RNA Transfection Is a Versatile Tool to Investigate Endothelin-1 Posttranscriptional Regulation

IMTIAZ A. MAWJI* AND PHILIP A. MARSDEN†,[1]

*Division of Cancer Genomics and Proteomics, Ontario Cancer Institute, University Health Network, University of Toronto, Toronto, Canada; and †Renal Division and Department of Medicine, St. Michael's Hospital and University of Toronto, Toronto, Canada

Endothelin-1 (ET-1) is a potent endothelial-derived vasoconstrictor and cellular mitogen. Perturbations in ET-1 levels have been observed in a number of cardiovascular and renal disorders. Steady-state ET-1 mRNA expression is regulated in the vascular endothelium by an inducible promoter and a constitutively short mRNA half-life. Recent studies have identified mRNA stabilization as a pathophysiologically relevant mechanism of ET-1 induction in vascular endothelial cells. However, mechanistic studies on posttranscriptional pathways in physiologically relevant postconfluent primary endothelial cell monolayers have remained challenging because of endothelial resistance to DNA-based gene transfer and expression. To overcome these challenges, we developed an RNA transfection method to study ET-1 posttranscriptional regulation. Reporter transcripts transfected into either preconfluent or postconfluent primary endothelial cells were rapidly and robustly expressed. RNA transfection reconstituted poly(A)-tail–dependent protein expression and ET-1 3′-UTR–dependent mRNA destabilization, suggesting that the transfected RNA accessed endothelial cell posttranscriptional pathways. Because RNA transfection uncouples transcription from expression, the influence of the ET-1 3′-UTR on posttranscriptional expression kinetics could also be monitored. Taken together, our results suggest that RNA transfection is a versatile tool to investigate ET-1 posttranscriptional regulation in endothelial cell culture models. Exp Biol Med 231:704–708, 2006

Key words: 3′-untranslated region; endothelin; endothelium; gene regulation; mRNA stability; RNA transfection

Introduction

Endothelin-1 (ET-1) is a potent endothelial-derived vasoconstrictor and cellular mitogen (1). *In vitro* and *in vivo* studies have implicated aberrant ET-1 expression as a marker of endothelial dysfunction, inflammatory vascular disease, and cardiac and vascular remodeling (2). ET-1 expression is regulated by an inducible promoter and a constitutively short mRNA half-life (30 min), enabling ET-1 mRNA and protein expression to mirror acute changes in transcriptional activity (3, 4).

Recent studies have identified a number of pathophysiologic cell stimuli that stabilize the ET-1 mRNA (4–7). For example, we previously observed that verotoxin, a type-A:B_5 toxin implicated in hemorrhagic colitis and hemolytic uremic syndrome, enhances endothelial cell ET-1 mRNA and protein expression through a mechanism of mRNA stabilization (5). Despite these advances, mRNA stabilization remains a poorly understood pathway of ET-1 biosynthesis. Moreover, mechanisms operative in the control of mRNA stability and translation in the vascular endothelium, in health and in disease, are poorly understood. In this regard, studies with the ET-1 mRNA offer a relevant model to dissect these pathways.

Mechanistic studies of a specific gene's mRNA typically involve the genetic uncoupling of mRNA expression from native transcriptional activity. For example, wild-type or mutant transcripts can be ectopically expressed from mammalian expression plasmids bearing constitutively active promoters and mRNA processing signals. Our laboratory and other laboratories have successfully used this approach to identify functionally important regions necessary for rapid ET-1 mRNA turnover (4, 8). Using transient transfection of expression plasmids into primary bovine aortic endothelial cells (BAECs), we localized an mRNA destabilizing activity to two regions of the ET-1 3′-UTR, termed DE1 and DE2 (destabilizing elements 1 and 2) (4). Although this method facilitates robust mechanistic studies in subconfluent BAEC and human nonendothelial cell lines, studies in postconfluent cell monolayers or

This research is supported by a grant from the Kidney Foundation of Canada. P.A.M. is a Heart and Stroke Foundation of Canada Career Investigator. I.A.M. is the recipient of a Canadian Institute of Health Research/Heart & Stroke Foundation of Canada Doctoral Research Award.

[1] To whom correspondence should be addressed at University of Toronto, Medical Sciences Building, Room 7358, 1 King's College Circle, Toronto, ON M5S 1A8. E-mail: p.marsden@utoronto.ca

Received September 30, 2005.
Accepted December 16, 2005.

1535-3702/06/2316-0704$15.00

transfection-resistant human primary endothelial cells remains challenging, owing in part to low transfection efficiencies. In addition, there is considerable difficulty in developing stable cell lines from cultured human umbilical vein endothelial cells (HUVECs) because of senescence and phenotypic loss beyond early passages (9). To overcome these challenges, we developed and optimized an RNA-based transfection methodology to study ET-1 posttranscriptional regulation in confluent endothelial cell monolayers. We present evidence that this method recapitulates the ET-1 3′-UTR destabilizing activity previously observed using transient or stable plasmid DNA–based RNA expression methods (4). RNA transfection may therefore be a versatile tool to investigate ET-1 posttranscriptional gene regulation.

Materials and Methods

Cell Culture. All cell culture reagents were obtained from Invitrogen unless otherwise indicated. Primary BAECs and HUVECs were isolated and cultured as previously described (4, 10). BAECs and HUVECs were used at passages 3–7. Human cervical epithelial carcinoma (HeLa) cells were obtained from ATCC (Manassas, VA) and cultured as previously described (4, 10).

Plasmids. Restriction enzymes, DNA modifying enzymes, and Taq polymerase were obtained from New England Biolabs (Beverly, MA) and Invitrogen (Burlington, Canada). All plasmids were generated using standard recombinant DNA methodologies and sequenced with an ABI Prism 377 DNA sequencer (ABI, Foster City, CA). *In vitro* transcribed reporter transcripts were synthesized from either pLuc-Ctrl or pLuc-Ctrl-A60 containing a bacteriophage T7 promoter directing the transcription of a firefly luciferase reporter RNA with a 0 or 60 nt poly(A) tail. An *Eco*RI site immediately downstream of firefly luciferase allowed for insertion of sequences within the 3′-UTR (see Fig. 1). Synthesis of pLuc-Ctrl was performed as described elsewhere (4). pLuc-Ctrl-A60 was synthesized by *Hin*dIII/*Xho*I excision of the poly(A)-containing luciferase from pSP-Luc^{+}-A60 (11) and into identical sites within pcDNA3 (Invitrogen). Wild-type ET-1 3′-UTRs were excised from host plasmids (4) by partial *Eco*RI digestion followed by ligation into the *Eco*RI site of pLuc-Ctrl-A60. pRSV-β-gal-A60 was used to synthesize β-gal-A_{60} transcripts, as previously described (11).

***In Vitro* Transcription.** Plasmids were linearized downstream of 3′-UTRs and/or A_{60} tails with *Xho*I (Fig. 1). ^{m}7Gppp-capped transcripts were transcribed *in vitro* with mMessage mMachine (Ambion, Austin, TX) with 1 μg of linearized DNA template and either T7 (pLuc-series) or Sp6 (pRSV-β-gal-A60) polymerase in the presence of a 1:4 ratio of GTP:cap analog. The size, integrity, and quantification of *in vitro* transcribed RNA was confirmed by agarose gel elctrophoreses.

Transfections. Unless stated otherwise, 1×10^5 BAECs were seeded in gelatin-coated 60-mm dishes, cultured to confluence for 48 hrs, transfected with equimolar amounts of capped RNAs (0.35 μg/kb), 1.5 μg of capped β-gal-A_{60} RNA (transfection efficiency control), and sufficient total cellular BAEC RNA to a total of 3 μg transfected RNA per plate. Transfections were performed with Lipofectin Reagent (Invitrogen) according to the manufacturer's protocol at a lipid:RNA mass ratio of 2:1 for 6 hrs, unless indicated otherwise. Total cellular protein was extracted in triplicate and measured for luciferase activity (test transcripts) (10), β-galactosidase activity (cotransfected efficiency control) (11), and protein concentration (12). Total cellular RNA was extracted in pooled duplicates and analyzed by Northern blot, as previously described (4). RNA transfection experiments were repeated two to five times in independent BAEC or HUVEC clones with at least two independent lots of *in vitro* synthesized RNA.

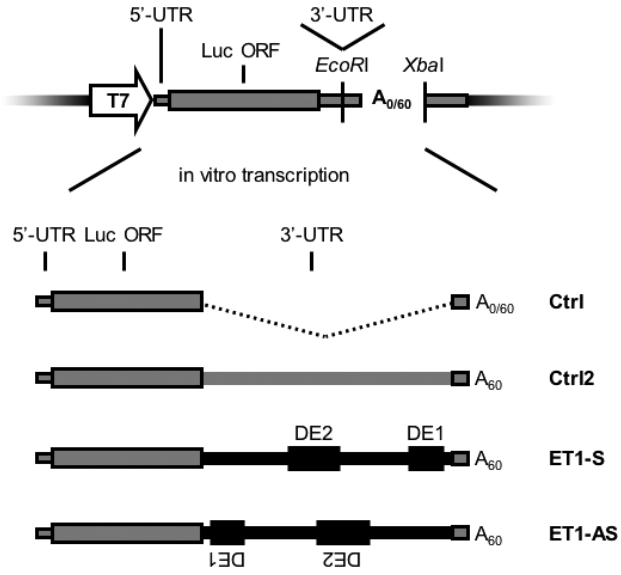

Figure 1. *In vitro* transcribed reporter transcripts. A schematic representation of the DNA template (top) engineered for *in vitro* transcription of luciferase (Luc) reporter transcripts (bottom). Reporter transcripts feature a very short 3′-UTR (Ctrl), a 1-kb size control 3′-UTR (Ctrl2), or a wild-type ET-1 3′-UTR in the sense or antisense direction (ET1-S or ET1-AS, respectively). Black lines represent ET-1 3′-UTR sequences with the relative positions of two conserved mRNA destabilizing elements labeled DE1 and DE2 (4). A_0 and A_{60} represent the presence or absence of a 60-nt poly(A) tail.

Data Analysis. Data points represent the mean ± SEM of a representative or multiple independent experiments. Statistically significant differences represent $P < 0.05$ calculated by either the unpaired Student's t test or analysis of variance (Dunnet or Bonferroni posttests; GraphPad Software Inc., San Diego, CA).

Results

Development and Optimization of RNA Transfection Methods. We developed an RNA transfection system to study posttranscriptional gene regulation in postconfluent endothelial cell cultures. Plasmids were

engineered for *in vitro* transcription of capped luciferase reporter transcripts with either a 0 or 60 nt poly(A) tail and control or ET-1 3′-UTRs (Fig. 1). Luciferase reporter transcripts were efficiently transcribed *in vitro* and robustly expressed in transfected confluent BAECs (Fig. 2). Transcripts with a poly(A) tail 60 nt in length exhibited protein expression that was 50-fold greater than that for transcripts without a poly(A) tail, whereas steady-state RNA levels were not different, at least as measured 6 hrs after transfection (Fig. 2B and C). This finding suggests RNA stability was not affected and that reporter transcripts were translationally enhanced, which is consistent with a previously established role for poly(A) tails in enhancing mRNA translational efficiency (13, 14).

Rapid, Robust, and Regulated Expression in RNA-Transfected Postconfluent Primary Endothelial Cells. To determine whether RNA transfection could be used to monitor mRNA expression in postconfluent primary endothelial cells, BAECs were plated at various densities and transfected with Ctrl-A_{60} RNA 48 hrs later (Fig. 3A). Reporter RNA was robustly expressed in postconfluent RNA-transfected BAECs with a 4-fold increase in expression *versus* that for preconfluent cells. In contrast, reporter RNA in postconfluent DNA-transfected BAECs was poorly expressed, yielding a 100-fold decrease in expression *versus* that for preconfluent cells (data not shown). In terms of specific activity in postconfluent BAECs, 1 μg of transfected luciferase RNA (Ctrl-A_{60}) typically yielded at least 10^5 light units per mg of protein, whereas 1 μg of transfected luciferase DNA plasmid (pLuc-Ctrl) typically only yielded 10^2 light units per mg of protein (data not shown). We next determined the contribution of the ET-1 3′-UTR in BAECs by means of RNA transfection. A 1-kb control 3′-UTR (Ctrl2) and an antisense ET-1 3′-UTR control (ET1-AS) were included to ensure that RNA size or structure did not account for differences in reporter expression levels (Fig. 1). The ET-1 3′-UTR conferred a 3-fold repression on reporter protein expression relative to control 3′-UTRs in both pre- and postconfluent BAECs

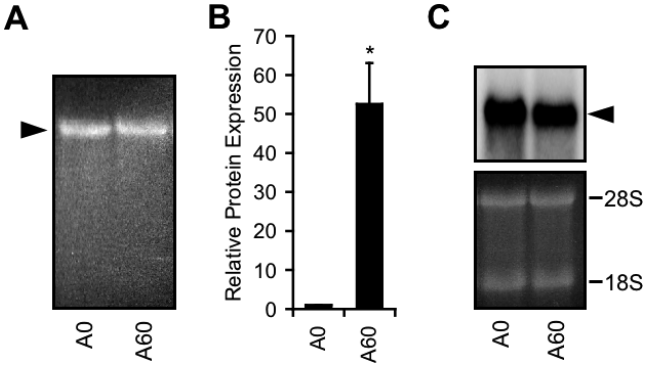

Figure 2. Reporter transcripts are efficiently expressed in BAECs and are subject to poly(A) tail-mediated regulation. (A) A representative ethidium bromide–stained gel of *in vitro* transcribed capped luciferase reporter transcripts with a Ctrl 3′-UTR (arrow) and poly(A) tails of either 0 or 60 nt. (B) Reporter transcripts were transfected into confluent BAEC for 6 hrs and assayed for luciferase activity. Data points represent the mean ± SEM of 3 independent experiments (*$P < 0.05$ vs. A_0). (C) Total cellular RNA was harvested from BAECs transfected with reporter transcripts described in panels A and B and analyzed for luciferase RNA expression (arrow) by Northern blotting.

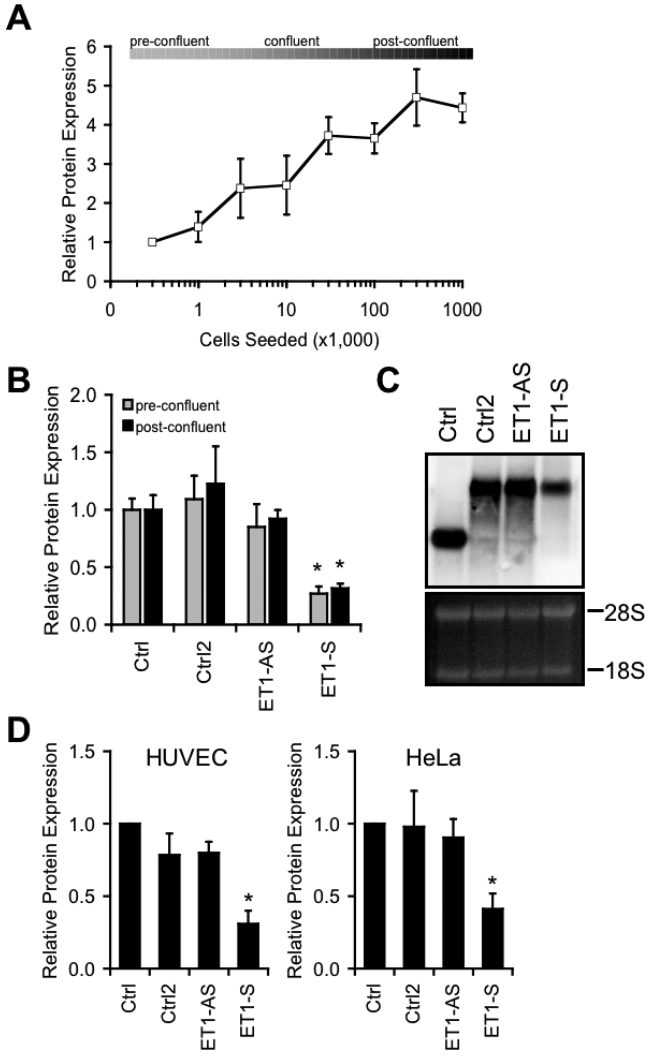

Figure 3. Reporter transcripts are robustly expressed in postconfluent BAECs and are subject to 3′-UTR-mediated posttranscriptional regulation in primary endothelial cells and nonendothelial cell lines. (A) Ctrl-A_{60} transcripts were transfected into BAEC with increasing cell density ranging from 20% confluent to postconfluent. Preconfluent cells had an elongated morphology characteristic of proliferating BAECs, whereas postconfluent cells had a cobblestone appearance characteristic of intact vascular endothelium (data not shown). BAEC were harvested 6 hrs after transfection and assayed for luciferase activity and normalized for protein content. Data points represent the mean ± SEM for 2 independent experiments. (B) Reporter transcripts with control (Ctrl), size control (Ctrl2), structure control (ET1-AS), or a wild-type ET-1 3′-UTR (ET1-S), all with an A_{60} tail, were transfected into pre- and postconfluent BAECs and assayed for luciferase activity. Data points represent the mean ± SEM for triplicate samples of a representative experiment (*$P < 0.05$ vs. Ctrl 3′-UTR). (C) Total cellular RNA was harvested from BAECs transfected with reporter transcripts and analyzed for luciferase reporter expression by Northern blotting. (D) Reporter transcripts were transfected into postconfluent HUVECs and HeLa cells. Data points represent the mean ± SEM for triplicate samples of a representative experiment (*$P < 0.05$ vs. Ctrl 3′-UTR).

(Fig. 3B). Because reporter expression is uncoupled from transcription, we concluded that the ET-1 3′-UTR destabilized the reporter mRNA, similar to our previous findings with DNA-based reporter RNA systems (4). We also tested whether RNA transfection could be used to monitor reporter protein expression in postconfluent HUVECs, which are notoriously difficult to transfect regardless of confluence. We noted robust reporter expression with greater than 10^3 and 10^5 light units per mg of protein in HUVECs and HeLa cells, respectively. Furthermore, the ET-1 3′-UTR repressed reporter expression in all cell types tested (Fig. 3B–D) (4).

We next studied the kinetics of reporter expression as a function of time in postconfluent BAECs. The ET-1 3′-UTR significantly suppressed reporter expression as a function of protein production rate (initial slope) and total protein production (area under the curve) as measured 6 hrs after transfection (Fig. 4). Future studies will be necessary to define the relative contributions of ET-1 mRNA stability *versus* translational efficiency in the presence of disease.

Discussion

Preconfluent and postconfluent primary endothelial cell cultures have markedly different phenotypes and gene expression profiles (3, 15, 16). Postconfluent endothelial cells (BAECs or HUVECs) attain a quiescent and pseudodifferentiated phenotype that resembles healthy vascular endothelium, whereas proliferating preconfluent endothelial cells resemble an activated endothelium typically seen in the presence of inflammation or vascular remodeling. These differences in phenotype are also represented by changes in gene expression. For example, postconfluent endothelial cells feature low-level ET-1 transcription and a stable eNOS mRNA (half-life, >24 hrs) whereas proliferating preconfluent endothelial cells feature activated ET-1 transcription and an unstable eNOS mRNA (half-life, <4 hrs) (3, 15).

In this study, we describe the development and optimization of an RNA transfection method to study posttranscriptional regulation in postconfluent primary endothelial cells, namely BAECs and HUVECs. DNA transfection and expression requires that plasmids access cellular nuclei for transcription; this process primarily occurs in proliferating cells (17). However, postconfluent endothelial cell monolayers have a very small percentage of proliferating cells (15), resulting in significantly decreased transfection efficiency. In contrast, RNA transfection bypasses the requirement for transcription and enables direct transcript expression in the cytoplasm.

We investigated the RNA destabilizing activity of the ET-1 3′-UTR and found that RNA transfection recapitulates the posttranscriptional activity previously reported using DNA-based transfection methods in BAECs, HUVECs, and HeLa cells (Fig. 3; Refs. 4, 8). We did not observe differences in ET-1 3′-UTR activity in preconfluent *versus* postconfluent BAECs, suggesting that cell density does not regulate ET-1 3′-UTR activity under basal conditions. These findings recapitulate our earlier studies involving DNA-based reporter methods to determine endogenous ET-1 expression in BAEC (data not shown) (4). These findings are also consistent with results of studies showing that increased ET-1 expression in proliferating vascular endothelial cells is primarily due to increased ET-1 mRNA transcription, rather than mRNA stability (15). However, we cannot exclude the possibility that endothelial cell density or expression does not influence ET-1 mRNA stability in pathophysiologic settings *in vitro* or *in vivo*. RNA transfection may therefore enable future studies on ET-1 mRNA stability in endothelial injury models requiring postconfluent endothelial cell monolayers.

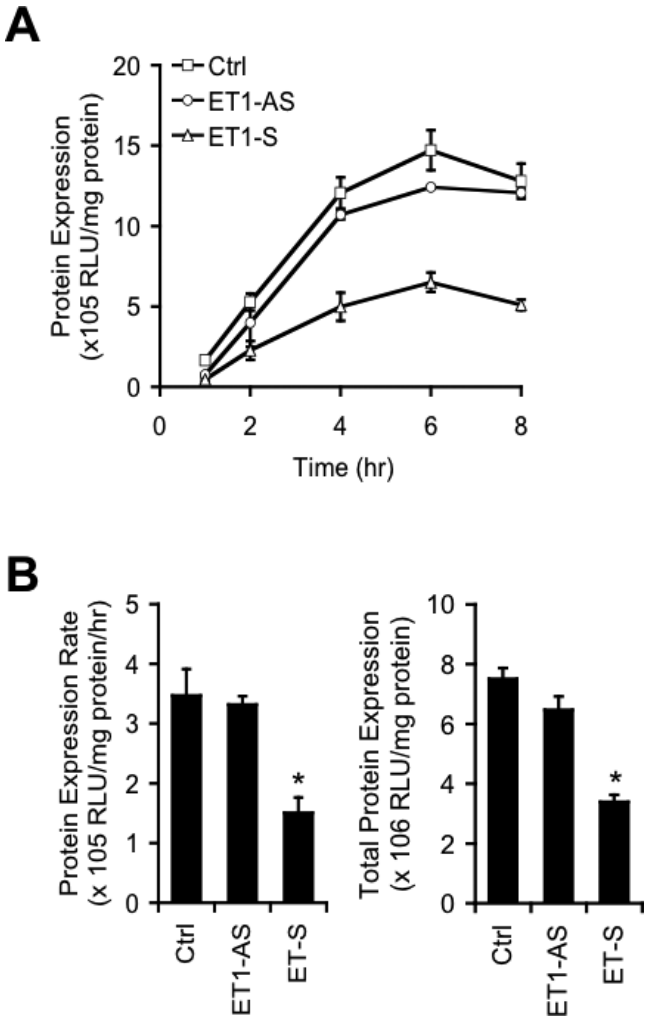

Figure 4. The ET-1 3′-UTR kinetically suppresses reporter transcript expression. (A) Reporter transcripts with an A_{60} tail and either a control (Ctrl, ET1-AS) or a wild-type ET-1 3′-UTR (ET1-S) were transfected into postconfluent BAECs and assayed for luciferase activity at the indicated time points. Data are presented as relative light units (RLU) per mg of protein. (B) Kinetic analysis of data in panel A reveals ET-1 3′-UTR suppression of expression rate (initial slope, left panel) and total expression (area under the curve, right panel). Data points represent the mean ± SEM for two independent experiments (*$P < 0.05$, compared with Ctrl).

An important feature of our system is its sensitivity to poly(A) tail length (Fig. 2). Translational efficiency and mRNA stability are significantly influenced by the presence or absence of a poly(A) tail, because of the interaction of poly(A) binding protein, mRNA cap-binding proteins, and in some cases, functional 3′-UTR *cis*-elements (13, 18). It is now appreciated that 3′-UTR regulated poly(A) tail length plays a critical role in pathophysiologic gene regulation (19–21). Therefore, synthetic control over poly(A) tail lengths could be an important tool for uncovering poly(A) tail–regulated pathways in health and disease.

A significant aspect of ET-1 biology is the transcriptional induction of ET-1 and the subsequent rate, magnitude, and persistence of ET-1 biosynthesis. Our kinetic studies (Fig. 4) recapitulate kinetic studies by others that used a DNA-based ET-1 promoter/3′-UTR reporter system (8). Together, results of these studies suggest the ET-1 3′-UTR may limit ET-1 expression, thereby attenuating the potentially harmful effects of excessive ET-1 production (1).

Our study defines RNA transfection as a versatile tool for investigating ET-1 posttranscriptional regulation in physiologically relevant postconfluent primary endothelial cell monolayers. This method may also be extended to the study of ET-1 mRNA stability in other complex cell culture models and in *ex vivo*/*in vivo* vascular models. The development of robust tools to understand gene expression in primary vascular endothelial cells will facilitate the discovery of gene regulatory networks that contribute to the health and disease of the vascular endothelium.

1. Kedzierski RM, Yanagisawa M. Endothelin system: the double-edged sword in health and disease. Annu Rev Pharmacol Toxicol 41:851–876, 2001.
2. Endemann DH, Schiffrin EL. Endothelial dysfunction. J Am Soc Nephrol 15:1983–1992, 2004.
3. Mawji IA, Marsden PA. Perturbations in paracrine control of the circulation: role of the endothelial-derived vasomediators, endothelin-1 and nitric oxide. Microsc Res Tech 60:46–58, 2003.
4. Mawji IA, Robb GB, Tai SC, Marsden PA. Role of the 3′-untranslated region of human endothelin-1 in vascular endothelial cells: contribution to transcript liability and the cellular heat shock response. J Biol Chem 279:8655–8667, 2004.
5. Bitzan MM, Wang Y, Lin J, Marsden PA. Verotoxin and ricin have novel effects on preproendothelin-1 expression but fail to modify nitric oxide synthase (ecNOS) expression and NO production in vascular endothelium. J Clin Invest 101:372–382, 1998.
6. Douthwaite JA, Lees DM, Corder R. A role for increased mRNA stability in the induction of endothelin-1 synthesis by lipopolysaccharide. Biochem Pharmacol 66:589–594, 2003.
7. Lee SD, Lee DS, Chun YG, Paik SH, Kim WS, Kim DS, Kim WD, Tuder RM, Voelkel NF. Transforming growth factor-beta1 induces endothelin-1 in a bovine pulmonary artery endothelial cell line and rat lungs via cAMP. Pulm Pharmacol Ther 13:257–265, 2000.
8. Reimunde FM, Castanares C, Redondo-Horcajo M, Lamas S, Rodriguez-Pascual F. Endothelin-1 expression is strongly repressed by AU-rich elements in the 3′-untranslated region of the gene. Biochem J 387:763–772, 2005.
9. Yang J, Chang E, Cherry AM, Bangs CD, Oei Y, Bodnar A, Bronstein A, Chiu CP, Herron GS. Human endothelial cell life extension by telomerase expression. J Biol Chem 274:26,141–26,148, 1999.
10. Karantzoulis-Fegaras F, Antoniou H, Lai SL, Kulkarni G, D'Abreo C, Wong GK, Miller TL, Chan Y, Atkins J, Wang Y, Marsden PA. Characterization of the human endothelial nitric-oxide synthase promoter. J Biol Chem 274:3076–3093, 1999.
11. Wang Y, Newton DC, Robb GB, Kau CL, Miller TL, Cheung AH, Hall AV, VanDamme S, Wilcox JN, Marsden PA. RNA diversity has profound effects on the translation of neuronal nitric oxide synthase. Proc Natl Acad Sci U S A 96:12,150–12,155, 1999.
12. Singh S, Bevan SC, Patil K, Newton DC, Marsden PA. Extensive variation in the 5′-UTR of Dicer mRNAs influences translational efficiency. Biochem Biophys Res Commun 335:643–650, 2005.
13. Wilusz CJ, Wormington M, Peltz SW. The cap-to-tail guide to mRNA turnover. Nat Rev Mol Cell Biol 2:237–246, 2001.
14. Wells SE, Hillner PE, Vale RD, Sachs AB. Circularization of mRNA by eukaryotic translation initiation factors. Mol Cell 2:135–140, 1998.
15. Flowers MA, Wang Y, Stewart RJ, Patel B, Marsden PA. Reciprocal regulation of endothelin-1 and endothelial constitutive NOS in proliferating endothelial cells. Am J Physiol 269:H1988–H1997, 1995.
16. Searles CD, Miwa Y, Harrison DG, Ramasamy S. Posttranscriptional regulation of endothelial nitric oxide synthase during cell growth. Circ Res 85:588–595, 1999.
17. Lechardeur D, Lukacs GL. Intracellular barriers to non-viral gene transfer. Curr Gene Ther 2:183–194, 2002.
18. Wilkie GS, Dickson KS, Gray NK. Regulation of mRNA translation by 5′- and 3′-UTR-binding factors. Trends Biochem Sci 28:182–188, 2003.
19. Conne B, Stutz A, Vassalli JD. The 3′ untranslated region of messenger RNA: a molecular 'hotspot' for pathology? Nat Med 6:637–641, 2000.
20. Crawford EK, Ensor JE, Kalvakolanu I, Hasday JD. The role of 3′ poly(A) tail metabolism in tumor necrosis factor-alpha regulation. J Biol Chem 272:21,120–21,127, 1997.
21. Waggoner SA, Liebhaber SA. Regulation of alpha-globin mRNA stability. Exp Biol Med (Maywood) 228:387–395, 2003.