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Rapid communication

Regulation of TGF-β-mediated endothelial-mesenchymal transition by microRNA-27

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Multiple microRNAs (miRNAs) regulate epithelial-mesenchymal transition and endothelial-mesenchymal transition (EndMT). Here we report that microRNA-27b (miR-27b) positively regulates transforming growth factor-β (TGF-β)-induced EndMT of MS-1 mouse pancreatic microvascular endothelial cells. TGF-β induced miR-23b/24-1/27b expression, and inhibition of miR-27 suppressed TGF-β-mediated induction of mesenchymal genes. Genome-wide miRNA target analysis revealed that miR-27 targets Elk1, which acts as a competitive inhibitor of myocardin-related transcription factor-serum response factor signalling and as a myogenic repressor. miR-27b was also found to regulate several semaphorin receptors including Neuropilin 2, Plexin A2 and Plexin D1. These results suggest important roles of miR-27 in TGF-β-driven EndMT.

Keywords: TGF-β; EndMT; microRNA; α-SMA; Elk1.

MicroRNAs (miRNAs) are important regulators of differentiation and cell fate decisions. Multiple miRNAs, such as microRNA-200 (miR-200) and miR-155, have been identified as suppressors or promoters of epithelial-mesenchymal transition (EMT) (*1*). In addition, several studies have described roles of miRNAs in endothelial-mesenchymal transition (EndMT), which is important for heart development and various pathological processes (*2*). We have previously reported that transforming growth factor-β (TGF-β) induces mesenchymal genes including α-smooth muscle actin (α-SMA) by activating Rho signals and myocardin-related transcription factor A (MRTF-A), and that constitutively active miR-31 is a positive regulator of TGF-β-induced EndMT and EndMT-associated secretory phenotype (EndMT-SP) in MS-1 mouse pancreatic microvascular endothelial cells (*2*, *3*).

miR-23, miR-24 and miR-27, members of miR-23/24/27 clusters, are highly conserved vertebrate miRNAs and are involved in various biological processes (*4*). Mammals have two miR-23/24/27 clusters: miR-23a∼27a∼24-2 and miR-23b∼27b∼24-1. Members of miR-23/24/27 have been proposed to have important roles in angiogenesis (*5*–*8*). In addition, deregulated expression of these miRNAs and cancer-related roles have been described in many studies with varying results (*9*). miR-23 and miR-27 are shown to facilitate EMT in several types of cancer including lung cancer (*9*). We also previously reported that TGF-β induces the expression of miR-23a in A549 lung cancer cells (*10*). However, in contrast to their roles in EMT, the roles of miR-23/24/27 in EndMT have not been well characterized. In the present study, we studied the effects of TGF-β on expression levels of miR-23/24/27 and potential contribution of miR-27 to TGF-β-driven EndMT in MS-1 endothelial cells.

miRNA microarray analysis (H.I.S. & A.K., article in preparation) showed that TGF-β increased the expression of miR-23b, miR-24 and miR-27b in MS-1 cells (Fig. 1a). The effects on miR-23a and miR-27a were not remarkable. Next, we investigated the roles of endogenous miR-27b in EndMT by inhibiting endogenous miR-27 activity. Locked nucleic acid (LNA) miR-27b inhibitor (LNA-miR-27b) abolished miR-27b activity in MS-1 cells (Fig. 1b). miR-27b inhibitor suppressed induction of mesenchymal markers, α-SMA and SM22α, by TGF-β (Fig. 1c). In contrast, miR-27b inhibitor did not attenuate induction of conventional Smad target genes, Smad7, Fibronectin1, and PAI-1 and downregulation of endothelial markers, VEGFR2 and CD34 (Fig. 1d and e). These results demonstrated that TGF-β induces the expression levels of miR-23b/24-1/27b but not of miR-23a/24-2/27a in MS-1 cells and that miR-27 positively regulates mesenchymal gene induction by TGF-β.

In order to identify miR-27 targets during TGF-β-mediated EndMT at a transcriptome-wide level, we analysed the RNAseq data using MS-1 cells treated with miR-27b inhibitor and/or TGF-β, reported in our previous study (*3*). In consistent with widespread inhibitory effects of miRNAs on target gene expression, mRNA levels of potential miR-27 target genes with both conserved and poorly conserved target sites generally increased by miR-27 silencing (Fig. 2a and Supplementary Table S1). Gene ontology (GO) analysis showed that LNA-miR-27b-sensitive miR-27 target genes are related to diverse molecular functions, such as ion binding, transcriptional regulation, and regulation of phosphorylation (Fig. 2b and Supplementary Table S2).

To analyse functions of miR-27 targets in the context of known biological pathways, we performed pathway map analysis using MetaCore software. Representative miR-27 target genes frequently observed in the pathway maps enriched for LNA-

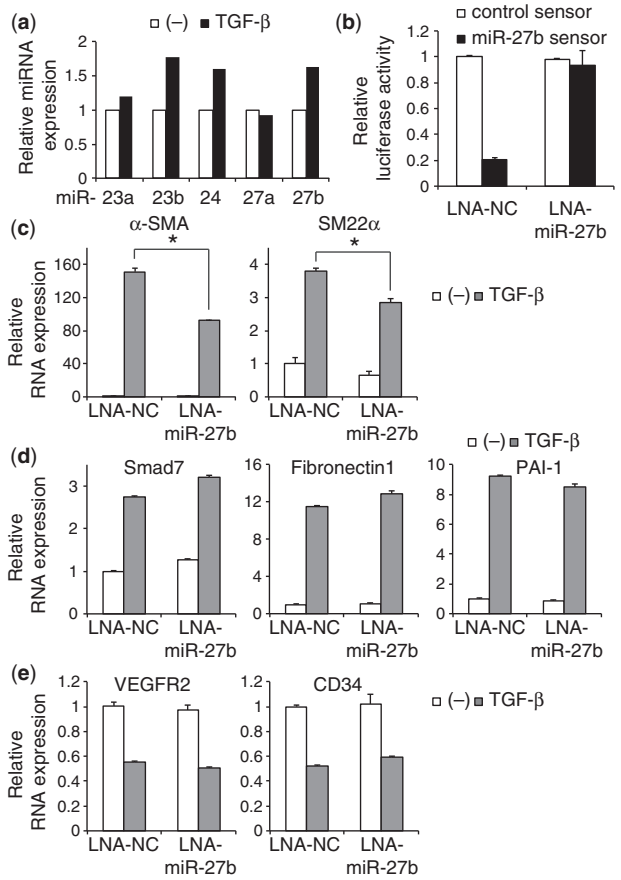

Fig. 1 miR-27b is a positive regulator of EndMT induced by TGF-β in MS-1 endothelial cells. (a) Effects of TGF-β on miR-23/24/27 expression levels, determined by miRNA microarray analysis (TGF-β2, 1 ng/ml, 72 h). **(b)** Inhibition of miR-27b activity by miR-27b inhibitor in MS-1 cells. MS-1 cells were transfected with LNA control miRNA inhibitor (LNA-NC) or LNA miR-27b inhibitor (LNA-miR-27b, 50 nM) and miRNA sensor vectors, and subjected to dual luciferase reporter assay after 48 h. **(c–e)** Effects of miR-27 inhibition on TGF-β-mediated gene expression changes (c, EndMT genes; d, conventional TGF-β target genes; and e, endothelial cell-specific genes), determined by qRT-PCR analysis. MS-1 cells transfected with LNA miRNA inhibitors were stimulated with TGF-β2 (1 ng/ml, 72 h). Error bars represent SDs. $*P < 0.05$. Experimental procedures and primer information are described in Supplementary Materials and Supplementary Tables S5 and S6.

miR-27b-sensitive miR-27 targets included several transcriptional factors such as CREB1 and Elk1 and various signaling regulators, including phosphoinositide 3-kinase, c-Jun N-terminal kinase and p38 mitogen-activated protein kinase (Fig. 2c and Supplementary Table S3). Such miR-27 target genes also included multiple transcription factors, such as Elk1, EPAS1, ARNT, CREB1, SP1 and GATA2, important for endothelial function (Supplementary Fig. S1). Gene set enrichment analysis (GSEA) also uncovered an association between LNA-miR-27b-dependent gene expression changes and gene sets associated with hypoxia and Elk1 paralogue, Elk3 (Fig. 2d). Since Elk1 is a ternary complex factor of the ETS-domain family, which competes with MRTFs for binding to serum response factor (SRF) and acts as a myogenic repressor (*14*, *15*), we next focused on Elk1. Reporter assay of Elk1 3' untranslated region (3'UTR) confirmed that miR-27b targets Elk1 3'UTR depending on the predicted target site (Fig. 2e and f). These results suggest that miR-27 may facilitate TGF-β-driven EndMT through Elk1 suppression and subsequent MRTF-A activation in MS1 cells.

In addition, we found that miR-27 targets VAV3, a regulator of actin remodeling and MRTF-A activity (Supplementary Table S1). Considering our previous report describing that VAV3 is regulated by another EndMT-promoting miRNA, miR-31 (*3*), VAV3 may be combinatorially regulated by miR-27 and miR-31. Although miR-31 is required for induction of EndMT-SP, i.e. induction of multiple inflammatory chemokines and cytokines including CCL17, CX3CL1, IL-6 and Angptl2 (*3*), the effects of miR-27 inhibition on EndMT-SP were weak relative to miR-31 inhibition (Supplementary Fig. S2).

We further systematically analysed TGF-β-dependent suppression of miR-27 target genes, which may be accompanied with induction of miR-27b by TGF-β. Comparison of GO terms enriched in genes downregulated by TGF-β and GO terms enriched in miR-27 target genes downregulated by TGF-β and upregulated by LNA-miR-27b suggested that genes associated with semaphorin receptor activities are suppressed by TGF-β in a miR-27-dependent fashion (Fig. 3a and Supplementary Table S4). This gene set included Neuropilin 2 (Nrp2), Plexin A2 (PlxnA2) and Plexin D1 (PlxnD1). We confirmed that miR-27b targets 3'UTRs of Nrp2, PlxnA2 and PlxnD1 as well as Elk1 (Fig. 3b and Supplementary Fig. S3). These findings suggest that TGF-β-induced EndMT is associated with miRNA-mediated alteration of semaphorin receptor activities.

In conclusion, we demonstrated that miR-27b is a positive mediator of EndMT induced by TGF-β, adding a novel function of miR-27 in vascular biology. Relationships between miR-27 and some of the targets identified in this report, Elk1, Nrp2, PlxnA2, and PlxnD1, are maintained in mammals and zebrafish (see Supplementary Materials), suggesting that regulation of these targets by miR-27 may contribute to conserved developmental processes. In contrast to EndMT-promoting activity of miR-27 observed in our study, a previous report described that miR-23 restricts differentiation from endocardial cells to endocardial cushion cells and cardiac valve formation (*16*). In the developing heart, miR-27b expression is observed in only myocardium but not endocardial cushions and increases in the later developmental stages (*17*). Thus, miR-23 and miR-27 may differentially modulate EndMT in embryonic heart development and adult pathology.

We identified Elk1 as a novel target of miR-27. Another report has shown that miR-143 regulates plasticity and fate of vascular smooth muscle cells by targeting Elk1 (*18*). These findings suggest that multiple miRNAs are tightly integrated into a core transcriptional network involved in smooth muscle differentiation and proliferation. Our analysis also demonstrated that miR-27b regulates several semaphorin receptors Nrp2, PlxnA2 and PlxnD1, suggesting

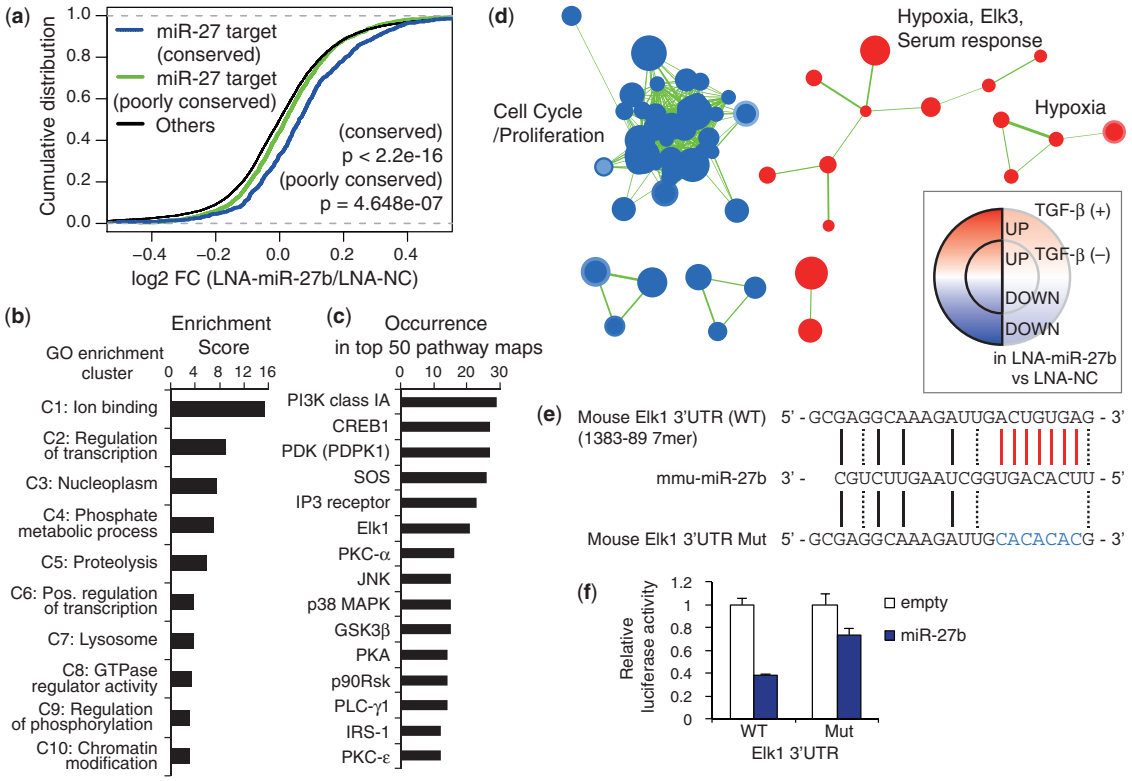

Fig. 2 Transcriptome-wide identification of miR-27 target genes. (a) Cumulative distribution plots of mRNA fold changes of miR-27 target genes predicted by TargetScan (right, conserved; middle, poorly conserved) and other expressed genes (left) by miR-27 inhibition in MS-1 cells. *P* values were calculated by one-sided Kolmogorov-Smirnov test. **(b)** GO analysis of miR-27 target genes upregulated by LNA-miR-27b. **(c)** Occurrence of representative LNA-miR-27b-sensitive miR-27 targets in top 50 pathway maps from MetaCore Pathway Map analysis. **(d)** Connectivity maps of gene sets obtained from GSEA for LNA-miR-27b-induced gene expression changes using C2CGP gene set collection (FDR < 0.0001). Right and left nodes represent gene sets with enrichment in LNA-miR-27b samples and in LNA-NC samples, respectively. Enrichments with and without TGF-β treatment are mapped to the node borders and inner node area, respectively. Gene sets without edges are not shown. **(e)** Sequence alignment between miR-27b and its putative-binding site in mouse Elk1 3'UTR. **(f)** Suppression of Elk1 3'UTR by miR-27b. Dual luciferase assay was performed in HEK293T cells using pri-miR-27b expression vector and wild-type or mutated Elk1 3'UTR reporter vector (shown in panel (e)) according to previous reports (*11–13*).

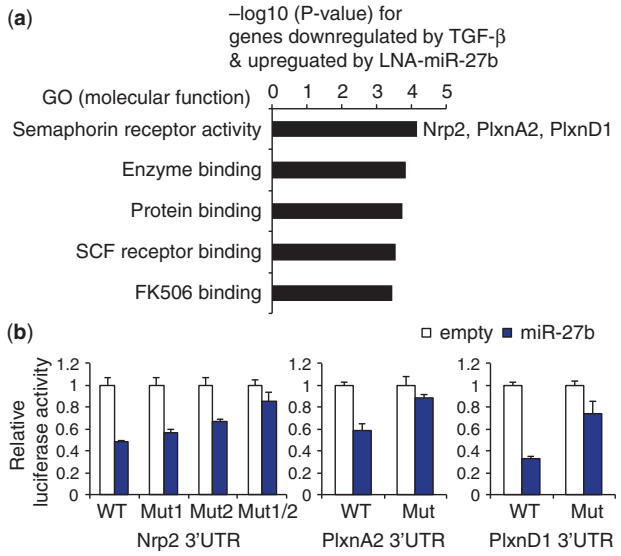

Fig. 3 miR-27b regulates semaphorin receptors. (a) Top 5 GO (molecular function) terms enriched in miR-27 target genes downregulated by TGF-β and upregulated by LNA-miR-27b, obtained from MetaCore analysis. **(b)** Suppression of 3'UTRs of Nrp2, PlxnA2 and PlxnD1 by miR-27b, analysed as in Figure 2f. Sequences of mutated 3'TR are shown in Supplementary Figure S3.

another novel feature of TGF-β-induced EndMT. miR-23 and miR-27 have been shown to promote endothelial cell sprouting and angiogenesis by targeting various angiogenesis regulators, Sprouty2, SEMA6A and Dll4 (*6–8*). Since Plexin A2 is a receptor for SEMA6A, coordinate regulation of both semaphorin ligands and semaphorin receptors may underlie angiogenic phenotypes of miR-27. Furthermore, Nrp2 is shown to negatively regulate contractility of vascular smooth muscle cells (*19*). Thus, suppression of Nrp2 by miR-27 may be associated with acquisition of contractile phenotype during EndMT. Further analysis would shed light on the importance of miR-27 and related members in EndMT and EndMT-associated pathological processes. These findings may offer development of therapeutic approach to manipulate EndMT.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of Interest
None declared.

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