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Endothelial Specificity of ICAM2, Flt-1, and Tie2 Promoters *In Vitro and In Vivo*

Jing Lei, Yoram Vodovotz, Timothy R. Billiar

Abstract**—**To identify an endothelial cell-specific promoter suitable for vascular-specific targeting, we tested five promoters *in vitro*--Tie2SE, Tie2LE, ICAM2, Flt-1 and vWF--for promoter activity and specificity in endothelial cells, smooth muscle cells and non-vascular resident cells as well as tissues. These promoters, except for vWF, exhibited good endothelial activity and specificity *in vitro*. In a syngenic heart transplantation model, the ICAM2 promoter was variably functional in coronary endothelial cells of donor hearts. Thus, the ICAM2, Flt-1, Tie2SE and Tie2LE promoters hold promise for endothelial-specific targeting, but *in vitro* expression may not predict *in vivo* expression.

Keywords**—**vascular-specific targeting, endothelial cell-specific promoter, endothelial specificity

List of Abbreviation:
Tie2SE: the murine Tie2 promoter with 1.6kb enhancer
Tie2LE: the murine Tie2 promoter with 10kb enhancer
ICAM2: the human intercellular molecular 2 promoter (-292 ~ +44 bp)
Flt-1: the human *fms*-like tyrosine kinase -1 promoter (-748 ~ +284 bp)
vWF: the human von Willebrand factor promoter with enhancer (-487 ~ +246 bp)

I. Introduction

VASCULAR-targeted gene therapy is a promising option for vascular diseases, including tumor angiogenesis, atherosclerosis, intimal hyperplasia [1]. However, the major limitation in this field has been the lack of vessel wall-specific targeting. To facilitate vascular-specific targeting, one strategy is to identify promoters capable of directing endothelial cell-specific expression following gene transfer. Several endothelial cell-specific promoters have been identified: the murine Tie2 promoter with a 1.6 kb enhancer [2] (defined as Tie2SE herein), the murine Tie2 promoter with a 10 kb enhancer [2] (defined as Tie2LE herein), the human intercellular adhesion molecular 2 promoter (ICAM2, -292 ~ + 44 bp) [3], the human von Willebrand factor promoter with enhancer (vWF, -487 ~ + 246 bp) [4], the human *fms*-like tyrosine kinase -1 promoter (Flt-1, -748 ~ + 284 bp) [5], the murine vascular endothelial cadherin promoter with enhancer (-2486 ~ +24 bp) [6], the murine preproendothelin-1 promoter with enhancer (5.9 kb 5' flank region + first exon and first intron) [7], and the murine preproendothelin-1 promoter with enhancer (9.2kb 5' flank region + first exon and first intron) [8]. The endothelial-specificity of these promoters, except for the Flt-1 promoter, was established in transgenic mice [2-4, 6-8]. The endothelial-specificity of Flt-1 was identified *in vitro* by serial deletions of the promoter region of the Flt-1 gene [5]. It was also confirmed in two other studies *in vitro* and *in vivo* [9, 10], in which Flt-1 was utilized in adenoviral vectors.

Conflicting results have also been reported. For example, the ICAM2 promoter was reported to drive endothelial-specific expression in transgenic mice [3, 11-13], but also led to leaky expression in human primary vascular smooth muscle cells, human primary foreskin fibroblasts, HeLa cells, and HepG2 cells when these cells were infected by an adenoviral vector driving the expression of *lacZ* i*n vitro* [9]. Moreover, these nominally endothelial-specific promoters were identified by different research groups, and were not compared directly in a single study. According, we tested five endothelial cell-specific promoters *in vitro*, and the two optimal promoters, Flt-1 and ICAM2, *in vivo*.

II. Materials and Methods

DNA plasmids and adenoviruses

The DNA plasmids pTie2SE-lacZ and pTie2LE-lacZ were kindly provided by Dr. Thomas N. Sato **(**University of Texas Southwestern Medical Center), and are referred to as pHHSDKXK and pT2HLacZpA1I.7, respectively. The ICAM2, vWF, Flt-1 and cytomegalovirus (CMV) promoters were constructed in E1-deleted adenoviruses referred to as AdICAM2-lacZ, AdvWF-lacZ, AdFlt-1-lacZ, and AdCMV-lacZ, respectively. The Flt-1 promoter was also constructed in a DNA plasmid upstream of the luciferase gene, referred to as pFlt-1-luc (provided by Dr. Andrew H Baker, University of Glasgow with the permission of the original authors). The Tie2SE, ICAM2, vWF, and CMV promoters were constructed in DNA plasmids upstream of the FretMT gene and are referred to as pTie2SE-FretMT, pICAM2-FretMT, pvWF-FretMT, and pCMV-FretMT, respectively. FretMT is a fusion protein that is composed of Enhanced Cyan Fluorescent Proteins (ECFP), human metallothionein (MT) and Enhanced Yellow Fluorescent Proteins (EYFP) genes. The four DNA plasmids were kindly provided by Dr. Bruce Pitt (University of Pittsburgh). The Rous sarcoma virus promoter (RSV) was

Jing Lei is with Cellular and Molecular Pathology Department, University of Pittsburgh, Pittsburgh, PA 15261 USA (phone: 412-648-2081; fax: 412-647-5959; e-mail: jinglei2009@ gmail.com).
Yoram Vodovotz is with Department of Surgery, University of Pittsburgh, Pittsburgh, PA 15261 USA (e-mail: vodovotzy@upmc.edu).
Timothy R Billiar is with Department of Surgery, University of Pittsburgh, PA 15216 USA (e-mail: billiartr@upmc.edu).
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constructed in a DNA plasmid upstream of the luciferase gene and is referred to as pRSV-luc. The DNA plasmid pCMV-EGFP was purchased from Clontech (Mountain view, CA). The adenovirus AdCMV-EGFP was kindly provided by Dr. Andrea Gambotto (University of Pittsburgh).

Cell Culture

Smooth muscle cells were isolated from Lewis rat (Harlan Sprague-Dawley, Indianapolis, IN) aortic strips and cultured as previously described (16). The cells from passages 3 to 7 were used for transfection. Bovine aortic endothelial cells (BAEC) were purchased from BioWhittaker (Walkersville, MD) and cultured in EGM2-MV medium. Hepatocytes were isolated from Lewis rats (200-250g, Harlan Sprague-Dawley) and cultured as previously described (17). HEK293 cells were cultured in Dulbecco's Modified Eagles medium (high glucose, Cellgro, Herndon, VA) supplemented with 10% FBS and 100 U/mL penicillin, and 100 μg/mL streptomycin.

Transfection

Transfections were performed in BAEC, rat smooth muscle cells, rat hepatocytes and HEK293 cells. All cells were plated in 12-well plates at a density of 5×10^4 cells/well except for HEK293 cells (5×10^5 cells/well).

Detection of reporter gene expression

FretMT expression was detected by green fluorescence using an Olympus Provis AX70 microscope (Tokyo, Japan). *LacZ* expression was detected by X-gal staining (beta-galactosidase staining kit, Mirus Bio). Luciferase activity was measured using a Luciferase assay system (Promega, San Luis Obispo, CA) in an AutoLumat LB 953 Luminometer (Berthold, Bad Wildbad, Germany). Luciferase activity was normalized to the amount of protein and expressed as relative luciferase activity (LU/μg protein).

Ex vivo transduction of rat aorta, cardiac and skeletal muscle and X-gal staining of the tissues

Rat aortas were cut into 5 mm-long strips and cardiac and skeletal muscles into 3 mm × 3 mm pieces. 2×10^9 PFU of AdFlt-1-lacZ, AdICAM2-lacZ, AdvWF-lacZ, AdCMV-lacZ, or AdCMV-EGFP were diluted in 200 μl of Opti-MEM. The tissue samples were incubated in the adenoviral mixture at 37°C for 2 hours, then cultured in 5 ml culture medium (Dulbecco's Modified Eagles medium (low glucose)/Ham's F12, 1:1 vol:vol; BioWhittaker; Walkersville, MD with 10% FBS) in 30-mm dishes for 72 hours with daily changes of culture medium.

The tissues were fixed in 2% paraformaldehyde, washed with PBS and stained for X-gal (Mirus Bio). Cryostat sections (6 μm thickness) were assessed for positive staining using a reflected light differential interference contrast microscope (Olympus Provis AX70, Tokyo, Japan).

Ex vivo transduction of donor heart using adenoviral vector and heterotopic heart transplantation

Syngenic heart transplantation was performed using PVG rats (200~250g, Harlan Sprague Dawley). The donor heart was perfused *in situ* using 10 ml Lactated Ringer's solution (Rxveterinary Products, Porterville, CA). After ligation of the inferior vena cava, superior vena cava and pulmonary vessels, the adenoviral vectors AdFlt1-lacZ, AdICAM2-lacZ or AdCMV-lacZ, at a dose of 10^{10} pfu (diluted in Lactated Ringer's solution to make the total volume 1000 μl), was injected slowly into the coronary arteries through the brachiocelephilic artery. The aortic root and the pulmonary artery were then clamped and the donor heart was harvested. The donor heart was kept in the Lactated Ringer's solution at 4ºC for 2 hours. Heterotopic syngenic heart transplantation was performed as previously described (18).

X-gal staining of the donor heart

Three days after transplantation, explanted donor hearts were fixed in 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) for 2 hours, and then in 30% sucrose (Sigma-Aldrich) overnight. Cryostat sections were cut to a thickness of 6 μm. X-gal staining was performed on every 10th section. X-gal staining was detected using a reflected light differential interference contrast microscopy (Olympus Provis AX70, Tokyo, Japan).

III. RESULTS AND DISCUSSIONS

To compare the activity and specificity of Tie2SE, ICAM2, and vWF, the DNA plasmids pTie2SE-FretMT, pICAM2-FretMT, and pvWF-FretMT were used to transfect endothelial cells (BAEC) and non-endothelial cells (primary rat smooth muscle cells and HEK293 cells). As shown in TABEL 1 and Fig. 1, both Tie2SE and ICAM2 exhibited good reporter gene expression and endothelial-specificity. Although ICAM2 was also activated in approximately 5% of HEK293 cells, this is much less compared to the positive control CMV (activated in 60% of the cells). No gene expression was detected when the vWF promoter or the negative control pCMV-lacZ was used (data not shown).

TABLE I
DETERMINATION OF ENDOTHELIAL SPECIFICITY OF PROMOTERS

	Rat SMC	BAEC	HEK293	Rat Hepatocytes
pTie2SE-FretMT	0	3%	0.5%	/
pICAM2-FretMT	0.4%	5%	5%	/
pvwF-FretMT	0	0.5%	0.5%	/
pCMV-FretMT	10%	15%	60%	/
pTie2LE-lacZ	/	7%	0.3%	0.1%
pTie2SE-lacZ	/	30%	50%	10%

To determine the endothelial specificity of Tie2SE, ICAM and vWF, smooth muscle cells, BAEC, and HEK293 cells were transfected with pTie2SE-FretMT, pICAM2-FretMT, pvWF-FretMT, or pCMV-FretMT. To determine the endothelial specificity of Tie2LE, BAEC, HEK293 cells, and rat hepatocytes were transfected with pTie2LE-lacZ or pCMV-lacZ. The percentage of cells showing green fluorescence (for FretMT) or blue staining (for lacZ) was calculated. Each transfection was performed in three wells of a 12-well plate. This experiment was repeated three times. The data shown represent the average from one experiment.

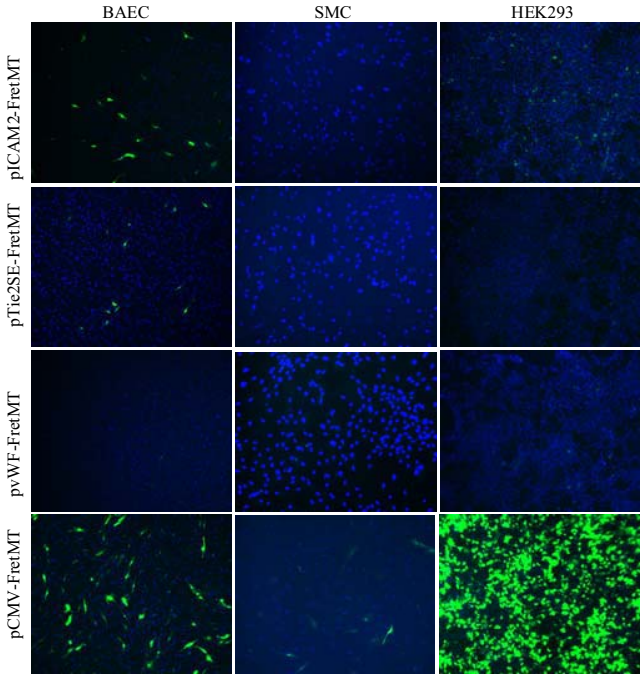

Fig. 1 Green fluorescence in cells transfected by pICAM2-FretMT, pTie2SE-FretMT, pvWF-FretMT or pCMV-FretMT. The left panel shows green fluorescence in BAEC, the middle panel shows in smooth muscle cells and the right panel shows in HEK293 cells. The blue fluorescent spots were nuclei stained by Hoechst. Each transfection was performed in three wells of a 12-well plate. This experiment was repeated three times

To test Tie2LE, pTie2LE-lacZ, pCMV-lacZ (positive control) and pCMV-FretMT (negative control) were used for transfection. TABEL 1 and Fig. 2 show that the Tie2LE promoter led to good X-gal staining in BAEC while exhibiting very low activity in hepatocytes and HEK293 cells. No staining was seen with the negative control (data not shown). For Flt-1, the DNA plasmids pFlt-1-luc, pRSV-luc (positive control), and pCMV-EGFP (negative control) were transfected into cells. At 48 hrs after transfection, the Flt-1 promoter demonstrated good luciferase activity in BAEC, although it also exhibited some expression in hepatocytes (Fig. 3).

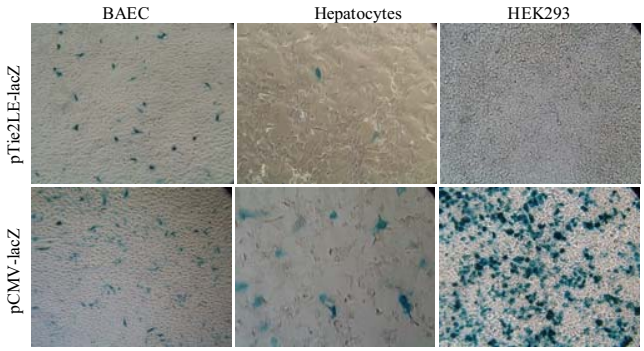

Fig. 2 X-gal staining in cells transfected by pTie2LE-lacZ or pCMV-lacZ. The left panel shows X-gal staining in BAEC, the middle panel shows in rat hepatocytes and the right panel shows in HEK293 cells. Each transfection was performed in three wells of a 12-well plate. This experiment was repeated three times

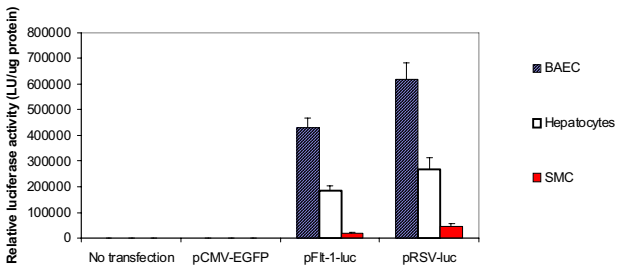

Fig. 3 Relative luciferase activity in cells transfected by pFlt-1-luc, pRSV-luc and pCMV-EGFP. Each transfection was performed in three wells of a 12-well plate, which generates the mean and standard deviation of the relative luciferase activity. This experiment was repeated three times and the data shown represent the result from one experiment

Tissue specificity was assessed by transducing tissues *ex vivo* using the adenoviral vectors AdFlt-1-lacZ, AdICAM2-lacZ, or AdvWF-lacZ at a dose of 2 x 10^9 pfu. AdCMV-lacZ and AdCMV-EGFP served as positive and negative controls, respectively. As shown in Fig 4, approximately 10% of the endothelial cells and 8% of the adventitial cells exhibited X-gal staining in the AdFlt-1-lacZ-transduced aorta, but no staining was observed in smooth muscle cells. Low number of X-gal-positive cells was seen in transduced cardiac and skeletal muscle. In the AdICAM2-lacZ transduced tissues, 7% of the endothelial cells and 2% of the adventitial cells, but few other cells exhibited X-gal staining. The vWF promoter did not lead to X-gal staining in any of the tissues examined. The negative control AdCMV-EGFP did not exhibit any positive X-gal staining (data not shown). Our results in cells and tissues are consistent with the result of Stuart *et al.* [9] and Renolds *et al.* [10] showing the endothelial specificity of Flt-1. Stuart et al. [9] showed that ICAM2 lacked endothelial specificity when AdICAM2 was used to infect cells. We also have similar results (data not shown). However, our *in vitro* data show that ICAM2 exhibited endothelial specificity when pICAM2-EGFP was used to transfect cells and AdICAM2-lacZ was used to transduce intact tissue, which is consistent with Cowan *et al.* [3, 11-13] who showed its endothelial specificity in transgenic mice lines [3, 11-13]. The lack of activity by vWF *in vitro* is consistent with Stuart *et al.*, although some papers showed its endothelial specificity in transgenic mice [4, 14, 15].

To assess AdFlt-1-lacZ and AdICAM2-lacZ *in vivo*, donor hearts were transduced *ex vivo* at a viral dose of 10^{10} pfu prior to transplantation. AdCMV-lacZ and AdCMV-EGFP were used as positive and negative controls, respectively. As shown in Fig 5, good *lacZ* expression was observed on day 3 in the endothelial cells of the coronary arteries and veins in 4 of 22 donor grafts that were transduced with AdICAM2-lacZ. In the 4 positive hearts, 22%, 33%, 51% and 57% of coronary vessels, respectively, exhibited positive X-gal staining with almost no positive cardiomyocytes.

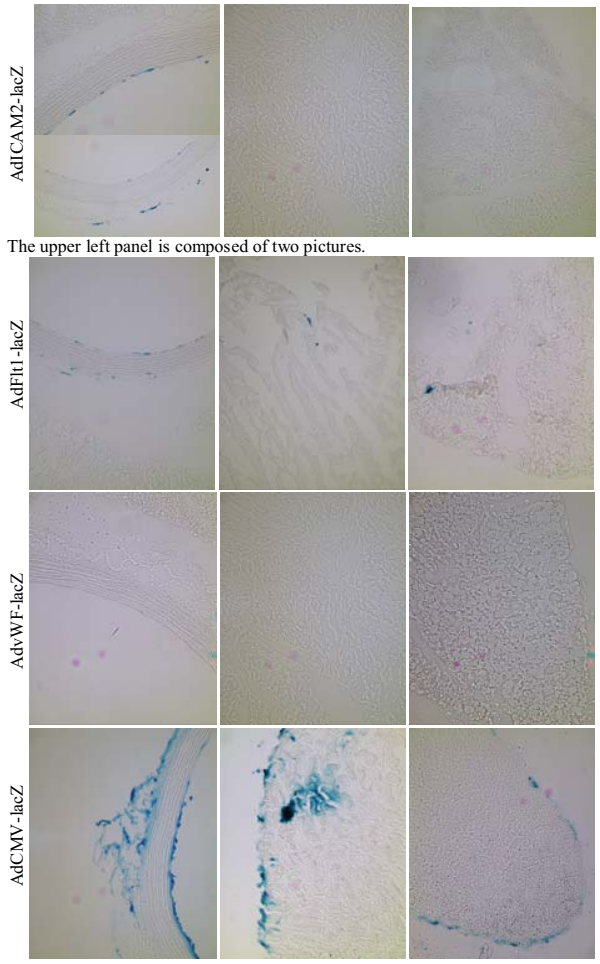

Fig. 4 X-gal staining of tissues transduced by AdICAM2-lacZ, AdFlt-1-lacZ, AdvWF-lacZ and AdCMV-lacZ. The left panel shows X-gal staining in aorta, the middle panel shows in cardiac muscle and the right panel shows in skeletal muscle. This experiment was repeated three times

The Flt-1 promoter directed positive staining in a low number of endothelial cells (<5% of coronary vessels). The CMV promoter drove extensive *lacZ* expression in cardiomyocytes with scattered and varied gene expression in endothelial cells. The negative control AdCMV-EGFP did not lead to positive staining in any cells (data not shown). Thus, the transduction efficacy in endothelial cells when using both AdICAM2-lacZ and AdCMV-lacZ is variable and likely reflects the poor endothelial transduction efficiency by adenovirus *in vivo*.

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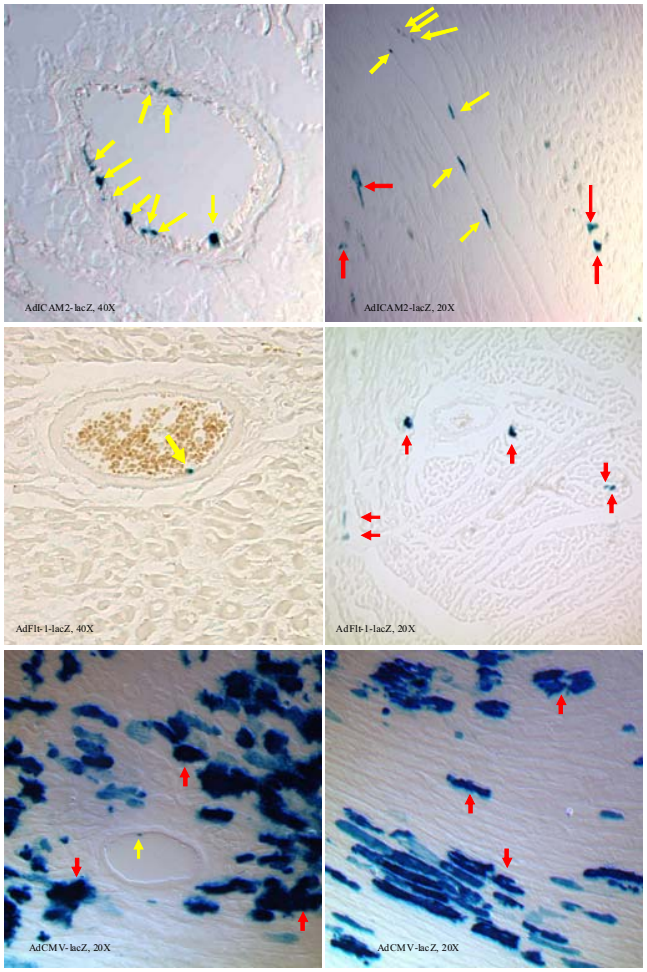

Fig. 5 X-gal staining of donor heart that was transduced by AdFlt-1-lacZ, AdICAM2-lacZ or AdCMV-lacZ *ex vivo*. The top two panels show that the ICAM2 promoter directed blue staining in endothelial cells of coronary arteries (top left), veins and cardiomyocytes (top right). The middle two panels show that the Flt-1 promoter directed a few blue staining in coronary artery (middle left) and cardiomyocytes (middle right). The bottom two panels show that the CMV promoter directed a few blue staining in coronary artery (bottom left) and extensive staining in cardiomyocytes (bottom left and right). The yellow arrows represent positive staining in endothelial cells and the red arrows represent positive staining in cardiomyocytes. Magnification (20X or 40X) of the tissue is indicated in the picture. In each group, transduction/transplantation was repeated at least six times

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