuli. Multiple repressors that participate in VWF promoter activity differentially in distinct organs, and different regulatory elements that overcome these repressors in an organ-specific manner, may indicate the transcriptional regulatory mechanisms that are susceptible to diverse and differential vascular bed-specific regulation.

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Disclosures
None.

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*Generation and analyses of transgenic mice*—Construct LacZK containing the wild type VWF promoter sequences –487 to +247 fused to the LacZ gene and the SV40 poly-A signal was generated as previously described 1. Construct LacZK<sup>NFI</sup>, containing 3 bp substitution mutations in the NFI-binding site was generated by replacing the HindIII fragment of LacZK that spans sequences -487 to -90 of the VWF, with the similar HindIII fragment from plasmid HGHK<sup>rm3</sup> 2 that contains the desired mutation in the NFI-binding site. Constructs LacZK<sup>NFY</sup> and LacZK<sup>NFI-NFY</sup> were generated as follows. The VWF promoter fragment -90 to +247 was amplified using primers that correspond to sequences -90 to -60 (with an additional 9 bp sequence containing the HindIII site); and +213 to +247 with base substitution mutations at +226 to +228 [CCG to GAA, as previously described 3] as well as an additional 9 bp sequence containing the PstI site. The plasmid LacZK was digested with HindIII and PstI and sequences corresponding to VWF fragment -487 to +247 were removed. The PCR generated VWF sequences -90 to +247 containing the mutation in NFY-binding site were then ligated to the HindIII/PstI digested LacZK fragment. This resulted in generation of a construct containing VWF sequences -90 to +247 with mutation in NFY binding site. This construct was then linearized with HindIII, followed by insertion of HindIII fragments containing either wild-type VWF sequences -487 to -90 (obtained from LacZK) to generate construct LacZK<sup>NFY</sup>, or NF1 mutant VWF sequences -487 to -90 (obtained from LacZK<sup>NFI</sup>) to generate construct LacZK<sup>NFI-NFY</sup>. Sequences of all constructs were confirmed. DNA fragments containing VWF-LacZ and poly-A were isolated from constructs LacZK, LacZK<sup>NFI</sup>, LacZK<sup>NFY</sup> and LacZK<sup>NFI-NFY</sup> by Sall digestions of these constructs, purified
and used for microinjections to generate C57BL/6 transgenic mice by Ozgene (Bentley, WA, Australia). The Health Sciences Animal Policy and Welfare Committee at the University of Alberta approved all animal housing and experimentation. A minimum of two independent lines (and two littermates of each line) for each transgene were analyzed.

**RNA preparation and real-time polymerase chain reaction (RT-PCR) analyses.** RNA was prepared from organs (heart, lung, liver, kidney and brain) using the Qiagen RNeasy minikit (Valencia, CA) as recommended by manufacturer and 1 μg of RNA was used to generate cDNA using Invitrogen SuperScript® Reverse Transcriptase (Carlsbad, CA) as recommended by manufacturer. The cDNA was diluted (1:50) and 10 μl was used as template for RT-PCR analyses using power SYBR® PCR Master Mix (Applied Biosystems, Foster Green CA) as previously described. The primers used for detection of mouse VWF, GAPDH and transgene LacZ were as shown in Table 1.

**Immunohistochemical detection of β-galactosidase-** Tissue macro-arrays of organs (heart, lung, liver, kidney and brain) were prepared by Histobest inc. (Edmonton, Alberta) and subjected to immunohistochemistry using primary anti-β galactosidase antibody (Abcam ab116-100) as previously described. The primers used for detection of mouse VWF, GAPDH and transgene LacZ were as shown in Table 1.

**Immunofluorescent detection of LacZ, NFI and PECAM-** Organs (lung, liver, kidney and brain) from control C57Bl/6 and LacZK^{NFI} were frozen in OCT and 5 micron sections were subjected to immunofluorescent analysis as previously described. All anti- NFI (A,
B, C and X) primary antibodies were used at dilution 1:100 while anti PECAM antibody was used at either 1:100 (when costaining was with anti-NFI antibodies) or at 1:200 (when costaining with anti b-galactosidase antibody). Anti b-galactosidase was also used at 1:200. All secondary antibodies [goat anti rabbit Alexa 594 and goat anti rabbit Alexa 488 (Molecular Probes), donkey anti-goat Alexa 594 and donkey anti-goat 488] were used at 1:2000 dilutions. Primary antibodies used were polyclonal rabbit antibodies specifically detecting NFIA, NFIB, NFIC, NFIX (Aviva System Biology, San Diego, CA), anti-β galactosidase antibody (Fitzgerald, Concord, MA) and goat polyclonal anti-mouse PECAM antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Cell culture and Chromatin Immunoprecipitation (ChIP). HUVECs and HEK 293 cells were maintained as previously described. Human microvascular lung endothelial cells were purchased from Lonza and maintained as described by manufacturer. ChIP and RT-PCR analyses were as previously described with primers detecting NFI and YY1 binding sequences of the VWF gene shown in Table 1.


