Brief report

MicroRNAs modulate the angiogenic properties of HUVECs

Laura Poliseno, Andrea Tuccoli, Laura Mariani, Monica Evangelista, Lorenzo Citti, Keith Woods, Alberto Mercatanti, Scott Hammond, and Giuseppe Rainaldi

MicroRNAs (miRNAs) have recently come into focus as key posttranscriptional modulators of gene expression. In this work, we addressed whether in vitro angiogenesis is an miRNA-regulated process. We performed large-scale analysis of miRNA expression in human umbilical vein endothelial cells (HUVECs) and found that 15 highly expressed miRNAs have the receptors of angiogenic factors as putative targets. In particular, we demonstrated that miR-221 and miR-222 affect c-Kit expression and, as a consequence, the angiogenic properties of its ligand stem cell factor. Interaction between miR-222 and c-Kit is likely to be part of a complex circuit that controls the ability of endothelial cells to form new capillaries. (Blood. 2006;108:3068-3071)

© 2006 by The American Society of Hematology

Introduction

Angiogenesis is performed in the adult organism by the mobilization of endothelial cells from preexisting capillaries and requires degradation of extracellular matrix, migration, proliferation, and organization in new capillary networks.[1] MicroRNAs (miRNAs) have recently come into focus as a powerful mechanism to control gene expression after transcription. They are short, single-stranded RNA molecules transcribed from noncoding genes, which, after maturation and entry into the RNA interference pathway,[2,3] bind by imperfect pairing to the 3'UTR of mRNAs and inhibit translation.[4] A growing body of evidence suggests a key regulatory role for miRNAs[5-8] during embryogenesis and differentiation of adult tissues, with each differentiation grade characterized by a specific miRNA signature.[9]

Human umbilical vein endothelial cells (HUVECs) are a valuable model of in vitro angiogenesis because of their ability to form capillarylike structures called tubes in response to appropriate stimuli.[10] In this study, we asked whether miRNAs are involved in the posttranscriptional modulation of angiogenesis-related genes. To this end, the miRNA signature of HUVECs was acquired, and several highly expressed miRNAs were found to have angiogenic growth factor receptors as putative targets. In particular, we demonstrated that the miR-221/miR-222 family modulates the angiogenic activity of stem cell factor (SCF) by targeting its receptor, c-Kit.

Study design

Reagents, cells, and culture conditions

Reagents are listed in Document S1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Endothelial cells were grown in M199 + 20% FBS, 12.5 U/mL heparin, and 20 ng/mL EGF on 1% gelatin-coated plates (Document S1, isolation protocol and purity assessment). HEK 293T cells were grown in DMEM + 10% FBS. Cells were grown at 37°C in a humidified atmosphere containing 6% CO_2.

Transfections

For details on cell transfection, please refer to Document S1.

Molecular assays

miRNome analysis was performed according to Thomson et al.[9] For immunodetection of c-Kit by immunohistochemistry, immunofluorescence, or Western blot, RT-PCR, plasmid construction, and EGFP reporter assay, please refer to Document S1.

Cellular assays

Tube formation and wound healing assay were performed according to Matsui et al,[10] and migration assay was performed according to Poliseno et al.[11] For details and for cell viability protocol, please refer to Document S1.

Statistical analysis

Reported data are the mean ± SD of at least 3 independent experiments and were compared using the 2-tailed unpaired *t* test.

Results and discussion

HUVECs, when seeded on Matrigel and stimulated with the appropriate factors, organize themselves into tubes that resemble capillaries. VEGF, bFGF, HGF, SCF, Ang-1, and SDF-1 are angiogenic factors able to

The online version of this article contains a data supplement.

An Inside *Blood* analysis of this article appears at the front of this issue.

induce tube formation and promote survival and migration of HUVECs; only the first 3 increase cell proliferation.[10,12,13]

To investigate whether the organization into tubes is an miRNA-regulated process, the miRNA signature of HUVECs was obtained and 27 highly expressed miRNAs were identified. Four published prediction algorithms[14-17] were used to determine whether these miRNAs are predicted to target the receptors (Flt-1, Flk-1, Nrp-1, Nrp-2, FGF-R, c-Met, c-Kit, Tie-2, CXCR4) of the angiogenic factors listed. Receptors were chosen because they represent the initial step of the signal transduction cascade connecting each growth factor to the final biologic response. Results show (Table 1) that miR-23b, miR-23a, miR-let-7a, miR-let-7b, miR-125b, miR-221, miR-222, miR-125a, miR-24, miR-106a, miR-20, miR-16, miR-let-7c, miR-103, and miR-133a can potentially target 5 of these receptors.

Given that they share the same seed, miR-221 and miR-222 belong to the same family.[15] Their genes are located in close proximity on Xp11.3 chromosome.[18,19] Interestingly, in c-Kit–positive HUVECs (Figure S1A), they are expressed in a common pri-miRNA (Figure S1B, third panel), suggesting a coordinated transcriptional regulation.[20] According to the prediction algorithms considered,[14-17] both miR-221 and miR-222 target c-Kit 3′UTR. Given that the activation of c-Kit by SCF promotes the tube formation, migration, and cell survival of HUVECs,[10] we sought to determine whether the miR-221/miR-222 family modulates the levels and biologic activity of c-Kit. On transfection of the miR-221/miR-222 mix, c-Kit protein was decreased (Figure 1A-B) without a significant change in mRNA level (Figure 1C-D), a clear indication of posttranscriptional regulation. A decrease in receptor

level was expected to dampen the biologic effects of SCF. In keeping with this hypothesis, we found that miR-221/miR-222–transfected cells were no more able to form tubes (Figure 1E, left panel) or to heal wounds (Figure 1E, right panel) in response to SCF. In addition, cell migration potential was reduced (Figure 1F). We also considered the effect of miR-221/miR-222 transfection on SCF-induced cell survival.[10] The total number of ssEGFP-transfected cells exposed to SCF for 48 hours was higher than that of miR-221/miR-222–transfected cells cultured in the same conditions but still lower than that expected after one cell doubling (Figure 1G), whereas the fraction of dead cells was similar in both samples before and after SCF addition (Figure 1G, black bars). Taken together these results support the conclusion that miR-221/miR-222 indeed reduced SCF-induced survival without affecting the death rate. Such a deep change in biologic properties caused by a partial protein reduction might be explained by ligand-receptor binding triggering a signal that is highly amplified within the cells, and it confirms the importance of a fine modulation of receptor expression for the final biologic outputs.

To demonstrate that the interaction between miR-221/miR-222 and c-Kit is direct, a heterologous system was set up. On *c-kit* 3′UTR, miRanda predicts target site 215 for miR-221, and TargetScan predicts target sites 1030 and 1959 for miR-221 and miR-222 (Figure S2A). Target site 1959 is not present in all the published *c-kit* mRNA sequences. To ensure its presence in HUVECs, we performed reverse transcription–polymerase chain reaction (RT-PCR) with the appropriate pair of primers. Because the sequencing of the PCR product (Figure S2B) demonstrated the presence of the 1959 target site in *c-kit* mRNA, a *c-kit* 3′UTR

Table 1. Transmembrane receptors predicted to be targets of miRNAs

miRNA	Flt-1	Nrp-2	FGF-R	c-Met	c-Kit
21	—	—	—	—	—
23b	—	—	—	b + d	—
126	—	—	—	—	—
23a	—	—	—	b + c + d	—
100	—	—	—	—	—
let-7a	—	c	—	—	—
let-7f	—	—	—	—	—
99a	—	—	—	—	—
let-7b	—	—	—	c	—
125b	c	—	—	—	—
221	—	—	—	—	a, a, c
223	—	—	—	—	—
222	—	c	—	—	a, a
31	—	—	—	—	—
125a	—	c	—	—	—
24	b + d	c	c	—	—
29a	—	—	—	—	—
29b	—	—	—	—	—
let-7d	—	—	—	—	—
106a	b + c + d	c + d	—	—	—
20	b + d	c + d	—	—	—
let-7e	—	—	—	—	—
16	—	b + c	—	—	—
let-7c	—	—	—	c	—
103	—	b + c	—	—	—
29c	—	—	—	—	—
133a	—	c	b + d, d	—	—

MicroRNAs expressed at the highest level and receptors of angiogenic factors are listed in the first column and the first row of the table, respectively. Flt-1/VEGF-R1 receptor and Nrp-2 receptor are bound by vascular endothelial growth factor (VEGF). FGF-R, c-Met, and c-Kit are the receptors of basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), and stem cell factor (SCF), respectively. TargetScan[14] (a), TargetScanS[15] (b), miRanda[17] (c), and PicTar[16] (d) prediction algorithms were used to identify miRNAs that putatively targeted the 3′UTR of reported receptors. The letter is repeated when the same algorithm predicts more than one target. + indicates that the same target is predicted by more than one algorithm.
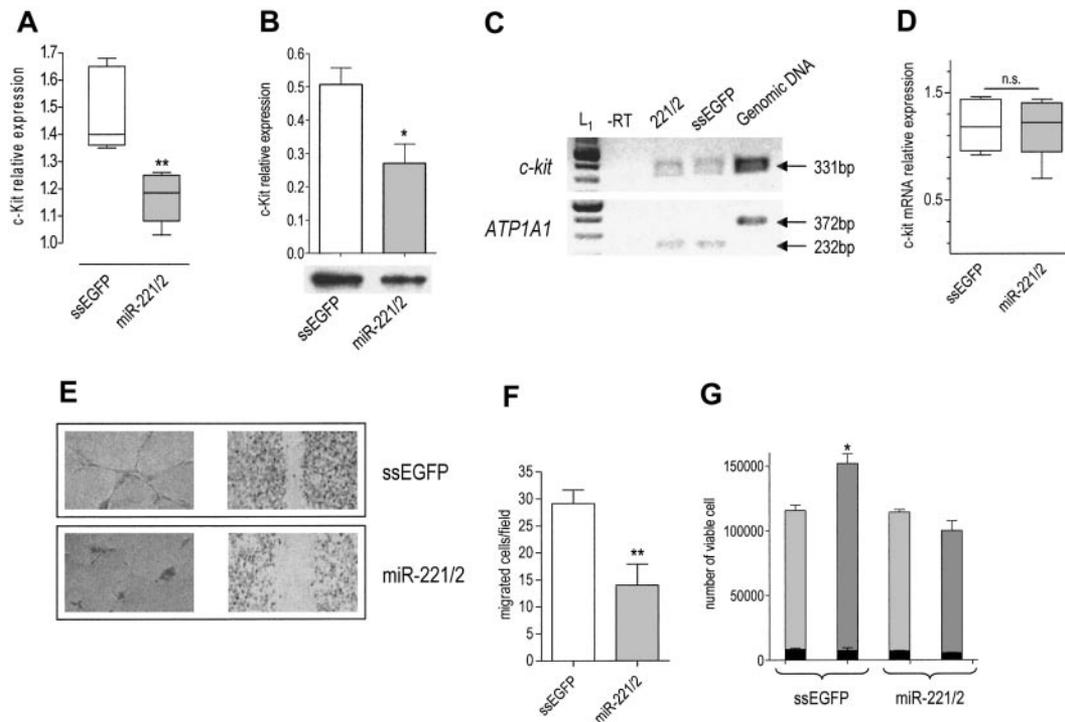
Figure 1. Effects of miR-221 and miR-222 transfection in HUVECs. c-Kit protein expression in cells transfected with ssEGFP or with the miR-221/miR-222 mix was detected 48 hours after transfection by (A) FACS (**$P < .01$) or (B) by Western blot analysis (band intensity normalized to that of tubulin; *$P < .05$). (C) RT-PCR of c-Kit. (top) c-kit mRNA level was detected by RT-PCR in control and miR-221/miR-222 mix–transfected cells by amplification of a 331-bp fragment. (bottom) A 232-bp fragment of ATP1A1 mRNA was amplified from the same RT samples to ensure equal loading per lane. The absence of the 372-bp fragment in lanes 1 to 3 indicates that the cDNA is not contaminated by genomic DNA. A representative experiment of 3 performed is reported. L_1 indicates 100-bp ladder. (D) Quantification of c-Kit mRNA. RT-PCR band intensity of c-Kit and ATP1A1 was quantified using image analysis software, and their ratio was used to compare relative expression. (E) SCF-induced tube formation (left panel): 48 hours after transfection, HUVECs were seeded onto Matrigel and were exposed to 100 ng/mL SCF + 2% FBS for 6 hours. Images of wound healing were taken after staining with crystal violet. An EPSON Expression 1680 Pro scanner (Seiko Epson, Nagano, Japan) equipped with a transparent unit was used to acquire the images. EPSON Twain Network version 2.00E and Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA) were used for image acquisition and elaboration. Results of 1 of 3 representative experiments are reported. SCF-induced wound healing (right panel): HUVECs were collected 24 hours after transfection, counted, and seeded at a density of 2×10^5 on a coated 12-well multiplate. Six hours later, the monolayers were wounded and healing was observed after overnight incubation. The tube formation was photographed after staining with crystal violet. Images were acquired with a Wilovert inverted microscope (Hund, Wetzlar, Germany) equipped with $10\times/0.25$ objective lenses and a CoolPix 4500 digital camera (Nikon, Tokyo, Japan). (F) SCF-induced migration: HUVECs transfected with ssEGFP or miR-221/miR-222 mix were allowed to migrate through type I collagen–coated filters in response to 100 ng/mL SCF for 5 hours. The number of migrated cells per field (magnification, $\times 200$) is reported. **$P < .01$. (G) Cell viability: cells were collected at the end of transfection (light gray bars) and after 48 hours of exposure to SCF (gray bars), stained with trypan blue, and counted. Total number of viable cells is reported. Dead cells (black bars) in control and miR-221/miR-222–transfected plates are also reported. *$P < .05$.

fragment that encompasses all 3 targets was amplified and cloned downstream of EGFP ORF[21] within the pEGFP-C1 plasmid to obtain the p3′UTR plasmid (Figure S2C). p3′UTR was transfected in HEK 293T cells, together with the aspecific miR-26a expression plasmid (p26a) or with the specific expression plasmids p221 and p222 (Figure S2D), which showed a strong relative activity on the p3′UTR plasmid (Figure S2E) that was absent when single target sites (p215, p1030, p1959; Figure S2C) were used. These data indicate a direct interaction of miR-221 and miR-222 with c-Kit 3′UTR and suggest that cooperation among the 3 target sites for miR-221 or between the 2 target sites for miR-222 is necessary to obtain a significant effect. However, we cannot rule out the possibility that other unknown target sites for the miR-221/miR-

222 family could cooperate with those predicted by the algorithms considered.

Taken together, these results indicate that miR-221 and miR-222 modulate the angiogenic activity of SCF and of the level of its receptor c-Kit. We believe that the interaction between miR-221/miR-222 and c-kit, recently shown to play an important role in erythropoiesis,[22] might also have a role in the formation of new vessels that occur in physiological and pathologic conditions (tumor growth). Further validation of the miRNA target pairs reported in Table 1 should help to discover the miRNA network responsible for the fine-tuning of the angiogenic process and, in the long term, to find new therapeutic approaches to modulate angiogenesis.

References

1. Carmeliet P. Angiogenesis in health and disease. Nat Med. 2003;9:653-660.

2. Kim VN. MicroRNA biogenesis: coordinated cropping and dicing. Nat Rev Mol Cell Biol. 2005;6:376-385.

3. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116:281-297.

4. Pillai RS, Bhattacharyya SN, Artus CG, et al. Inhi-

bition of translational initiation by Let-7 MicroRNA in human cells. Science. 2005;309:1573-1576.

5. Yang WJ, Yang DD, Na S, Sandusky GE, Zhang Q, Zhao G. Dicer is required for embryonic angiogenesis during mouse development. J Biol Chem. 2005;280:9330-9335.

6. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. Science. 2004;303:83-86.

7. Zhao Y, Samal E, Srivastava D. Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. Nature. 2005;436:214-220.

8. Fazi F, Rosa A, Fatica A, et al. A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPα regulates human granulopoiesis. Cell. 2005;123:819-831.

9. Thomson JM, Parker J, Perou CM, Hammond

SM. A custom microarray platform for analysis of microRNA gene expression. Nat Methods. 2004; 1:47-53.

10. Matsui J, Wakabayashi T, Asada M, Yoshimatsu K, Okada M. Stem cell factor/c-kit signaling promotes the survival, migration, and capillary tube formation of human umbilical vein endothelial cells. J Biol Chem. 2004;279:18600-18607.

11. Poliseno L, Evangelista M, Ricci F, Bonotti A, Nannipieri M, Rainaldi G. Identification of active siRNAs against IGF-IR of porcine coronary smooth muscle cells in a heterologous cell line. Int J Mol Med. 2005;15:713-718.

12. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J. 1999;13:9-22.

13. Broudy VC, Kovach NL, Bennett LG, Lin N, Jacobsen FW, Kidd PG. Human umbilical vein endothelial cells display high-affinity c-kit receptors and produce a soluble form of the c-kit receptor. Blood. 1994;83:2145-2152.

14. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. Cell. 2003;115:787-798.

15. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 2005;120:15-20.

16. Krek A, Grun D, Poy MN, et al. Combinatorial microRNA target predictions. Nat Genet. 2005;37: 495-500.

17. John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS. Human MicroRNA targets. PLoS Biol. 2004;2:e363.

18. Calin GA, Sevignani C, Dumitru CD, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci U S A. 2004;101: 2999-3004.

19. Altuvia Y, Landgraf P, Lithwick G, et al. Clustering and conservation patterns of human microRNAs. Nucleic Acids Res. 2005;33:2697-2706.

20. Baskerville S, Bartel DP. Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. RNA. 2005; 11:241-247.

21. Poliseno L, Evangelista M, Mercatanti A, Mariani L, Citti L, Rainaldi G. The energy profiling of short interfering RNAs is highly predictive of their activity. Oligonucleotides. 2004;14:227-232.

22. Felli N, Fontana L, Pelosi E, et al. MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor downmodulation. Proc Natl Acad Sci U S A. 2005;102: 18081-18086.

23. Basta G, Lazzerini G, Massaro M, et al. Advanced glycation end products activate endothelium through signal-transduction receptor RAGE: a mechanism for amplification of inflammatory responses. Circulation. 2002;105:816-822.

24. Yarden Y, Kuang WJ, Yang-Feng T, et al. Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. EMBO J. 1987;6:3341-3351.

25. Sun L, Lee J, Fine HA. Neuronally expressed stem cell factor induces neural stem cell migration to areas of brain injury. J Clin Invest. 2004; 113:1364-1374.

MicroRNAs modulate the angiogenic properties of HUVECs

Laura Poliseno, Andrea Tuccoli, Laura Mariani, Monica Evangelista, Lorenzo Citti, Keith Woods, Alberto Mercatanti, Scott Hammond and Giuseppe Rainaldi

---

Updated information and services can be found at:
http://www.bloodjournal.org/content/108/9/3068.full.html

Articles on similar topics can be found in the following Blood collections
Free Research Articles (5431 articles)
Gene Therapy (602 articles)

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml