

**TITLE: Platelet microparticle delivered microRNA-Let-7a promotes the angiogenic switch**

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**Highlights**

- Platelet microparticles deliver the microRNA Let-7a to endothelial cells.
- Let-7a directly targets and reduces production of thrombospondin-1.
- The loss of anti-angiogenic thrombospondin-1 promotes *in vivo* capillary like formation.

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**Abstract**

Platelet microparticle (PMP)-induced angiogenesis plays a key role in tumour metastasis and has been proposed to contribute towards cardiovascular disease by enhancing atherosclerotic plaque vulnerability. However, the mechanisms underlying PMP induced angiogenesis are ill defined. Recent reports demonstrate that PMPs deliver micro-RNAs (miRNAs) to recipient cells, controlling gene expression. We therefore evaluated whether miRNA transfer was a key regulator of PMP-induced angiogenesis. Co-culturing PMPs with human umbilical vein endothelial cells (HUVEC) on extracellular matrix gel induced robust capillary like structure formation. PMP treatment altered the release of angiogenesis modulators from HUVEC, including significantly reducing production of anti-angiogenic thrombospondin-1 (THBS-1). Both functional responses were abrogated by treating PMPs with RNase, suggesting the transfer of PMP-derived RNA was a critical event. PMPs were an abundant source of miRNA Let-7a, which was transferred to HUVEC following co-incubation. Using luciferase reporter assays we have shown that Let-7a directly targets the 3'UTR of the THBS-1 mRNA. HUVEC transfection with a Let-7a anti-sense oligonucleotide reduced the ability of PMPs to inhibit THBS-1 release, and significantly decreased PMP induced *in vitro* angiogenesis. Antibody neutralisation of THBS-1 reversed the anti-angiogenic effect of let-7a inhibition in PMP treated HUVEC, highlighting Let-7a dependent translational repression of THBS-1 drives angiogenesis. Importantly, plasmid overexpression of Let-7a in HUVEC alone induced robust tubule formation on extracellular matrix gel. These data reveal a new role for Let-7a in promoting angiogenesis and show for the first time PMPs induced angiogenic responses occur through miRNA regulation of HUVEC.

**Key words:**

Angiogenesis, Platelet microparticles, Thrombospondin-1, Let-7a

## Introduction

Platelet microparticles (PMPs) are small vesicles, 0.1-1 $\mu$ m in diameter, shed from activated or apoptotic platelets [1]. PMPs represent the majority of circulating MPs [2], with levels increasing in inflammatory and cardiovascular disease states.[3, 4] Through delivery of bioactive molecules including cytokines, mRNAs and miRNAs to recipient cells, PMPs participate in intercellular communication. There is growing evidence that these signalling mechanisms promote a pro-inflammatory environment [1], contributing to atherosclerosis [5] and tumour metastasis.[6]

MicroRNAs (miRNAs) are small non-coding RNAs 19-24 nucleotides in length that post-transcriptionally regulate gene expression. By binding to near complementary sites within the 3'UTR of target mRNAs, miRNAs block gene translation or induce mRNA degradation [7]. MiRNAs are predicted to regulate most mammalian genes, playing a vital role in cellular development and function. [8] Importantly, the miRNA distribution pattern is tissue specific and is altered in a range of cancers [9-11] and cardiovascular diseases [12-15]. Platelets contain a diverse array of over 490 miRNAs. [16, 17] Seminal work by Gidlof et al demonstrated activated platelets release functional miR-320b in PMPs, which is taken up by endothelial cells to repress intercellular adhesion molecule 1 (ICAM 1) expression. [18] Subsequent studies have confirmed that PMP-delivered miRNAs translationally repress a range of target genes in recipient cells.[19-22]

PMPs are strongly pro-angiogenic.[23-26] Inflammatory states including cancer and atherogenesis are accompanied by robust angiogenesis, with newly formed blood vessels helping to regulate tumour growth and plaque progression.[27, 28] However, the mechanisms behind PMP-induced angiogenesis have not been fully defined. Given the documented role of PMPs in both disease states,[29, 30] coupled with the abundance of PMPs within the circulation[2] and their propensity to transfer miRNAs,[19, 21] we hypothesised that PMP-induced angiogenesis is driven by miRNA regulation of endothelial cell function. In the present study we report that PMPs are internalised by human umbilical vein endothelial cells (HUVEC), transferring miRNA-Let-7a. Through targeting the 3'UTR of thrombospondin-1 (THBS-1) mRNA, Let-7a significantly reduces THBS-1 protein release from endothelial cells. Our data show this inhibition of THBS-1 production is a key mechanism driving PMP mediated capillary like formation *in vitro*.

## Materials and Methods

### Platelet and PMP isolation

Human blood was taken by venepuncture from healthy young adult volunteers into acid citrate dextrose (ACD; 29.9 mmol L<sup>-1</sup> sodium citrate, 113.8 mmol L<sup>-1</sup> glucose, 72.6 mmol L<sup>-1</sup> sodium chloride and 2.9 mmol L<sup>-1</sup> citric acid, pH 6.4). Platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at 200 × *g* at 20 °C, 20 min. Washed platelets (WP) were isolated from the PRP by centrifugation at 800 × *g* at 20 °C, 12 min in the presence of prostaglandin E<sub>1</sub> (PGE<sub>1</sub>; 50 ng mL<sup>-1</sup>). Platelets were resuspended in Tyrodes buffer (150 mmol L<sup>-1</sup> NaCl, 5 mmol L<sup>-1</sup> HEPES, 0.55 mmol L<sup>-1</sup> NaH<sub>2</sub>PO<sub>4</sub>, 7 mmol L<sup>-1</sup> NaHCO<sub>3</sub>, 2.7 mmol L<sup>-1</sup> KCl, 0.5 mmol L<sup>-1</sup> MgCl<sub>2</sub>, 5.6 mmol L<sup>-1</sup> glucose). For DiO6 stained microparticles, PRP was incubated with 10 μmol DiO6 dye for 15 minutes before the isolation of WP. PMP were isolated as reported by Laffont.[21] WP suspensions were activated with thrombin (0.1 U/ml) for 60 minutes. Platelet activation was stopped by EDTA (20mM) and platelets pelleted by centrifugation (3200*g*;10 minutes). PMPs were isolated by centrifugation at 20,000*g*; 90 minutes at 18°C, then resuspended in Tyrodes buffer. Protein content was measured using Bradford assay. For RNase assay, PMPs were treated with RNase A (1U/ml) at 37°C for 60 minutes and reaction stopped by RNase inhibitor (10 U/ml) for 5 minutes.[18] PMPs were diluted 100-fold in PBS and analysed by flow cytometry as previously reported [21, 31] for phosphatidylserine exposure detected by Fluorescein (FITC)-Annexin-V (BD Biosciences) binding or platelet specific marker CD41a expression detected with APC-conjugated anti human CD41a antibody or APC-conjugated mouse IgG isotope control (BD Biosciences). Following incubation for 30minutes, samples were diluted 5-fold in PBS and analysed using a Beckman Coulter – CyAn™ ADP analyser (Beckman Coulter, UK) calibrated using BD cytometer setup and tracking beads (BD Biosciences) gated for the reported PMP size of 0.1–1.0μm.

### Cell culture

Primary HUVEC from 7 different healthy donors (Bradford University Ethical Tissue biobank), were cultured in Endothelial cell growth medium (EGM) supplemented with endothelial cell growth supplement kit (PromoCell), maintained at 37°C in a humidified atmosphere (5% CO<sub>2</sub>). At least 3 different donor HUVEC were used between passages 2-6 in all experiments.

### Extracellular matrix gel *in vitro* angiogenesis assays

Angiogenesis on extracellular matrix gel was quantified using Endothelial Tube Formation Assay Kits (Cell Biolabs). Transfected or control HUVEC (2 × 10<sup>4</sup>cells/ml) in EGM supplemented with 2% serum

were added to each coated well and treated with PMPs (100µgPMP protein/ml) or Tyrodes buffer control at 37°C for 18 hours. Tube formation was observed with an Evos fluorescent microscope (x40;Life Technologies) after staining with 1µM CalceinAM (Cell Biolabs). Four microscope fields were selected at random and photographed. Total tube length and individual branch point number per field under was recorded as tube-forming ability. AngioTool (National Cancer Institute, United States) was used for quantification.

### **Angiogenesis cytokine array assays**

The Proteome Profile Human Angiogenesis Array kit, (R&D Systems) was used according to the manufacturer's instruction. Briefly, conditioned media from serum starved HUVEC (2x10<sup>5</sup>cells/ml) cultured with 100µgPMP protein/ml or Tyrodes buffer control at 37°C for 24 hours was collected and centrifuged to remove cellular debris. After blocking of non-specific binding at room temperature antibody array membranes were incubated with conditioned media at 4°C overnight then incubated with diluted horseradish peroxidase-conjugated streptavidin (room temperature, 30 min). Chemiluminescent visualization was analysed densitometrically by G-Box (Gel documentation and analysis, Syngene). Quantification was carried out using the Gel plugin on Image J v1.49 software (National Institutes of Health, United States).

### **THBS-1 ELISA**

Conditioned media was collected as in angiogenesis cytokine array assays. The concentration of thrombospondin-1 (THBS-1) in media was analysed using the Human Thrombospondin-1 Quantikine ELISA Kit (R&D Systems), according to manufacturer's instructions.

### **THBS-1 Western Blotting**

HUVEC were transfected with Let-7a inhibitor or non-targeting control (Active motif) as below before treatment with 100µg/ml PMP. THBS-1 levels were then analysed by Western Blotting [32] using Anti-Thrombospondin 1 antibody (ab85762, abcam) at a 1:1000 dilution. Membranes were re-probed with anti-Beta tubulin antibody ab6046.

### **Cell transfection with anti-Let-7a antisense oligonucleotides or precursor expression plasmid**

Cells were seeded in supplemented EGM for 24-hours prior to transfection. HUVEC were transfected with Let-7a inhibitor or non-targeting control (Active motif) using Lipofectamine 3000 (Life

Technologies). Transfection complexes were added to confluent cells to a final concentration of 1.8nM. HUVEC were transfected with Let-7a pmir expression plasmid or empty pmir plasmid using FuGENE HD reagent. For expression plasmid, transfection complexes were added to confluent cells to a final concentration of 200µg. Transfected cells were cultured for 24 hours before removing transfection media. Cells were starved before trypsinisation and treatment with 100µg/ml PMP.

#### **RNA extraction and quantitative RT-PCR of microRNA**

Total RNA was extracted from PMPs, or PMP treated HUVEC following three rounds of PBS washes, using the Aurum total RNA Mini kit (BIO-RAD) according to the manufacturer's instructions. To assess PMP transfer, HUVEC were treated with 0.25ng/µl Actinomycin D for 4-hours before treatment with PMP and total RNA extraction. Stem loop cDNA was synthesised from total RNA with iScript select cDNA synthesis kit (BIO-RAD) using specific stem loop primers for:

Let-7a, 5' GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGAT ACGACA ACTA 3',

U6s, 5' GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCACGA 3'.

Quantitative real time PCR reactions for detection of Let-7a expression was performed using the IQ SYBR® green supermix (BIO-RAD) as previously reported.[33] The primers were designed as follows:

Let-7a forward, 5' AAAAAAAAAAGCCCGCTGAGGTAGTAGTTGTA 3',

U6s forward, 5' AAAAAAAAAAGCCCGCTGCGCAAGGATGAC 3',

Universal reverse 5' AAAAAAAAAAGTGCAGGGTCCGAGGT 3'.

#### **Reporter gene activity assays**

HEK-293T cells (2 x 10<sup>4</sup>cells/ml) were transfected with 600µg Reporter luciferase vector including, 3'UTR of THBS-1 or empty 3'UTR reporter vector, using FuGENE HD reagent. After 12-hours cells were transfected with 1.8nM of Let-7a inhibitor or non-targeting control, using Lipofectamine 3000. Following 2-hours culture, transfected cells were treated with 100µg/ml PMP for 15 hours in normal culture conditions. Renilla activity was measured by the LightSwitch Luciferase Assay (ActiveMotif) using the The GloMax Explorer system (Promega).

#### **Immunofluorescent tracking of PMP internalisation by HUVEC**

1 x 10<sup>5</sup> HUVEC seeded on a glass cover slips in supplemented EGM were treated with 100µg/ml of DiOC6 stained PMPs for 6, 12 or 18 hours. Cells were washed with PBS and fixed with 4%

paraformaldehyde for 10 minutes before staining with DAPI and visualisation with Evos fluorescent microscopy (x400) (Life Technologies).

### **Statistical analysis**

Results are expressed as mean $\pm$  SEM using at least three independent experiments using PMPs isolated from different blood donors and analysed by Students t-test or one-way ANOVA as detailed in the figure legends. Differences between means was considered significant when  $P < 0.05$  (\*) or  $P < 0.001$  (\*\*) between groups.

## Results

### PMPs induce HUVEC tubule formation

Thrombin, produced at heightened levels around atherosclerotic plaques and tumours, strongly promotes PMP production. Given the reported role of angiogenesis in these pathologies we initially sought to confirm the pro-angiogenic nature of PMPs produced by thrombin stimulation. Treating HUVEC with PMPs induced capillary like structure formation on extracellular matrix gel, increasing both mean tube length and number of branch points ( $p < 0.05$ ) (Figure 1). Recent studies by Laffont [20] and Liang [19, 22] demonstrated PMPs deliver miRNAs into recipient cells to modulate target gene expression. To determine if PMP-induced tubule formation was reliant on delivered RNA, we first depleted total PMP RNA with RNase [34, 35] before incubation with HUVEC for *in vitro* angiogenesis assays. RNase treatment reduced total PMP RNA to undetectable levels (Figure 1d). Prior to co-incubation with HUVEC, residual RNase was inhibited with RNase inhibitor and removed by washing. RNase treatment limited PMP stimulated tube formation, with mean tube length and number of branch points decreasing (figure 1A); suggesting PMP-induced *in vitro* angiogenesis in part requires transfer of an RNA species.

### PMPs alter the profile of angiogenic modulators released by HUVEC

Since angiogenesis is driven by growth factor signalling we assessed if the angiogenic signature of HUVEC was altered by PMP treatment. Conditioned media from serum starved or PMP treated HUVEC were analysed with Proteome Profiler Human Angiogenesis Arrays.[36] PMP treatment significantly enhanced HUVEC release of several proangiogenic factors including endothelin-1 (ET-1), monocyte chemoattractant protein 1 (MCP-1), angiogenin (Ang) and placental growth factor (PIGF) (Figure 2). PMP treatment also significantly reduced the release of the potent antiangiogenic factor thrombospondin-1 (THBS1) ( $p < 0.05$ ). RNase treatment of PMPs partially reversed their ability to alter ET-1 and Ang release, and fully blocked their ability to alter MCP-1, PIGF and THBS-1 levels. These data show PMPs induce a unique cytokine profile from HUVEC, which is partially dependent upon PMP-derived RNA content.

### The miRNA Let-7a targets THBS-1

Given the recent reports that PMPs alter recipient cell function through miRNA delivery,[18-22] and that RNase treatment altered the pro-angiogenic effect of PMP treatment, we investigated the link. The array data showed the only factor strongly downregulated by PMPs was THBS-1; this effect was abrogated by RNase treatment, fitting the pattern of direct miRNA targeting. We used TargetScan and miRanda, to search potential miRNAs targeting THBS-1, identifying the miRNA Let-7a (Figure 3a). The



Let-7 family are the most abundant miRNAs within platelets[16] with Let-7a being one of the most highly expressed[18]. qRT-PCR confirmed the presence of Let-7a within PMPs, which was degraded by RNase treatment ( $p < 0.05$ ; Figure 3B). Importantly when RNA was spiked into these samples after RNA isolation it was not degraded, meaning the RNase was successfully neutralised prior to RNA isolation from PMP. This demonstrates RNase treatment effectively strips intact PMPs of their Let-7a rather than degrading RNA once PMPs have been lysed during RNA isolation. To validate that Let-7a directly targets THBS-1 mRNA, HEK293 cells were co-transfected with a reporter plasmid for the THBS-1 3'UTR and either a Let-7a expression plasmid or empty vector control. Let-7a expression completely inhibited the reporter signal of THBS-1 3'UTR, confirming direct targeting (Figure 3C). Together our data suggests the reduction in THBS-1 in PMP treated HUVEC could be due to repression by delivered Let-7a.

#### **PMPs can deliver Let-7a into HUVEC *in vitro***

Previous reports demonstrated PMPs are internalised by recipient cells[18, 19, 21] resulting in miRNA transfer. We initially verified with fluorescent microscopy that DiOC6 labelled PMPs were internalised by HUVEC, with the fluorescent signal diffusing through HUVEC cytoplasm over time (figure 4B). To confirm that PMPs delivered Let-7a into endothelial cells, HUVEC were co-cultured with or without 100 $\mu$ g protein PMP/ml for 24hours and qRT-PCR was used to assess relative expression. Upon co-culture, HUVEC Let-7a levels increased significantly ( $p < 0.05$ ; Figure 4A), an effect reduced by pre-treating PMPs with RNase. To confirm that the observed increase in Let-7a by PMP-treatment was dependent on active transfer, rather than altered HUVEC Let-7a production, transcription was inhibited with actinomycin D (Figure 4A). Under these conditions PMPs still significantly increased the expression of HUVEC Let-7a. These data confirm PMPs are internalised by HUVEC resulting in a delivery dependent increase in Let-7a, although a small proportion of Let-7a is also reliant on transcriptional activity.

#### **PMP-derived Let-7a downregulates THBS-1 protein production**

MiRNAs function as translational repressors through binding to the 3'-UTR of target mRNA molecules. MiRNA binding may additionally result in target mRNA degradation.[37] We therefore determined whether the exogenous Let-7a delivered by PMPs could affect THBS-1 production by the recipient HUVEC. Treating HUVEC with PMPs for 24 hours surprisingly had a small positive effect on THBS-1 mRNA levels (Figure 5A). MRNAs have previously been shown to be directly transferred from platelets to target cells.[38] We confirmed the presence of THBS-1 mRNA in PMPs (Figure 5B) and showed that treating PMPs with RNase reduced their ability to induce the small increase in HUVEC THBS-1 (Figure

5A). As in figure 3B we confirm RNase was degrading RNA in the intact PMPs (Figure 5B). It is likely the small increase in THBS-1 mRNA remaining in the presence of RNase treated PMPs is due to cytokines carried by PMPs such as PDGF known to regulate THBS-1.[23, 39] We next assessed the impact of PMP-Let-7a transfer on THBS-1 protein production by HUVEC. PMP treatment robustly decreased THBS-1 protein levels from  $221\pm 10$  to  $134\pm 3$  ng/ml ( $p<0.05$ ). This reduction was abolished by pre-transfection with the Let 7a antisense oligonucleotide (ASO) (Figure 5C). Transfection with a scrambled oligonucleotide had no effect on the reduction in THBS-1 production by PMPs. To confirm that PMP-Let-7a signalling was not just reducing THBS-1 release we measured total THBS-1 levels in HUVEC by Western blotting (Figure 5D), the data confirm PMP delivered Let-7a directly reduces the production of anti-angiogenic THBS-1 protein by HUVEC.

### **PMP-derived Let-7a dependent inhibition of THBS-1 promotes tube formation**

Next we investigated the role of PMP transferred Let-7a in the induction of angiogenesis. Serum depleted HUVEC were transfected with either Let 7a ASO or scrambled-oligonucleotide then co-cultured with  $100\mu\text{g}$  protein PMP/ml for 24hours on extracellular matrix gel and *in vitro* angiogenesis assessed. PMP treatment increased mean total tube length and number of branch points ( $p<0.05$ , Figure 6AaC). Let-7a inhibition partially, but significantly, reduced the ability of PMPs to stimulate angiogenesis, with mean tube length and number of branch points decreasing ( $p<0.05$ ). Scrambled-oligonucleotide has no effect on PMP mediated angiogenesis, showing for the first time PMP-induced angiogenesis in HUVEC is strongly supported by the transfer of PMP Let-7a. To further confirm this we used plasmid overexpression of Let-7a, which alone induced robust angiogenesis, increasing mean tube length and branch points to a similar level observed by PMP treatment (Figure 6D-F). Together our data suggest Let-7a helps drive *in vitro* tubule formation whilst repressing THBS-1 production.

Finally, we investigated if the Let-7a dependent decrease in THBS-1 was responsible for the observed PMP-induced Let-7a dependent angiogenesis. Initially we used blocking antibodies to the two main THBS-1 HUVEC receptors, CD29 and CD47.[40, 41] Unfortunately these antibodies interfered with the *in vitro* angiogenesis assay (data not shown). We therefore next neutralised THBS-1 with monoclonal anti-THBS-1 IgM antibody (Clone A 4.1).[42] Neutralising THBS-1 in HUVEC co-cultured with PMP had a small but significant positive effect on endothelial tube formation and branching; suggesting a low level of THBS-1 mediated anti-angiogenic activity was present. This observation is consistent with our data (Figure 7AC) that PMP treatment does not fully abrogate THBS-1 production. Importantly, the reduction in PMP induced angiogenesis by transfection with Let-7a ASO was reversed by the THBS-1 neutralising, but not control antibody. Let-7a inhibition reduced PMP stimulated mean tube length and branching from  $9020\pm 604\mu\text{m}$  and  $43\pm 5$  to  $6620\pm 192\mu\text{m}$  and  $25\pm 4$  respectively ( $p<0.05$ ). In the

presence of antibody Clone A 4.1 mean tube length and branching increased back to  $9056 \pm 296 \mu\text{m}$  and  $45 \pm 2$  ( $p < 0.05$  vs PMP treatment in Let-7a ASO transfected cells). Thus, neutralising THBS-1 rescues the PMP stimulatory effect on angiogenesis which had been blunted by Let-7a inhibition. Together these data show exogenous PMP-delivered Let-7a inhibits production of anti-angiogenic THBS-1 from HUVEC, thereby increasing tubule formation.

## Discussion

Platelets are the major source of microparticles in the bloodstream; circulating at concentrations up to 50µg/ml.[43] In response to agonist stimulation, inflammation and disease states, including arterial thrombosis and malignancy, PMP levels significantly increase.[44] The PMP concentration of 100µg/ml used in this study represents heightened levels found in these conditions.[44] PMPs support the pathogenesis of vascular disease and metastasis, regulating processes such as endothelial–leukocyte adhesion, inflammation[5] and production of matrix-remodelling enzymes.[45] A growing body of evidence suggests microparticles may drive these responses through participating in intercellular communication by transferring regulatory miRNAs. Gidlof et al used RNA-seq to show that nine miRNAs were differentially expressed in PMPs from myocardial infarction patients; PMP transfer of these miRNAs modulated endothelial cell intercellular adhesion molecule-1 (ICAM-1) expression.[18] Since then, PMP delivered miRNAs have been shown to target genes in HUVEC including FBXW7[21] and insulin like growth factor-1 receptor[19], in addition to regulating tumour suppressor EPB41L3 in A549 lung cancer cells[22] and the ATPase Na<sup>+</sup>/K<sup>+</sup> transporting subunit beta 1 in macrophages.[20] The physiological and functional impact of PMP-miRNA regulation of gene expression requires systematic investigation. Advances in this field will allow better understanding of the role of delivered miRNAs in pathologies, enabling them to be validated as therapeutic targets. In this study we investigated the mechanisms by which PMPs stimulate the angiogenic switch, an important step in cardiovascular disease and metastatic progression. Our data show for the first time that PMP delivered Let-7a helps drives endothelial tubule formation *in vitro* through translational repression of anti-angiogenic THBS-1, results which now need to be confirmed *in vivo*.

Angiogenesis plays a fundamental role in cancer development and has been linked to atherosclerosis.[27] Angiogenesis may both weaken atherosclerotic plaques and enable leukocyte infiltration to growing lesions.[46] Vulnerable plaques are more likely to rupture, thereby promoting thrombosis, whilst leukocytes play a central role in driving inflammation. Experimental support for a causative relationship still remains to be clearly defined however, in part due to the complexity of lesion development and progression and in part due to a lack of reliable animal models. Previous studies have reported that PMPs stimulate angiogenesis[23, 24, 26], promoting endothelial survival, proliferation and tube formation.[24] However the underlying mechanisms are still under investigation. Neutralising antibodies against VEGF and PDGF reduce the stimulatory effect of PMP treatment, suggesting a crucial role for growth factors in neo-vessel formation.[23] Our data show that PMP treatment directly alters the angiogenic profile of HUVEC. Importantly, many of these PMP-elicited responses were blocked by stripping PMPs of their RNA content. These findings suggest PMP

induced intercellular communication through RNA transfer may play a strong supportive role in regulating angiogenesis underpinning metastatic and cardiovascular diseases. Consistently, PMP stimulation of the lung cancer cell line A549 has been shown to upregulate mRNA levels of proangiogenic VEGF, IL-8 and HGF through miRNA transfer.[26] Moreover, global reduction of endothelial miRNA by siRNA silencing of Dicer, a key enzyme in the maturation of miRNAs, potentially decreases angiogenesis, [47] further demonstrating the critical supportive role miRNAs play in regulating blood vessel growth.

Believing the reduction in THBS-1 to be a key driving behind *in vitro* angiogenesis, we used Targetscan to identify Let-7a as a potential regulator of THBS-1. The Let-7 family are amongst the most highly expressed miRNAs in platelets, [16] and we show Let-7a is present in PMPs, which then efficiently deliver this miRNA into HUVEC. Using luciferase reporter assays we show PMP-Let7a targeted the 3'UTR of THBS-1 mRNA, potentially inhibiting THBS-1 protein synthesis in HUVEC. Consistently, synthesised Let-7a, alongside miRNAs-18a, 29b, 194 and 221, has been shown to directly target THBS-1 in HeLa cells.[48] Once bound, miRNAs silence target gene expression through translational repression, inducing mRNA destabilisation, or a combination of these events.[37] Our data that Let-7a represses THBS-1 protein, but not mRNA levels, is consistent with translational inhibition. Transfection of HUVEC with a Let-7a antisense oligonucleotide blunted the ability of PMPs to downregulate THBS-1 production and subsequent tube formation, further evidencing the importance of the Let-7a-THBS-1 axis. Crucially, plasmid over-expression of Let-7a induced robust *in vitro* angiogenesis alone, demonstrating for the first time to our knowledge the role this miRNA plays in endothelial tube formation. We acknowledge that this current study was limited to *in vitro* investigations, and need now to be confirmed *in vivo*. The Let-7 miRNA family plays a principal role in differentiation during tissue development and are often dysregulated in human cancers and cardiovascular diseases.[49] Given our data that PMPs efficiently transfer large amounts of Let-7a into recipient cells, we believe they are well situated to profoundly influence the progression of these pathologies.

Blood vessel growth is regulated by a balance between localised production of angiogenesis inhibitors and activators. Levels of THBS-1 are a key determinant of this 'angiogenic switch'.[50] Our angiogenesis cytokine array, ELISA and Western Blotting data show that PMP treatment potentially reduces THBS-1 production from HUVEC, which correlated with enhanced tube formation in *in vitro*. We confirmed with antibody neutralisation that PMP-Let 7a reduction in THBS-1 is partly responsible for the induction of angiogenesis, although it is likely other miRNAs and cytokines delivered by PMPs also contribute. THBS-1 exerts antiangiogenic effects through several mechanisms including

suppressing VEGF bioavailability and activity, blunting pro-angiogenic nitric oxide signalling, inducing endothelial apoptosis and by inhibiting endothelial motility and proliferation.[50] Thus, the removal of the THBS-1 'angiogenic brake' by PMP treatment would allow endogenous positive regulators such as VEGF to drive neo-blood vessel formation. *In vivo* THBS-1 levels are tightly controlled; alterations in its levels and activity are intimately linked with CVD and cancer progression.[51, 52] THBS-1 deficiency accelerates atherosclerotic plaque maturation in ApoE<sup>-/-</sup> mice, commonly used to model atherogenesis. The plaques in these mice are characterised by a higher immune infiltrate and a larger necrotic core,[53] suggesting loss of THBS-1 destabilises lesions. Our data showing PMPs profoundly regulate THBS-1 levels and tubule formation *in vitro*, implicate delivered Let-7a as playing a major supportive role in angiogenesis supported pathologies, a hypothesis which warrants further *in vivo* examination.

In summary, our results demonstrate the first evidence to our knowledge of the role played by Let-7a in angiogenesis, and describe a new molecular mechanism by which PMPs induce tubule formation. The documented increase in PMPs within cancer and atherosclerotic disease, coupled with dysregulated angiogenesis, suggests a link between these observations. Our study therefore provides evidence for a potential miRNA based therapeutic target to treat angiogenesis driven cardiovascular and metastatic disease.

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### **Authorship**

Contributions: A.C. performed experiments and analysed data. WR and J.B. co-designed the study and experiments and wrote the manuscript together. A.M.G. designed experiments and the study and edited the manuscript.

The authors declare no conflicts of interest.

## References

1. Burnouf, T., et al., *Platelet microparticles: detection and assessment of their paradoxical functional roles in disease and regenerative medicine*. *Blood Rev*, 2014. **28**(4): p. 155-66.
2. Lynch, S.F. and C.A. Ludlam, *Plasma microparticles and vascular disorders*. *Br J Haematol*, 2007. **137**(1): p. 36-48.
3. Skeppholm, M., et al., *Platelet-derived microparticles during and after acute coronary syndrome*. *Thromb Haemost*, 2012. **107**(6): p. 1122-9.
4. Tan, K.T., et al., *Clinically apparent atherosclerotic disease in diabetes is associated with an increase in platelet microparticle levels*. *Diabet Med*, 2005. **22**(12): p. 1657-62.
5. Wang, Z.T., Z. Wang, and Y.W. Hu, *Possible roles of platelet-derived microparticles in atherosclerosis*. *Atherosclerosis*, 2016. **248**: p. 10-6.
6. Dashevsky, O., D. Varon, and A. Brill, *Platelet-derived microparticles promote invasiveness of prostate cancer cells via upregulation of MMP-2 production*. *Int J Cancer*, 2009. **124**(8): p. 1773-7.
7. Bartel, D.P., *MicroRNAs: target recognition and regulatory functions*. *Cell*, 2009. **136**(2): p. 215-33.
8. Ambros, V., *The functions of animal microRNAs*. *Nature*, 2004. **431**(7006): p. 350-5.
9. Selth, L.A., et al., *Circulating microRNAs predict biochemical recurrence in prostate cancer patients*. *Br J Cancer*, 2013. **109**(3): p. 641-50.
10. Shapira, I., et al., *Circulating biomarkers for detection of ovarian cancer and predicting cancer outcomes*. *Br J Cancer*, 2014. **110**(4): p. 976-83.
11. Madhavan, D., et al., *Circulating miRNAs with prognostic value in metastatic breast cancer and for early detection of metastasis*. *Carcinogenesis*, 2016. **37**(5): p. 461-70.
12. Liu, W., et al., *Circulating microRNAs correlated with the level of coronary artery calcification in symptomatic patients*. *Sci Rep*, 2015. **5**: p. 16099.
13. Karakas, M., et al., *Circulating microRNAs strongly predict cardiovascular death in patients with coronary artery disease-results from the large AtheroGene study*. *Eur Heart J*, 2016.
14. Fichtlscherer, S., et al., *Circulating microRNAs in patients with coronary artery disease*. *Circ Res*, 2010. **107**(5): p. 677-84.
15. Wang, G.K., et al., *Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans*. *Eur Heart J*, 2010. **31**(6): p. 659-66.
16. Ple, H., et al., *The repertoire and features of human platelet microRNAs*. *PLoS One*, 2012. **7**(12): p. e50746.
17. Landry, P., et al., *Existence of a microRNA pathway in anucleate platelets*. *Nat Struct Mol Biol*, 2009. **16**(9): p. 961-6.
18. Gidlof, O., et al., *Platelets activated during myocardial infarction release functional miRNA, which can be taken up by endothelial cells and regulate ICAM1 expression*. *Blood*, 2013. **121**(19): p. 3908-17, s1-26.
19. Pan, Y., et al., *Platelet-secreted microRNA-223 promotes endothelial cell apoptosis induced by advanced glycation end products via targeting the insulin-like growth factor 1 receptor*. *J Immunol*, 2014. **192**(1): p. 437-46.
20. Laffont, B., et al., *Platelet microparticles reprogram macrophage gene expression and function*. *Thromb Haemost*, 2016. **115**(2): p. 311-23.
21. Laffont, B., et al., *Activated platelets can deliver mRNA regulatory Ago2\*microRNA complexes to endothelial cells via microparticles*. *Blood*, 2013. **122**(2): p. 253-61.
22. Liang, H., et al., *MicroRNA-223 delivered by platelet-derived microvesicles promotes lung cancer cell invasion via targeting tumor suppressor EPB41L3*. *Mol Cancer*, 2015. **14**: p. 58.
23. Brill, A., et al., *Platelet-derived microparticles induce angiogenesis and stimulate post-ischemic revascularization*. *Cardiovasc Res*, 2005. **67**(1): p. 30-8.
24. Kim, H.K., et al., *Platelet microparticles induce angiogenesis in vitro*. *Br J Haematol*, 2004. **124**(3): p. 376-84.

25. Hayon, Y., et al., *Platelet microparticles induce angiogenesis and neurogenesis after cerebral ischemia*. *Curr Neurovasc Res*, 2012. **9**(3): p. 185-92.
26. Janowska-Wieczorek, A., et al., *Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer*. *Int J Cancer*, 2005. **113**(5): p. 752-60.
27. Khurana, R., et al., *Role of angiogenesis in cardiovascular disease: a critical appraisal*. *Circulation*, 2005. **112**(12): p. 1813-24.
28. Nishida, N., et al., *Angiogenesis in cancer*. *Vasc Health Risk Manag*, 2006. **2**(3): p. 213-9.
29. Das, S. and M.K. Halushka, *Extracellular vesicle microRNA transfer in cardiovascular disease*. *Cardiovasc Pathol*, 2015. **24**(4): p. 199-206.
30. Varon, D., et al., *Involvement of platelet derived microparticles in tumor metastasis and tissue regeneration*. *Thromb Res*, 2012. **130 Suppl 1**: p. S98-9.
31. Rousseau, M., et al., *Detection and quantification of microparticles from different cellular lineages using flow cytometry. Evaluation of the impact of secreted phospholipase A2 on microparticle assessment*. *PLoS One*, 2015. **10**(1): p. e0116812.
32. Sultan, S.A., et al., *The Role of Maternal Gestational Diabetes in Inducing Fetal Endothelial Dysfunction*. *J Cell Physiol*, 2015. **230**(11): p. 2695-705.
33. Yoon, S.O., et al., *Deregulated expression of microRNA-221 with the potential for prognostic biomarkers in surgically resected hepatocellular carcinoma*. *Human pathology*, 2011. **42**(10): p. 1391-1400.
34. Risitano, A., et al., *Platelets and platelet-like particles mediate intercellular RNA transfer*. *Blood*, 2012. **119**(26): p. 6288-95.
35. Deregibus, M.C., et al., *Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA*. *Blood*, 2007. **110**(7): p. 2440-8.
36. Deng, Y., et al., *Prostacyclin-producing human mesenchymal cells target H19 lncRNA to augment endogenous progenitor function in hindlimb ischaemia*. *Nat Commun*, 2016. **7**: p. 11276.
37. Jin, H.Y. and C. Xiao, *MicroRNA Mechanisms of Action: What have We Learned from Mice?* *Front Genet*, 2015. **6**: p. 328.
38. Kirschbaum, M., et al., *Horizontal RNA transfer mediates platelet-induced hepatocyte proliferation*. *Blood*, 2015. **126**(6): p. 798-806.
39. Breitkopf, K., et al., *Thrombospondin 1 acts as a strong promoter of transforming growth factor beta effects via two distinct mechanisms in hepatic stellate cells*. *Gut*, 2005. **54**(5): p. 673-81.
40. Short, S.M., et al., *Inhibition of endothelial cell migration by thrombospondin-1 type-1 repeats is mediated by beta1 integrins*. *J Cell Biol*, 2005. **168**(4): p. 643-53.
41. Kaur, S., et al., *Thrombospondin-1 inhibits VEGF receptor-2 signaling by disrupting its association with CD47*. *J Biol Chem*, 2010. **285**(50): p. 38923-32.
42. De Luna, N., et al., *Role of thrombospondin 1 in macrophage inflammation in dysferlin myopathy*. *J Neuropathol Exp Neurol*, 2010. **69**(6): p. 643-53.
43. Antwi-Baffour, S., et al., *Human plasma membrane-derived vesicles inhibit the phagocytosis of apoptotic cells--possible role in SLE*. *Biochem Biophys Res Commun*, 2010. **398**(2): p. 278-83.
44. Franca, C.N., et al., *Microparticles as potential biomarkers of cardiovascular disease*. *Arq Bras Cardiol*, 2015. **104**(2): p. 169-74.
45. Varon, D. and E. Shai, *Platelets and their microparticles as key players in pathophysiological responses*. *J Thromb Haemost*, 2015. **13 Suppl 1**: p. S40-6.
46. Silvestre-Roig, C., et al., *Atherosclerotic plaque destabilization: mechanisms, models, and therapeutic strategies*. *Circ Res*, 2014. **114**(1): p. 214-26.
47. Suarez, Y., et al., *Dicer dependent microRNAs regulate gene expression and functions in human endothelial cells*. *Circ Res*, 2007. **100**(8): p. 1164-73.



48. Dogar, A.M., et al., *Multiple microRNAs derived from chemically synthesized precursors regulate thrombospondin 1 expression*. *Nucleic Acid Ther*, 2014. **24**(2): p. 149-59.
49. Lee, H., et al., *Biogenesis and regulation of the let-7 miRNAs and their functional implications*. *Protein Cell*, 2016. **7**(2): p. 100-13.
50. Lawler, P.R. and J. Lawler, *Molecular basis for the regulation of angiogenesis by thrombospondin-1 and -2*. *Cold Spring Harb Perspect Med*, 2012. **2**(5): p. a006627.
51. Choi, K.Y., et al., *Higher plasma thrombospondin-1 levels in patients with coronary artery disease and diabetes mellitus*. *Korean Circ J*, 2012. **42**(2): p. 100-6.
52. Jeanne, A., et al., *Original insights on thrombospondin-1-related antireceptor strategies in cancer*. *Front Pharmacol*, 2015. **6**: p. 252.
53. Moura, R., et al., *Thrombospondin-1 deficiency accelerates atherosclerotic plaque maturation in ApoE<sup>-/-</sup> mice*. *Circ Res*, 2008. **103**(10): p. 1181-9.

**Figure 1: PMPs induce HUVEC *in vitro* tubule formation:** (A) Representative images showing extracellular matrix gel-based, capillary-like tubule formation of: HUVEC alone (A-I), treated with 100µg/ml PMPs (A-II), or RNase then RNase inhibitor-treated PMPs (A-III), RNase inhibitor treated PMP (A-IV), RNase inhibitor only (A-V) or RNase only (A-VI) for 24-hours. Data shown are mean ± standard error of mean for (B) mean total number of branch points and (C) mean tube length (µm). (D) Total RNA was isolated from PMPs treated with RNase then RNase inhibitor or buffer control only, using TRIzol reagent. RNA concentration was quantified by spectrophotometry. Data shown are mean concentration (µg/ml) ± standard error of mean.  $n=3$ . Statistical significance was analysed using ANOVA (figures 1b-c) or students t test (figure 1d), \* $P<0.05$  \*\* $P<0.001$  between groups.

**Figure 2: PMP-induced cytokine release from HUVEC is partially dependent on RNA transfer.** (A) HUVEC were serum depleted for 24 hours followed by treatment with either 100µg/ml PMP or RNase then RNase treated PMP. Conditioned media was collected and analysed for angiogenesis related protein release with a proteome profiler kit. (A) Protein array images of supernatant from HUVEC treated with: (A-I) 1% serum (A-II) 100µg/ml PMPs and (A-III) 100µg/ml PMPs treated with RNase. THBS-1 is highlighted on the blots. (B): Quantitative data of pixel density. Data shown are mean ± standard error of  $n=3$  using 6 pooled samples of PMP for each experiment. Statistical significance was analysed using ANOVA, \* $P<0.05$  between groups.

**Figure 3: Direct regulation of THBS-1 by microRNA Let-7a.** (A) Targetscan analysis prediction of THBS-1 3'UTR targeting by Let-7a. (B) Relative Let-7a expression in PMPs treated with RNase then RNase inhibitor or buffer control only. In some experiments RNA was spiked back into samples after RNA isolation to check the RNase activity had been neutralised. Data shown are mean ± standard error of mean,  $n=3$ . (C) HEK293T cells transfected with THBS-1 3'UTR were transfected with Let-7a pmir plasmid or empty pmir plasmid and Let-7a miRNA inhibitor or non-targeting miRNA inhibitor. Luciferase signal for the 3'UTR was measured by Lightswitch luciferase reagent. Data percent luciferase activity, with cells transfected with THBS-1 3'UTR alone normalised to 100%  $n = 6$ . Statistical significance was analysed using ANOVA, \* $P<0.05$  \*\* $P<0.001$  between groups.

**Figure 4: PMPs deliver Let-7a microRNA to HUVEC *in vitro*** (A) Relative miRNA expression of Let-7a in HUVEC cultured in 1% serum only, or in 1% serum supplemented with 100µg/ml PMP or PMPs (pre-treated with RNase then RNase inhibitor) or HUVEC pre-treated with 0.25ng/ul of actinomycin D or control for 24 hours, followed by treatment with 100µg/ml PMPs or control for 24 hours in 1% serum. Data shown are mean ± standard error of mean,  $n=9$ . Statistical significance was analysed using ANOVA, \* $P<0.05$ , \*\* $P<0.001$  between groups (B) DiOC6 labelled PMP (100µg/ml PMP) were incubated

with HUVEC for 6 hours B-I, 12 hours B-II or 18 hours B-III before DAPI staining. Shown are representative images,  $n=3$ .

**Figure 5: PMP-mediated inhibition of THBS-1 expression in HUVEC requires Let-7a.** (A) Relative quantification of THBS-1 mRNA levels in HUVEC treated with 100 $\mu$ g/ml PMP. Data shown are mean  $\pm$  standard error of mean,  $n=3$  independent biological samples. (B) Relative expression of THBS-1 mRNA levels in PMP treated with RNase or control buffer. In some experiments RNA was spiked back into samples after RNA isolation to check the RNase activity had been neutralised. (C) ELISA measurement of THBS-1 protein concentration in HUVEC conditioned media after transfection with either Let-7a ASO or a scrambled oligonucleotide, followed by 24 hours treatment with 100 $\mu$ g/ml PMP or control buffer. Data shown are mean  $\pm$  standard error of mean,  $n=3$  independent samples. Statistical significance was analysed using ANOVA, \* $P<0.05$ , \*\* $P<0.001$ . between groups. (D) Representative images ( $n=3$ ) of immunoblots with an anti-THBS-1 antibody and re-probed with an anti-Beta tubulin antibody of HUVEC after transfection with either Let-7a ASO or a scrambled oligonucleotide, followed by 24 hours treatment with 100 $\mu$ g/ml PMP or control buffer for 24 hours.

**Figure 6- delivered Let-7a is sufficient to promote tubule formation in HUVEC** (A) Representative images ( $\times 40$  magnification) showing extracellular matrix gel-based, capillary-like tube formation of: HUVEC alone (A-I) or after treatment with 100 $\mu$ g/ml PMPs for 24-hours (A-II) or transfected with either Let-7a ASO (A-III) or scrambled oligonucleotide (A-IV) for 24 hours, followed by stimulation with PMPs. Data shown are mean  $\pm$  standard error of mean for (B) mean tube length ( $\mu$ m) and (C) mean total number of branch points. (D) Representative images ( $\times 40$  magnification) showing extracellular matrix gel-based, capillary-like tube formation of HUVEC (D-I) transfected with Let-7a (D-II) or empty expression plasmid (D-III) for 24 hours. Data shown are mean  $\pm$  standard error of mean for (E) mean tube length ( $\mu$ m) and (F) mean total number of branch points.  $n=3$  independent biological repeats. Statistical significance was analysed using ANOVA, \* $P<0.05$ , \*\* $P<0.001$ . between groups.

**Figure 7: Targeting of THBS-1 by PMP-delivered Let-7a is required for PMP-induced tubule formation.** (A) Representative images showing extracellular matrix gel-based, capillary-like tube formation of: HUVEC alone (A-I) HUVEC treated with 100 $\mu$ g/ml PMPs for 24-hours (A-II) HUVEC transfected with Let-7a ASO followed by PMP treatment (A-III) HUVEC transfected with Let-7a ASO then treated with THBS-1 neutralising antibody, followed by PMP (A-IV) HUVEC transfected with Let-7a ASO then treated with control antibody, followed by PMP (A-V). Data shown are mean  $\pm$  standard error of mean for (B) mean tube length ( $\mu$ m) and (C) mean total number of branch points. Data shown are mean  $\pm$  standard error of mean,  $n=3$  independent biological samples of PMPs. Statistical significance was analysed using ANOVA, \* $P<0.05$ , \*\* $P<0.001$ . between groups.

Figure 1

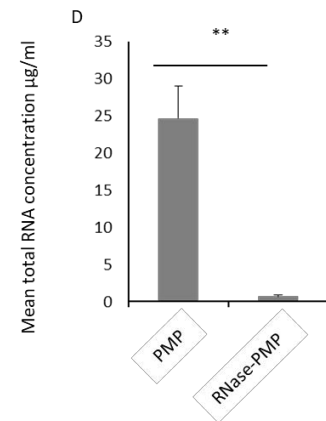
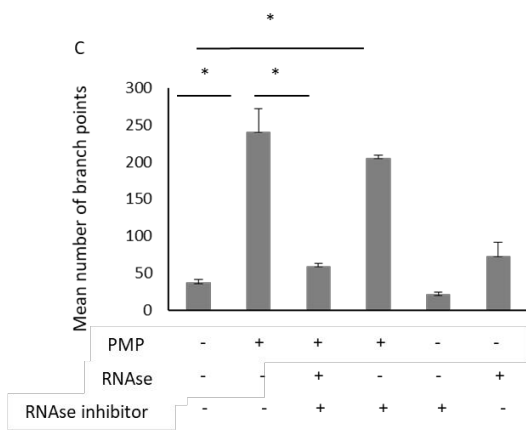
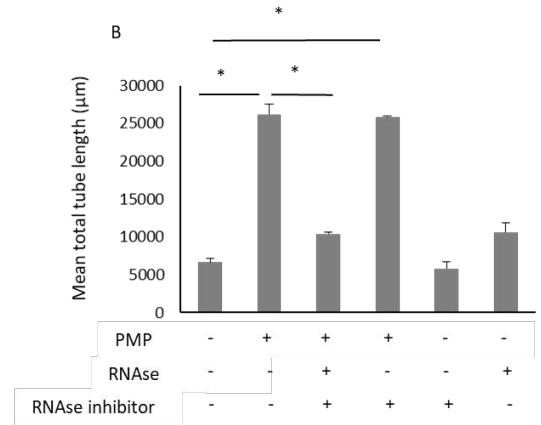
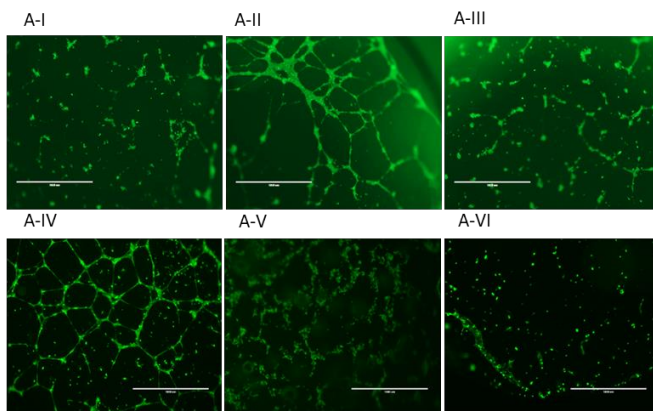


Figure 2

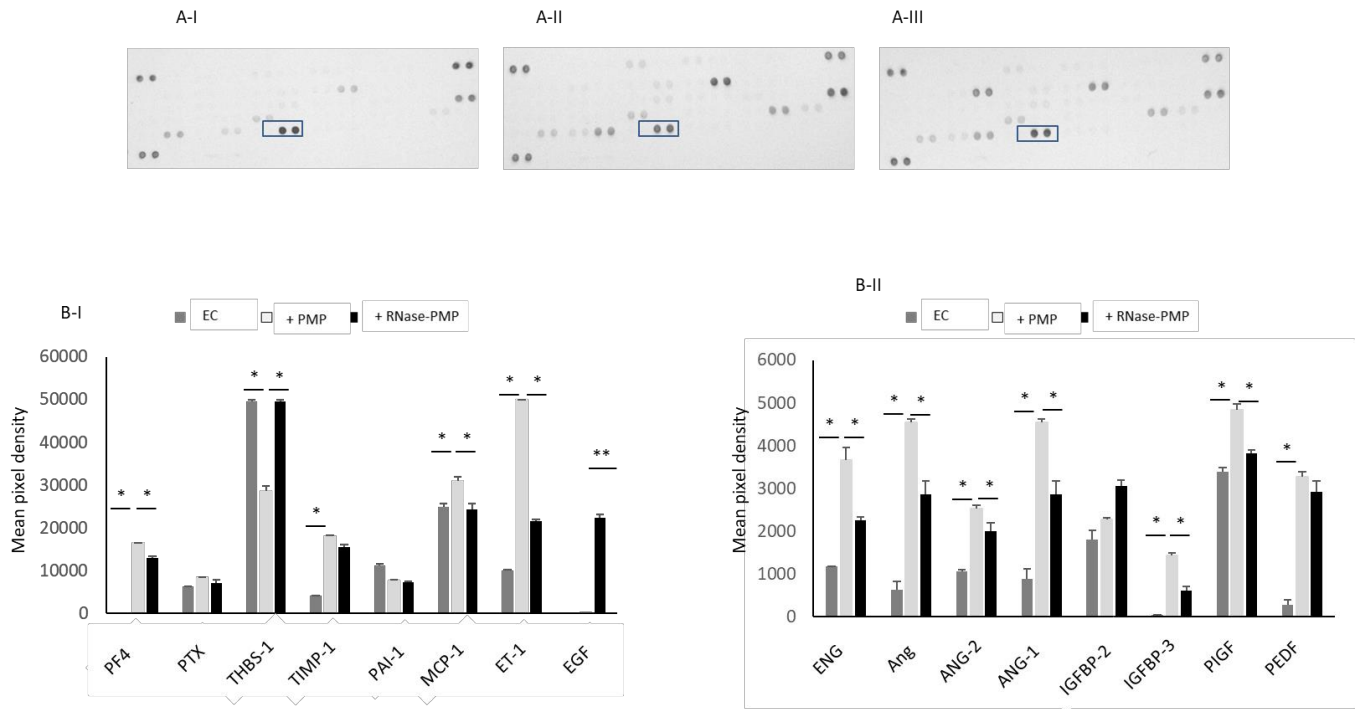


Figure 3

A

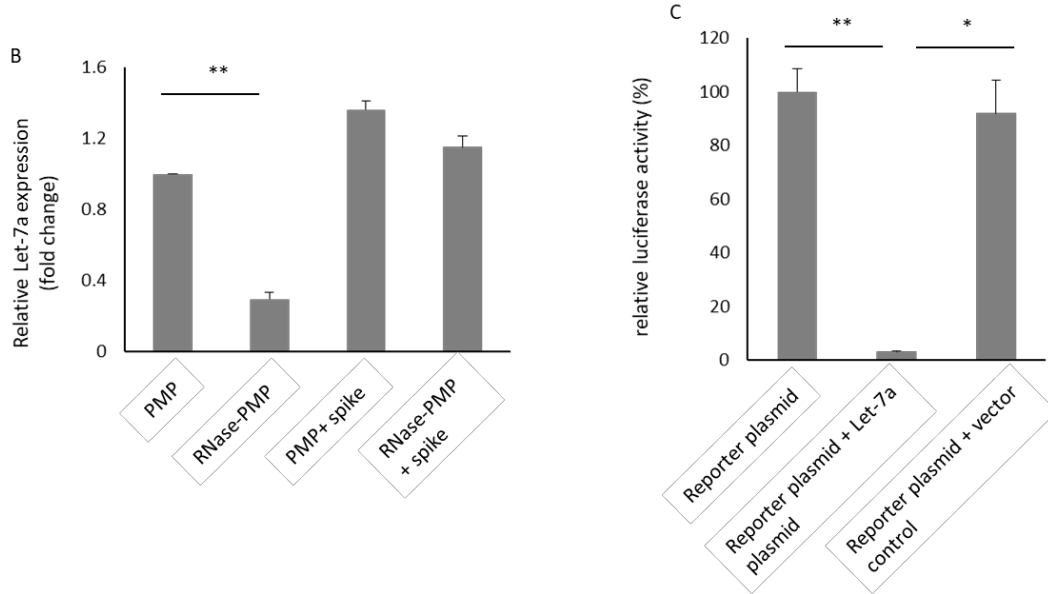
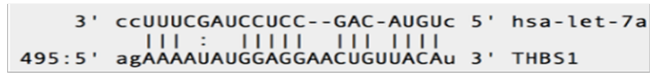


Figure 4

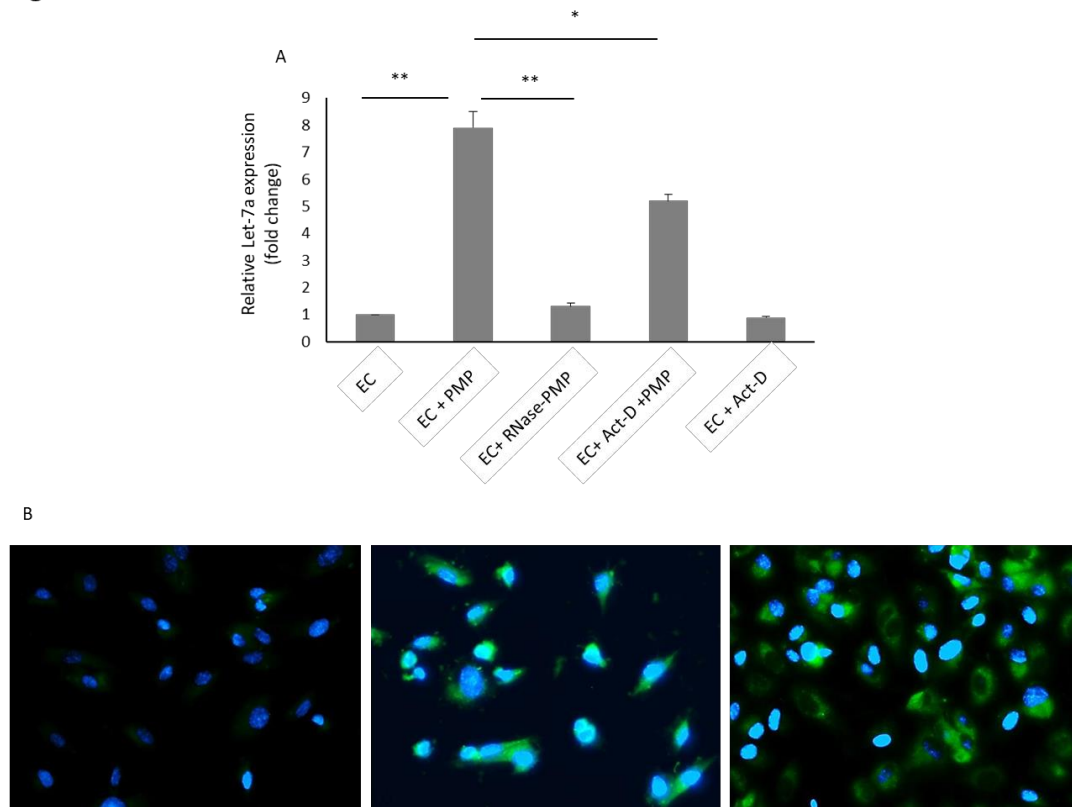


Figure 5

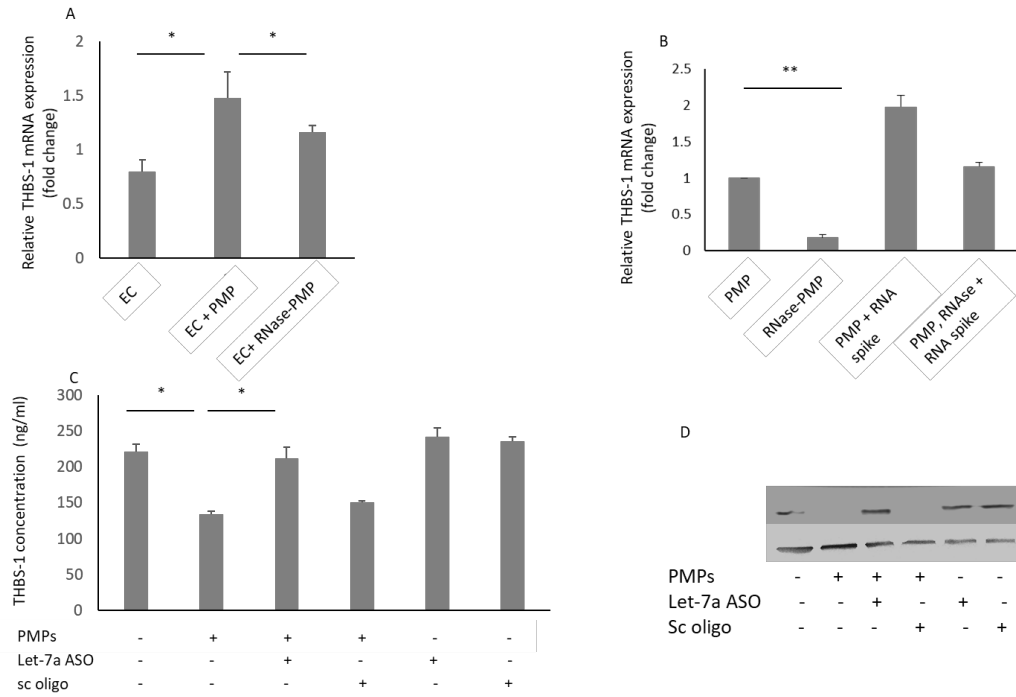




Figure 6

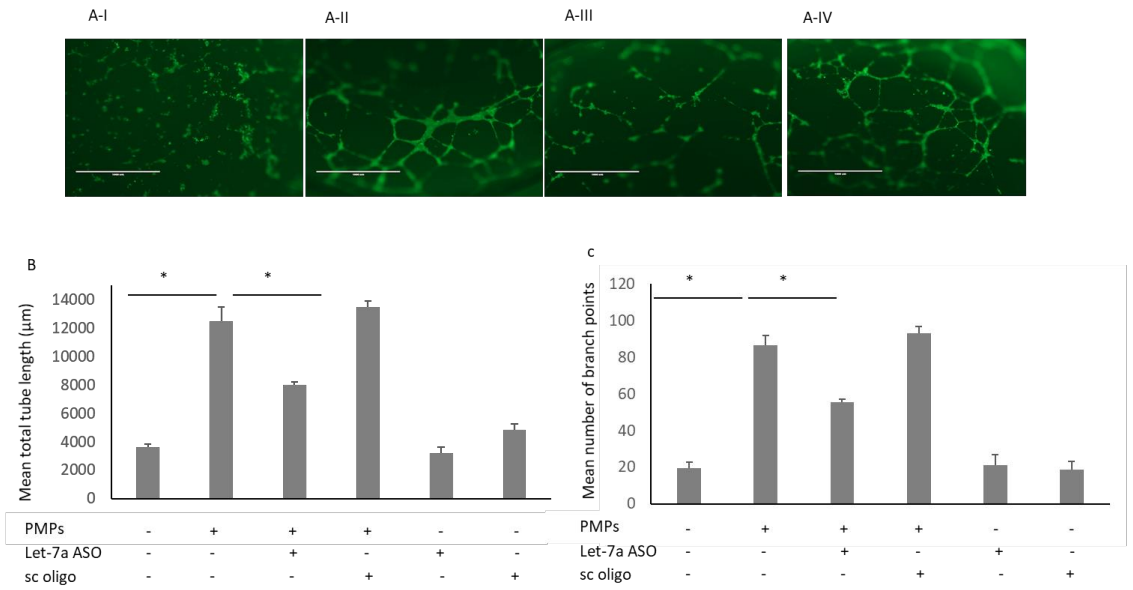


Figure 6

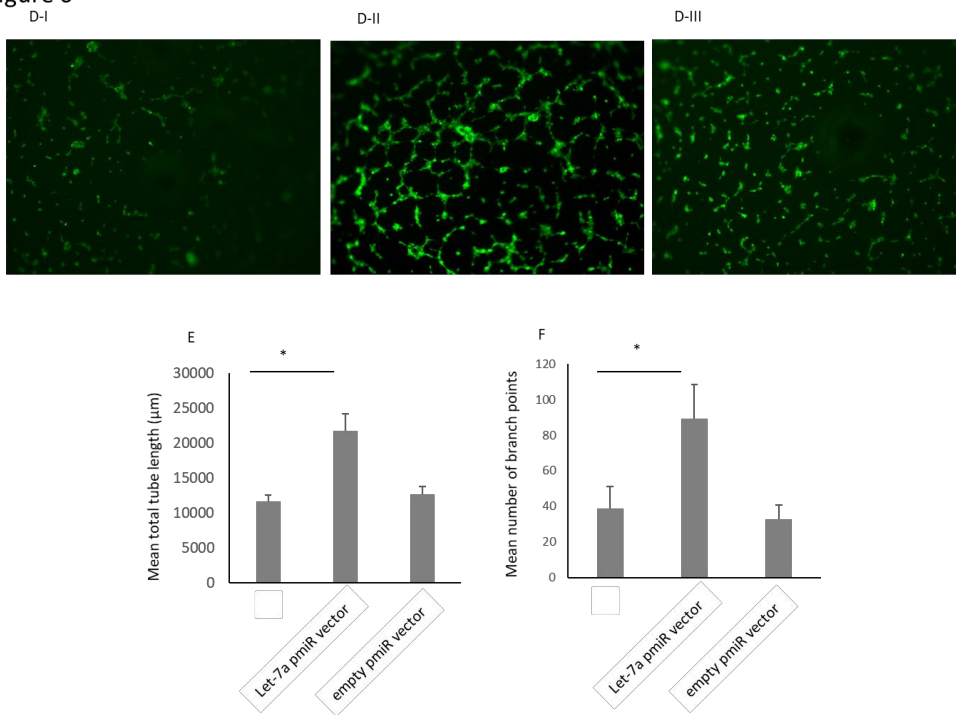


Figure 7

