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Horizontal RNA transfer goes deep: platelet consumption and microRNA utilization by vascular smooth muscle cells.

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Short Title: Neighborhood Watch Article on Zeng et al.

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Introduction

MicroRNAs (miRNAs), short non-coding RNAs that repress translation of cognate sequence-matched mRNAs through multiple mechanisms, are established as both important regulators and key biomarkers of cardiovascular function. miRNAs have been shown to modulate gene expression resulting in altered development and/or functional responses in every type of vascular cell. miRNAs also circulate in plasma, either complexed to lipoproteins or contained within extracellular vesicles. Circulation of miRNA enables its functionality both as biomarkers for altered gene expression to indicate and describe pathological conditions, and as mediators of horizontal gene transfer between vascular cells in autocrine or paracrine fashion. miRNAs are well-suited to these roles due to their relatively long half-life (~5 days) that can support long-term gene regulation in distal cells. Horizontal transfer of intercellular RNA extends the reach of miRNAs beyond their cell of origin and adds new dimensions to gene regulation *in vivo*. Horizontal miRNA transfer may be of particular relevance in post-mitotic and senescent vascular cells, in which modulation of gene expression by miRNAs can affect cellular activation in acute or chronic settings. In particular, platelets are highly enriched in miRNAs, and platelets are emerging as major sources and transfer hubs mediating miRNA exchange in blood. Platelet-derived microvesicles (PMVs) constitute a major fraction of plasma MVs, and these structures have been shown to deliver platelet miRNAs to other vascular and extravascular cells in multiple contexts. Moreover, phagocytosis of intact platelets has been demonstrated in several vascular cell types including endothelial cells, neutrophils and monocytes, providing yet another opportunity for miRNA exchange by engulfment of whole platelets. The mechanisms, cellular and molecular targets, and functional outcomes of horizontal transfer of platelet miRNAs are an active area of investigation. Most recently, Zeng *et al.* reported a new cellular target of platelet miRNAs: vascular smooth muscle cells (VSMCs) *in vitro* following co-incubation with platelets, and *in vivo* following wire injury to the femoral artery in mice, with effects on VSMC injury response [1]. Even more

surprising is the apparent engulfment of intact platelets by vascular smooth muscle cells, representing not only exchange of genetic material, but exchange of complex cellular content between these vascular cells.. In this Neighborhood Watch, we discuss the background, implications, unanswered questions, and future perspectives of this study.

Platelet RNA transfer

The recent report by Zeng *et al.* [1], builds upon an exciting area of research in the cardiovascular field: the ability of platelets to modulate other cells in contact with the blood by transferring (mi)RNAs, which then modulate the physiology of the recipient cells. This ability to transfer RNAs was first reported in 2012, when the delivery of mRNAs in platelet-like particles from the Meg01 cell line to human umbilical vein endothelial cells (HUVECs) and the THP-1 monocytic cell line was described. In addition, after transfusion of wildtype platelets into *TLR2*-deficient mice, *TLR2* mRNA was detected in the peripheral blood mononuclear cells (PBMCs), suggesting *in vivo* mRNA transfer [2]. The following year, Laffont *et al.*, reported that PMVs contain functional Ago2:miR-223 complexes which could be transferred to HUVECs, leading to down-regulation of the levels of *FBXW7* and *EFNA1* mRNAs [3]. In the subsequent years, PMV delivery of miRNAs to many cell types in culture was reported, including hepatocellular carcinoma cells, macrophages, lung epithelial cells, natural killer cells, and VSMCs [4]. Utilizing metabolically labeled megakaryocyte/platelet RNAs, we recently demonstrated transfer of platelet RNA to heterologous cells *in vivo.* We observed the delivery of multiple platelet-derived miRNAs including miR-24 and miR-223 to lung carcinoma cells in ectopic solid tumors *in vivo*, leading to miR-24-dependent tumor growth suppression. This effect was abrogated in the platelet thrombin receptor PAR4-null mouse, which harbor 50% fewer plasma-borne PMVs compared to wildtype, and was restored by PMV transfusion, demonstrating the dependence on PMVs for this process [5]. Zeng *et al.*, builds upon this background by demonstrating *in vivo* transfer of miR-223 to VSMCs

following endothelial denudation. They further report the engulfment of whole, activated platelets by VSMCs *in vitro*, and *in vivo* at the site of mechanical injury to the femoral artery in mice, and they relate these phenomena to subsequent inhibition of neointimal hyperplasia.

miR-223 and cardiovascular disease

miRNA-223 is the most abundant microRNA present in platelets, and thus there has been much interest in studying its roles in cardiovascular function and disease. In one of the first studies describing the miRNA content of platelets, Landry *et al.*, identified miR-223-containing Ago2 complexes in human platelets and demonstrated that miR-223 could regulate the expression of *P2RY12*, the mRNA that encodes the platelet specific adenosine diphosphate (ADP) receptor, $P2Y_{12}$ [6]. While deletion of *miR-223* had no effect on *in vitro* assays of platelet function, Wang *et al.*, demonstrated that miR-223-deficient mice had prolonged occlusion times in a model of photochemical-induced carotid thrombosis[7, 8], confirming a major role for platelet miRNAs, in particular miR-223, in platelet reactivity *in vivo*. Interestingly, WT platelets or PMVs transfused into miR-$223^{-/y}$ mice were able to restore occlusion times and lowered arterial expression of IGF-1R - a miR-223 target - at the site of injury, suggesting delivery of miR-223 to cells of the vessel wall [8].

Because of its abundance, miR-223 has also been utilized as a biomarker for platelet function, cardiovascular disease, and diabetes (Reviewed in [9]). Due to its ability to regulate the level of $P2Y_{12}$, there have been several studies reporting that low levels of miR-223 are associated with enhanced platelet function in patients using the anti-P2Y12 drug clopidigrel[10]. Lower circulating miR-223 levels have also been associated with diabetes [11] and with increased myocardial infarction risk [12]. The inverse relationship between miR-223 levels and platelet reactivity and its contributions to cardiovascular disease risk is consistent with the finding of Zeng

et al., in which miR-223-deficient mice exhibit increased intimal hyperplasia in response to vascular injury, correlating with increased thrombotic risk.

Unanswered questions and challenges

One of the most significant challenges in understanding the effects of horizontal miRNA transfer is isolating the causes: are effects due to the exchanged miRNA, or to exposure of the target cells to other properties of the delivery vehicle? Add to this the question of whether delivery vehicles themselves are a singular species, and the challenge becomes even greater. In the study from Zeng *et al.*, miRNAs appear to be delivered by whole, internalized and activated platelets, although close inspection of the images suggests that a large portion of the internalized material in VSMCs consists of PMVs, platelet exosomes, or platelet fragments. Enrichment of PMVs following platelet activation *in vitro*, and near a damaged vessel where platelets are becoming activated *in vivo*, would be expected, and PMV uptake by heterologous cells and subsequent miRNA transfer from PMVs has been demonstrated in many contexts. Moreover, at least some of the internalized platelets in VSMCs appear granulated, suggesting that VSMCs may take up platelets at a range of activation states. A related question is the source of whole platelets for uptake by exposed VSMCs at sites of vascular damage: are the VSMCs internalizing activated platelets directly from the induced thrombus at the injury site or from circulation? The persistence of platelet-expressed GFP in the intimal zone seven days after injury could indicate deposition of new platelet material during or after thrombus clearance. Ongoing platelet or PMV uptake may also provide fresh sources of miRNAs, and this in turn could account for long-term effects on neointimal hyperplasia. Altogether, the relative contributions of PMVs and platelets to miRNA transfer to VSMCs in the context of vascular injury, the nature of the platelets involved, and potentially distinct or compound effects over time, await further study.

There are alternative explanations for the observations reported by Zeng. et al. Activated platelets are well known to contribute to neointimal hyperplasia, and in particular $P2Y_{12}$ has been

strongly implicated: hyperplasia in mice is substantially suppressed both by $P2Y_{12}$ deletion and by treatment with ticagrelor, a $P2Y_{12}$ inhibitor, suggesting a critical role for platelet activation [13, 14]. Whether exposed VSMCs are reacting to releasates from activated platelets in plasma, as well as reacting to phagocytosed platelets apart from their miRNA cargo, remains to be determined.

The authors hone in on miR-223, a miRNA highly enriched in platelets but also expressed in other cells, and shown previously to be internalized from the circulation by VSMCs, leading to growth inhibition [15]. Plasma miR-223 in healthy human donors was also shown to be predominantly contained in extracellular vesicle (EV)-free ribonucleoprotein complexes, and only partially contained in MVs or exosomes [16]. Thus, effects of miR-223 depletion, including global knockout or a background of induced diabetes mellitus, may reflect direct loss of blood-derived miR-223 in VSMCs independent from platelets as a source. As $P2Y_{12}$ is a miR-223 target, miR-223 may inhibit neointimal hyperplasia through multiple pathways including direct modulation of platelet activation by $P2Y_{12}$ knockdown in megakaryocytes and platelets, as well as effects on VSMCs.

The mechanisms of platelet uptake, miRNA transfer and utilization by the VSMCs are unknown. How VSMCs, beneath a thick intima in large arteries, become exposed directly to large plasma-borne cellular material following endothelial denudation is not clear, and to our knowledge phagocytic properties of VSMCs have not previously been described. Another question is how the encapsulated miRNAs, apparently entrapped in the endosome/lysosome system in VSMCs, are exposed to target cell mRNAs in the cytosol to regulate translation. Further studies with antibody-based labeling or live imaging of the platelet-encompassing compartments will go a long way towards mapping the trafficking patterns and the ultimate fates of internalized platelets or PMVs and may suggest modes by which released miRNAs can escape lysosomal RNase digestion.

Future perspectives

The relevance of RNA to short-lived anucleate platelet biology was and continues to be an open question. Platelet RNAs can serve as biomarkers of disease, as indicators of megakaryocyte gene expression, or as clues to identifying regulators of platelet function. Evidence of platelet protein translation under resting or stimulated conditions suggests that platelet RNAs could serve similar roles as they do in nucleated cells. However, (mi)RNA delivery from platelets to heterologous cells suggests that platelet RNA content can also have a profound impact on the biology of long-lived, nucleated cells undergoing continuous protein synthesis for cellular maintenance and in response to the environment. This, combined with the observation that platelets could take up circulating RNAs from the environment, make the platelet RNA profile both an indicator and effector of cardiovascular health.

More work along the lines of Michael *et al.* and Zeng *et al.*, needs to be performed to demonstrate and explain the *in vivo* relevance of platelet RNA transfer, particularly of long-lived miRNAs. In the circulation, blood and vascular cells are continuously exposed to circulating MVs, and *in vitro* experiments in cultured cells in static media may react to several stimuli when MV preparations are added, including bioactive lipids, surface proteins, or other non-vesicle plasma components. The array of vascular cells exposed to platelet RNAs, and the physiological and pathophysiological contexts in which exposure occurs, have not been fully mapped. Cellular mechanisms and molecular drivers of platelet, PMV, or extracellular vesicle-free ribonuclear protein complex internalization are still poorly understood. Conserved mechanisms have yet to emerge and conversely putative cell type-specific mechanisms may provide a further point of regulation. Moreover, the temporal dynamics of platelet miRNA transfer *in vivo* demand further exploration, as this speaks directly to the levels of platelet-derived miRNAs available to locate and modulate mRNA targets over time. Finally, the identities and quantities of transferred miRNAs, and those of their cognate mRNA targets in recipient cells, need to be determined in

each case. These issues lead straight to the central question of the physiological consequences of horizontal miRNA transfer: how does a cohort of transferred miRNAs affect the translational landscape of recipient cells to modulate function. Emergent technologies, in particular next generation RNA sequencing and better *in vivo* imaging, will continue to bring us closer to understanding the integrative physiology of horizontal platelet miRNA transfer in cardiovascular health and disease.

Addendum

L. C. Edelstein and L. E. Goldfinger wrote and edited the manuscript. Both authors approved the final version.

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Disclosure of Conflicts of Interest

The authors state that they have no conflicts of interest.

References

1 Zeng Z, Xia L, Fan X, Ostriker AC, Yarovinsky T, Su M, Zhang Y, Peng X, Yi X, Pi L, Gu X, Chung SK, Martin KA, Liu R, Hwa J, Tang WH. Platelet-derived miR-223 promotes a phenotypic switch in arterial injury repair. *J Clin Invest*. 2019. 10.1172/JCI124508.

2 Risitano A, Beaulieu LM, Vitseva O, Freedman JE. Platelets and platelet-like particles mediate intercellular RNA transfer. *Blood*. 2012; **119**: 6288-95. 10.1182/blood-2011-12-396440.

3 Laffont B, Corduan A, Ple H, Duchez AC, Cloutier N, Boilard E, Provost P. Activated platelets can deliver mRNA regulatory Ago2*microRNA complexes to endothelial cells via microparticles. *Blood*. 2013; **122**: 253-61. 10.1182/blood-2013-03-492801.
4 Xia L, Zeng Z, Tang WH. The Role of Platelet Microparticle Associated microRNAs in Cellular Crosstalk. *Front Cardiovasc Med*. 2018; **5**: 29. 10.3389/fcvm.2018.00029.
5 Michael JV, Wurtzel JGT, Mao GF, Rao AK, Kolpakov MA, Sabri A, Hoffman NE, Rajan S, Tomar D, Madesh M, Nieman MT, Yu J, Edelstein LC, Rowley JW, Weyrich AS, Goldfinger LE. Platelet microparticles infiltrating solid tumors transfer miRNAs that suppress tumor growth. *Blood*. 2017; **130**: 567-80. 10.1182/blood-2016-11-751099.
6 Landry P, Plante I, Ouellet DL, Perron MP, Rousseau G, Provost P. Existence of a microRNA pathway in anucleate platelets. *Nat Struct Mol Biol*. 2009; **16**: 961-6. 10.1038/nsmb.1651.
7 Leierseder S, Petzold T, Zhang L, Loyer X, Massberg S, Engelhardt S. MiR-223 is dispensable for platelet production and function in mice. *Thromb Haemost*. 2013; **110**: 1207-14. 10.1160/TH13-07-0623.
8 Wang H, Wang Q, Kleiman K, Guo C, Eitzman DT. Hematopoietic Deficiency of miR-223 Attenuates Thrombosis in Response to Photochemical Injury in Mice. *Scientific reports*. 2017; **7**: 1606. 10.1038/s41598-017-01887-x.
9 Pordzik J, Pisarz K, De Rosa S, Jones AD, Eyileten C, Indolfi C, Malek L, Postula M. The Potential Role of Platelet-Related microRNAs in the Development of Cardiovascular Events in High-Risk Populations, Including Diabetic Patients: A Review. *Front Endocrinol (Lausanne)*. 2018; **9**: 74. 10.3389/fendo.2018.00074.
10 Shi R, Ge L, Zhou X, Ji WJ, Lu RY, Zhang YY, Zeng S, Liu X, Zhao JH, Zhang WC, Jiang TM, Li YM. Decreased platelet miR-223 expression is associated with high on-clopidogrel platelet reactivity. *Thromb Res*. 2013; **131**: 508-13. 10.1016/j.thromres.2013.02.015.
11 Zampetaki A, Kiechl S, Drozdov I, Willeit P, Mayr U, Prokopi M, Mayr A, Weger S, Oberhollenzer F, Bonora E, Shah A, Willeit J, Mayr M. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. *Circ Res*. 2010; **107**: 810-7. 10.1161/CIRCRESAHA.110.226357.
12 Zampetaki A, Willeit P, Tilling L, Drozdov I, Prokopi M, Renard JM, Mayr A, Weger S, Schett G, Shah A, Boulanger CM, Willeit J, Chowienczyk PJ, Kiechl S, Mayr M. Prospective study on circulating MicroRNAs and risk of myocardial infarction. *J Am Coll Cardiol*. 2012; **60**: 290-9. 10.1016/j.jacc.2012.03.056.
13 Surer S, Toktas F, Ay D, Eris C, Yavuz S, Turk T, Vural AH, Goncu MT, Gul NY, Yalcinkaya U. Effect of the P2Y12 antagonist ticagrelor on neointimal hyperplasia in a rabbit carotid anastomosis modeldagger. *Interact Cardiovasc Thorac Surg*. 2014; **19**: 198-204. 10.1093/icvts/ivu087.
14 Evans DJ, Jackman LE, Chamberlain J, Crosdale DJ, Judge HM, Jetha K, Norman KE, Francis SE, Storey RF. Platelet P2Y(12) receptor influences the vessel wall response to arterial injury and thrombosis. *Circulation*. 2009; **119**: 116-22. 10.1161/CIRCULATIONAHA.107.762690.
15 Shan Z, Qin S, Li W, Wu W, Yang J, Chu M, Li X, Huo Y, Schaer GL, Wang S, Zhang C. An Endocrine Genetic Signal Between Blood Cells and Vascular Smooth Muscle Cells: Role of MicroRNA-223 in Smooth Muscle Function and Atherogenesis. *J Am Coll Cardiol*. 2015; **65**: 2526-37. 10.1016/j.jacc.2015.03.570.
16 Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, Mitchell PS, Bennett CF, Pogosova-Agadjanyan EL, Stirewalt DL, Tait JF, Tewari M. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc Natl Acad Sci U S A*. 2011; **108**: 5003-8. 10.1073/pnas.1019055108.