MicroRNA-19 (miR-19) Regulates Tissue Factor Expression in Breast Cancer Cells*

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Tissue factor has been recognized as a regulator of tumor angiogenesis and metastasis. The tissue factor gene is selectively expressed in highly invasive breast cancer cells, and the mechanisms regulating tissue factor expression in these cells remain unclear. This study demonstrates that microRNA-19 (miR-19) regulates tissue factor expression in breast cancer cells, providing a molecular basis for the selective expression of the tissue factor gene. Tissue factor protein was barely detectable in MCF-7, T47D, and ZR-75-1 cells (less invasive breast lines) but was expressed at a significantly higher level in MDA-MB-231 and BT-20 cells (invasive breast lines) as assayed by Western blot. The tissue factor gene promoter was activated, and forced expression of tissue factor cDNA was achieved in MCF-7 cells, implying that the 3'-UTR of the tissue factor transcript is responsible for the suppression of tissue factor expression. Bioinformatics analysis predicted microRNA-binding sites for miR-19, miR-20, and miR-106b in the 3'-UTR of the tissue factor transcript. Reporter gene assay using the TF-3'-UTR luciferase reporter construct confirmed that the 3'-UTR negatively regulates gene expression in MCF-7 cells, an effect reversed by deletion of the miR-19-binding site. Application of the miR-19 inhibitor induces endogenous tissue factor expression in MCF-7 cells, and overexpression of miR-19 down-regulates tissue factor expression in MDA-MB-231 cells. RT-PCR analysis using cDNA made from Ago2-immunoprecipitated RNA samples confirmed that Ago2 binds preferentially to tissue factor 3'-UTR in MCF-7 cells, as compared with MDA-MB-231 cells, consistent with the observation that miR-19 levels are higher in MCF-7 cells.

Tissue factor, an initiator of the extrinsic coagulation cascade, was recently recognized as an important regulator of tumor angiogenesis and metastasis, independent of its function in the blood coagulation pathway (1). This is particularly true during breast cancer progression (2). This effect of tissue factor is mainly achieved through the tissue factor-factor VIIa complex that activates the protease-activated receptor 2 signaling pathway (3, 4), a G protein-coupled receptor signaling event that generates second messages and alters transcription of the target genes (5). Tissue factor is highly overexpressed in many types of tumors, including breast cancer, especially at the late stage of disease progression (6–10). A recent report indicates that tissue factor is expressed only in breast carcinoma cells having the highest invasive potential (11). Furthermore, formation of the tissue factor-factor VIIa complex promotes cellular signaling and migration of human breast cancer cells (12). Convincing evidence obtained over the last 5 years suggests that tissue factor may stimulate vascular epithelial growth factor production possibly through the protease-activated receptor 2 pathway, resulting in enhanced tumor angiogenesis (13). Although targeting of the tissue factor signaling pathway has been explored as a strategy to inhibit tumor angiogenesis (1) and prevent breast cancer progression (14), the mechanisms responsible for selective tissue factor expression remain to be defined.

MicroRNAs are a family of ~22-nucleotide-long RNA molecules that are highly conserved and expressed in many organisms (15). They are transcribed from various genetic regions and processed in the nuclei through cytoplasm, becoming mature single strand microRNA molecules. It is well recognized that microRNAs regulate gene expression in eukaryotic cells (15). The principal mechanism of microRNA regulation of gene expression is through the formation of a microRNA-RNA-induced silencing complex in the cytoplasm, which binds to the 3'-untranslated region (3'-UTR) of target transcripts, thereby leading to repression of protein translation or destabilization of the mRNAs (16). Altered expression of microRNA species has been well established in human cancer cells (17, 18). Importantly, certain microRNA species have been demonstrated to regulate tumor invasion and metastasis of breast cancer (19, 20) and are involved in mediating tumor angiogenesis (21).

The 3'-UTR of the tissue factor transcript is around 1 kb long, indicating a strong possibility that this 3'-UTR would contribute to the stability of the transcript and the regulation of the protein translation process. MicroRNA regulation of tissue factor expression has never been examined in any model system. This study investigated the potential regulation of tissue factor expression by microRNAs in breast cancer cells. We report here that tissue factor expression is regulated...
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by miR-194 in our model systems, which may explain overexpression of the tissue factor gene in invasive breast cancer cells.

EXPERIMENTAL PROCEDURES

Materials—The PGL3 promoter vector and the Dual-Luciferase assay kit were purchased from Promega (Madison, WI). MicroRNA inhibitors and mimics were obtained from Dharmacon, Inc. (Lafayette, CO). The antibody for tissue factor was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from ProMab Biotechnologies, Inc. (Richmond, CA). Ago2 antibody was ChIP grade and from Abcam Inc. (Cambridge, MA). The human tissue factor promoter reporter construct was obtained from Dr. Nigel Mackman (22). All other reagents were analytic grade and purchased from Sigma.

Cell Culture—Human breast cancer cell lines MCF-7, MDA-MB-231, T47D, BT-20, and ZR-75-1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in RPMI 1640 (MCF-7, T47D, BT-20, and ZR-75-1) and DMEM (MDA-MB-231) media supplemented with 10% fetal bovine serum and antibiotics. Cells were grown under a humid environment at 37 °C, 5% CO2, and passed twice a week for MCF-7, MDA-MB-231, T47D, and once a week for BT-20 and ZR-75-1. Appropriate cell numbers were seeded to meet the demand of different assays.

Confocal Microscopy—The YFP-TF chimera (sYT-pcDNA3.1(+)) was generated by in-frame insertion of the monomeric form of yellow fluorescent protein (kind gift of Dr. Roger Tsien, University of California, San Diego (23)) between the signal and mature peptide sequences of the full-length tissue factor cDNA clone (MGC-13630 from ATCC) by splicing and overlapping extension PCR. The expressed protein was analyzed in transient and stable transfected endothelial cells by Western blot, immunofluorescence, and activity assays (two-stage Xa generation assay) and was similar to the endogenous counterpart. MCF-7 and MDA-MB-231 cells, in RPMI 1640 and DMEM with supplements, respectively, were plated at 2.5 × 105 cells per well in a 6-well plate and incubated overnight. The cells were then transfected with 3 μg of the sYT-pcDNA3.1(+) construct in 2 ml of serum-free medium containing 8 μl of FuGENE HD transfection reagent (Roche Applied Science). Five hours after incubation, the medium was replaced with fresh medium supplemented with 10% fetal bovine serum. 48 h after transfection, the cells were washed three times with Hanks’ balanced salt solution and viewed under a Leica TCS SP2 confocal microscope with LCS Lite imaging software (0.9 × 63 objective, with excitation at 517 nm and emission at 527 nm). The images were quantified using the same software.

Cloning of the Tissue Factor 3'-UTR—Total RNA was isolated from MCF-7 cells with TRIzol reagent and reverse-transcribed using the SuperScript II kit (Invitrogen). The cDNA was then subjected to PCR amplification with the primers covering the 3'-UTR of the tissue factor transcript (1013-bp fragment, NM_001993, 1013–2026). The primers used were as follows: forward, 5'-TGCTCTAGAGGCAAGCATTGTGGAGCAGCT-3', and reverse, 5'-TGCTCTAGACATGTCAACCATAGAGGAAGCACTGTTG-3'. The PCR products were separated on 1% agarose gel containing ethidium bromide, and the DNA fragment was visualized under ultraviolet light. The DNA fragment was purified from the gel using QIAquick gel extraction kit (Qiagen Sciences, Valencia, CA) and cloned into the PGL3 promoter luciferase reporter construct (Promega, Madison, WI) downstream of the luciferase cDNA in both sense and antisense orientations. The cloned constructs were named TF-3'-UTR-S (sense orientation) and TF-3'-UTR-A (antisense orientation) and were confirmed by DNA sequencing. The TF-3'-UTR-A construct was used as a control whenever the TF-3'-UTR-S was used. The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to delete the miR-19- and miR-106b-binding sites from the TF-3'-UTR-S construct. The primers for the deletions were as follows: miR-19 deletion forward, 5'-CACCACCTGGCCACCCATGCTTTAGAT-ATATCCG-3', and miR-19 deletion reverse, 5'-GCCGAATATAATCTAAGACATGGGGCCATTAGTGGTGTG-3'; miR-106b deletion forward, 5'-GGGACCCCTCTCATATGCTGACTTAATGTC-3', and miR-106b deletion reverse, 5'-GCCACTTAAGTCACTAGCATATTAGGAGGTGCC-3'. The deletions were confirmed by DNA sequencing.

Dual-Luciferase Activity Assay—MCF-7 or MDA-MB-231 cells, growing in RPMI 1640 or DMEM with supplements, were plated at a density of 2.5 × 105 cells per well in a 6-well plate. After 24 h, the medium was removed, and the transfection complex containing 2 μg of the reporter constructs (TF-3'-UTR-S, TF-3'-UTR-A, or hTF-P2kb), 200 ng of the pRL-TK Renilla luciferase reporter vector (Promega, Madison, WI), and 10 μl of FuGENE HD transfection reagent (Roche Applied Science) in 2 ml of serum-free medium was added. After 5 h of incubation, the medium was replaced with 2 ml of RPMI or DMEM with 10% fetal bovine serum, and cells were incubated overnight. The cells then were lifted and plated into a 24-well plate at a density of 250,000 per well. Luminescence was measured 72 h after transfection, using a Dual-Luciferase reporter system (Promega, Madison, WI) following the manufacturer’s protocol. The firefly luciferase activity was normalized by the Renilla luciferase activity.

Application of MicroRNA Inhibitors or Mimics—MicroRNA inhibitors and mimics for hsa-miR-19a and hsa-miR-106b were purchased from Dharmacon, Inc. (Lafayette, CO). The day before transfection, MCF-7 or MDA-MB-231 cells were plated at 2.5 × 105 cells per well in a 6-well plate. On the day of transfection, the cells were washed with PBS and incubated with 100 nM of the inhibitors or mimics for miR-19a or miR-106b in 2 ml of serum-free medium containing 8 μl of FuGENE HD transfection reagent. After 5 h of incubation, the medium was replaced with 2 ml of RPMI 1640 or DMEM with supplements. For some of the experiments, cells were cotransfected with the microRNA inhibitors or mimics with 2 μg of the TF-3'-UTR-S construct. After 72 h of incubation...
with the inhibitors or mimics, cells were harvested either for luciferase assay or for Western blot analysis.

**Western Blot Analysis**—Western blot was performed as we described previously (24). In brief, cells were lysed with the lysis buffer, sonicated on ice, and centrifuged at 15,000 × g for 15 min to remove insoluble material. 40 μg of cell lysate from each sample was separated in 10% SDS-polyacrylamide gel, transferred to PVDF membrane, and blotted with antibodies against human tissue factor, Ago2, and GAPDH.

**Reverse Transcription-PCR Analysis**—Total RNA was isolated from MCF-7 and MDA-MB-231 cells using TRIzol reagent and reverse-transcribed with the SuperScript II kit (Invitrogen) as we described (25). The cDNA was then subjected to PCR amplification with the following primers: GAPDH forward, 5′-GGGATCTCGCTCCTGGAAG-3′, and GAPDH reverse, 5′-TGGGGAAGGTGAAGGTCGG-3′, and GAPDH forward, 5′-TCAGGCCACTACAATACTGTGG-3′, and GAPDH reverse, 5′-GGGATCTCGCTCCTGGAAG-3′; tissue factor forward, 5′-TCAGGCCACTACAATACTGTGG-3′, and tissue factor reverse, 5′-TTCTCTGAATATCCCCCTTCTC-3′; YFP forward, 5′-GCAATCGAGCTGAAGGGCAT-3′, and YFP reverse, 5′-GTCCGGCATGATATAAGACCTGTG-3′. The PCRs and the thermal cycles were detailed in our previous study (25). The PCR products were separated in a 1% agarose gel containing ethidium bromide and visualized under UV light.

**MicroRNA Detection by Real Time PCR**—miR-19 levels in MCF-7 and MDA-MB-231 cells were determined by real time PCR analysis. Total RNA was isolated using TRIzol reagent (Invitrogen), and microRNAs were enriched using the mirVana™ miRNA isolation kit (Ambion, Austin, TX). The microRNAs were reverse-transcribed using the RT² miRNA first strand kit from SABiosciences (Frederick, MD). Expression of miR-19 was assayed by real time PCR using the ABPrism 7700 sequence detection system (Applied Biosystems, Foster City, CA). The U6 small nuclear RNA served as an internal control for microRNA amplification (26). Primers for miR-19 and U6 were obtained from SABiosciences (Frederick, MD). Relative quantification of expression level was performed following the manufacturer’s protocol.

**Co-immunoprecipitation**—Co-immunoprecipitation using Ago2 antibody was performed as described previously (24). In short, cells growing in 100-mm dishes were washed with PBS and harvested by adding 150 μl of IP buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5 mM EDTA, 1 mM PMSF, and 1% Triton X-100). After sonicating the cells for 1–3 min on ice, insoluble material was removed by centrifugation. Supernatants were collected and protein concentrations determined. Super- natants were removed by centrifuging at 2,000 g of cell lysate from each sample was separated in 10% SDS-polyacrylamide gel, transferred to PVDF membrane, and blotted with antibodies against human tissue factor, Ago2, and GAPDH.

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**RESULTS**

**Expression of Tissue Factor Gene in Breast Cancer Cells**—Previous studies reported that tissue factor is highly expressed in breast cancer cells having high invasive potential (11, 27).

To understand whether tissue factor is expressed differently among breast cancer cell lines, we examined its expression in five human breast cancer cell lines representing highly invasive and less invasive phenotypes. Reverse transcription-PCR assay revealed that tissue factor mRNA is detectable in all cell lines (Fig. 1A) tested, although it is expressed at relatively higher levels in MDA-MB-231 and BT-20 cells (highly invasive lines (28)). Western blot analysis demonstrated that tissue factor is barely detectable in MCF-7, T47D, and ZR-75-1 cells (less invasive cell lines (28)). However, tissue factor is highly expressed in more invasive breast cancer lines, MDA-MB-231 and BT-20 (Fig. 1B). These observations are consistent with previous reports (11, 27). A reporter gene assay using a tissue factor gene promoter construct, hTF-P2kb (22), indicated that tissue factor transcription is indeed activated in MCF-7 cells by 1 μM phorbol myristate acetate (PMA, Fig. 1C), suggesting that transcription regulation of the tissue factor gene is achievable in this cell line. A cDNA construct was established in which the intact tissue factor coding sequence (the 3′-UTR was not included) was fused to the C terminus of the YFP cDNA. Interestingly, when this construct was tran-

**FIGURE 1.** **Tissue factor expression in breast cancer cells.** A, total RNA was isolated from human breast cancer lines, reverse-transcribed, and PCR-amplified with specific primers covering tissue factor (TF) and GAPDH cDNA. The PCR products were separated on 1% agarose gel containing ethidium bromide. B, cellular lysates from MCF-7, MDA-MB-231, T47D, ZR-75-1, and BT-20 cells were subjected to Western blot using antibodies against human tissue factor and GAPDH (shown in A and B are representatives of three experiments). C, human tissue factor gene promoter activity (hTF-P2kb) is up-regulated by phorbol myristate acetate (PMA) in MCF-7 cells. Cells were transfected with the hTF-P2kb construct and treated with 1 μM phorbol myristate acetate for 4 h. Luciferase activity was analyzed as described under “Experimental Procedures.” Data (means ± S.E., n = 3) are expressed as percentages of the luciferase activity in untreated control cells. *, p < 0.05, compared with control cells, using one-way ANOVA analysis.
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Potential candidates of microRNA species that might interact with the 3′-UTR of human tissue factor mRNA

The sequence for tissue factor mRNA (TF) was obtained from the GenBank™ (NM_001993), and microRNA sequences were obtained from the website of Memorial Sloan-Kettering Cancer Center. Table 1. The sequence of the miR-19, miR-106b, and miR-20 target sites in the tissue factor 3′-UTR. The potential interaction of microRNAs with the 3′-UTR of a particular gene transcript can be predicted through established web programs (29). We used a well established microRNA-target predicting program developed at the Memorial Sloan-Kettering Cancer Center to predict microRNAs that may interact with the 3′-UTR of tissue factor transcript. As shown in Table 1, two regions of the tissue factor 3′-UTR were found to match the seed sequences (30) of miR-19, miR-106b, and miR-20 (Table 1). To experimentally confirm our prediction, the 3′-UTR of the tissue factor transcript was cloned into the PGL3 luciferase promoter reporter construct both in a sense and antisense orientation downstream of the luciferase cDNA sequence (TF-3′-UTR-S and TF-3′-UTR-A) (Fig. 3A). The TF-3′-UTR-A construct served as a control. As shown in Fig. 3B, inclusion of the 3′-UTR of the tissue factor transcript led to a significant reduction of luciferase activity in MCF-7 cells, but not in MDA-MB-231 cells, indicating that tissue factor expression is regulated by its own 3′-UTR in MCF-7 cells (Fig. 3B).

miR-19 Regulates Tissue Factor Expression—To further confirm our prediction, we generated reporter constructs with the deletion of the two microRNA-binding sites, respectively, from the TF-3′-UTR-S construct (Fig. 3C). These constructs, along with the TF-3′-UTR-S, were transfected into MCF-7 and MDA-MB-231 cells, and luciferase activity was analyzed. As shown in Fig. 3D, when the miR-19-binding site was deleted from the 3′-UTR, the luciferase activity increased to a level comparable with that of the control in MCF-7 cells. However, disruption of the binding site for miR-106b and miR-20 only had marginal effects on the luciferase activity, indicating that miR-19 is primarily responsible for the reduc-

![FIGURE 2. Forced expression of the tissue factor gene in MCF-7 and MDA-MB231 cells. A and B, cells were transfected with the YFP-TF chimera construct. 48 h after the transfection, the YFP expression was captured by a Leica TCS SP2 confocal microscope with LCS Lite imaging software (0.9 × 63 objective, excitation 517 nm and emission 527 nm). A phase contrast image of the cells was also captured and is shown on the right. Images are representative of three experiments. C, YFP mRNA level was determined by reverse transcription-PCR, showing a similar expression level in MCF-7 and MDA-MB-231 cells.](image)

![FIGURE 3. 3′-UTR of the tissue factor transcript suppresses luciferase activity in MCF-7 but not in MDA-MB-231 cells. A, diagram showing the tissue factor 3′-UTR cloned into PGL3 promoter vector in both sense (TF-3′-UTR-S) and antisense orientation (TF-3′-UTR-A). B, MCF-7 and MDA-MB-231 cells were transfected with the TF-3′-UTR-S or TF-3′-UTR-A constructs. Luciferase (Luc) activity was analyzed 48 h after transfection. Data (means ± S.E., n = 3) are expressed as percentages of the luciferase activity detected in TF-3′-UTR-A transfected cells. C, shown are miR-19- and miR-106b-binding sites in the tissue factor 3′-UTR. The underlined nucleotides were deleted in the TF-3′-UTR-S reporter construct. D, MCF-7 (left panel) and MDA-MB-231 (right panel) cells were transfected with the TF-3′-UTR-A, TF-3′-UTR-S, or the two deletion constructs (del-1, miR-19-binding site deletion; del-2, miR-106b-binding site deletion), respectively. Luciferase activity was analyzed 48 h after transfection. Data (means ± S.E., n = 3) are expressed as percentages of the luciferase activity detected in TF-3′-UTR-A transfected cells. *p < 0.05, compared with the 3′-UTR-A transfected cells, using one-way ANOVA followed by Dunnett analysis.)
miR-19 has been shown to be expressed at a higher level in MCF-7 cells. Consistent with previous reports, we also found that miR-19 is highly expressed in MCF-7 cells.
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in MCF-7 versus MDA-MB-231 cells, indicating that Ago2 targets tissue factor 3′-UTR preferentially in MCF-7 cells, as compared with MDA-MB-231 cells. Ago2 protein is expressed in both cell lines (Fig. 6B).

DISCUSSION

Proper regulation of tissue factor expression is critical for homeostasis. The tissue factor gene is classified as an immediate early response gene that is a target of many regulatory influences. Transcription regulation of the tissue factor gene has been well characterized, which is associated with DNA consensus binding sites that have been found within the tissue factor gene promoter (35). We have confirmed previous observations that expression of the tissue factor gene is barely detectable at the protein level in less invasive breast cancer cells yet is highly expressed in invasive breast cancer cells (11, 27). The lower expression of the tissue factor gene in MCF-7 cells does not seem to be related to abnormal transcription suppression of the gene, as our results from the tissue factor gene promoter studies showed that the promoter is active and can be regulated through the MAPK pathway in this cell line, observations similar to a previous report (27). Furthermore, tissue factor mRNA is detectable in MCF-7 cells, and the genome hypomethylation pattern is similar between MCF-7 and MDA-MB-231 cells (36), implying that the tissue factor gene is not transcriptionally silenced in MCF-7 cells. The fact that YFP-TF was equally expressed in both MCF-7 and MDA-MB-231 cells indicates that there is no repression mechanism present in these cells targeting the coding sequence of the tissue factor gene. These observations led to the hypothesis that post-transcriptional regulation of tissue factor expression in less invasive breast cancer cells involves the 3′-UTR of the gene transcript. This hypothesis was confirmed by the experiments presented in this study.

Tissue factor gene expression has been shown to be regulated at a post-transcriptional level in cultured fibroblasts and monocytic cells (37, 38), yet the detailed mechanisms remain unclear. Several lines of experimental evidence from this study support the conclusion that miR-19 regulates tissue factor expression at a post-transcriptional level in breast cancer cells. First, bioinformatics analysis using a well-established program indicated that several microRNAs have a perfectly matched seed sequence with two regions of the 3′-UTR of the tissue factor transcript. This provides a rationale (29) to look into the potential regulation of tissue factor expression by these microRNA species. Second, we demonstrated that the 3′-UTR of the tissue factor transcript down-regulates this gene expression in MCF-7 but not MDA-MB-231 cells as assayed with the reporter gene technique. The down-regulation of the reporter gene activity was reversed in MCF-7 cells, indicating that Ago2 targets tissue factor 3′-UTR preferentially in MCF-7 cells, as compared with MDA-MB-231 cells. Ago2 protein is expressed in both cell lines (Fig. 6B).

pressing a high level of tissue factor protein. Finally, the experiments with Ago2 immunoprecipitation indicated that the tissue factor 3′-UTR is favored by Ago2 protein binding in MCF-7 cells versus MDA-MB-231 cells. This is consistent with the result showing that miR-19 is expressed at a higher level in MCF-7 cells. Thus, the role of miR-19 in regulating tissue factor expression in our model systems is clearly demonstrated.

Previous observations also indicated that the miR-17–92 cluster, of which miR-19 is a member, is expressed at higher levels in MCF-7 cells compared with MDA-MB-231 cells (32), and miR-19 is expressed at higher levels among the members of this cluster in MCF-7 cells (31). Because the tissue factor mRNA levels were not significantly affected by miR-19 inhibitor in MCF-7 cells nor by miR-19 mimics in MDA-MB-231 cells, the main action of miR-19 seems to inhibit protein translation of the tissue factor gene in less invasive breast cancer cells. Repression of protein translation is one of the well-established mechanisms of microRNA action in suppressing gene expression (16). This mechanism of action, the post-transcriptional regulation of tissue factor expression by miR-19, could well explain the selective expression of the tissue factor gene in highly invasive breast cancer cells.

The up-regulation of the miR-17–92 cluster has been observed in human cancer cells, including breast cancer (39, 40). These microRNAs are considered as oncogenes (41–43), which promote proliferation, inhibit apoptosis, and induce tumor angiogenesis (42, 44). However, the contribution of each individual microRNA in the cluster to tumor progression remains to be characterized. As tissue factor is involved in tumor angiogenesis and metastasis (1), our finding that miR-19 regulates tissue factor expression in breast cancer cells provides novel information on the mechanisms of microRNA-induced tumor progression. The potential effect of miR-19 on breast cancer invasiveness and angiogenesis could be directly tested in the relevant model systems. In a more broad sense, regulation of tissue factor expression by microRNAs could be relevant to the advancement of homeostatic biology in which cell-specific expression of tissue factor plays a pivotal role (35). Model systems such as monocytes or endothelial cells may be used to further investigate the regulation of tissue factor expression by microRNAs.

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