miR-19a-3p containing exosomes improve function of ischemic myocardium upon shock wave therapy

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Abstract Aims: As many current approaches for heart regeneration exert unfavorable side-effects, the induction of endogenous repair mechanisms in ischemic heart disease is of particular interest. Recently, exosomes carrying angiogenic miRNAs have been described to improve heart function. However, it remains challenging to stimulate specific release of reparative exosomes in ischemic myocardium. In the present study, we sought to test the hypothesis that the physical stimulus of shock wave therapy (SWT) causes the release of exosomes. We aimed to substantiate the pro-angiogenic impact of the released factors, to identify the nature of their cargo, and to test their efficacy in vivo supporting regeneration and recovery after myocardial ischemia. Methods and results: Mechanical stimulation of ischemic muscle via SWT caused extracellular vesicle (EV) release from endothelial cells both in vitro and in vivo. Characterization of EVs via electron microscopy, nanoparticle tracking analysis and flow cytometry revealed specific exosome morphology and size with presence of exosome markers CD 9, CD81 and CD63. Exosomes exhibited angiogenic properties activating protein kinase b (Akt) and extracellular-signal regulated kinase (ERK) resulting in enhanced endothelial tube formation and proliferation. A miRNA array and transcriptome analysis via next-generation sequencing were performed to specify exosome content. miR-19a-3p was identified as responsible cargo, antimir-19a-3p antagonized angiogenic exosome effects. Exosomes and target miRNA were injected intramyocardially in mice after left anterior descending artery (LAD) ligation. Exosomes resulted in improved vascularization, decreased myocardial fibrosis and increased left ventricular ejection fraction as shown by transthoracic echocardiography. Conclusions: The mechanical stimulus of SWT causes release of angiogenic exosomes. miR-19a-3p is the vesicular

cargo responsible for the observed effects. Released exosomes induce angiogenesis, decrease myocardial fibrosis and improve left ventricular function after myocardial ischemia. Exosome release via SWT could develop an innovative approach for the regeneration of ischemic myocardium. 2. Translational Perspective SWT is a non-invasive therapy used in clinical practice for more than three decades for lithotripsy and improved wound healing with no observed major adverse events. Cardiac application for myocardial regeneration is a promising approach for patients with ischemic cardiomyopathy. First human applications show promising results, although the working mechanisms remained obscure. We show for the first time that the mechanical stimulus of SWT causes release of angiogenic exosomes containing miR-19a-3p. Treatment of ischemic myocardium resulted in improved cardiac function and decreased fibrosis. Elucidating molecular mechanisms could promote the clinical application of cardiac SWT.

5 3. Non -standard Abbreviations and Acronyms. CFSE . . . Carboxyfluorescein succinimidyl ester EV … extracellular vesicles HUVEC . . . human umbilical vein endothelial cells LAD . . . left anterior descending LDPI . . . laser Doppler perfusion imaging MVB . . . multivesicular bodies nSMase . . . neutral sphigomyelinase SCID . . . severe combined immunodeficient SW . . . shock wave SWT . . . shock wave therapy TLR3 … Toll -like receptor 3 TSP - 1 . . . thrombospondin 1 VEGF … Vascular endothelial growth factor VEGFR2 … Vascular endothelial growth factor receptor 2

4. Introduction

Exosomes were reportedly released from adult cardiomyocytes upon hypoxic exposure 1 , from rat hearts exposed to ischemia 2 , and were found to be elevated in plasma of humans subjected to remote ischemic preconditioning 2 , and as a result cardioprotective mechanisms are supposedly triggered 2-4 .

No method so far exists to stimulate the release of angiogenic exosomes in vivo. Our group gained experience with mechanical stimulation by shock waves earlier and we therefore hypothesized that SWT increases the release of angiogenic exosomes in ischemic myocardium.

Low-energy shock wave therapy (SWT) is a promising, non-invasive approach that induces an angiogenic response and thereby supports the regeneration of ischemic hearts and limbs 5-8 . Its efficacy at inducing angiogenesis and corresponding benefits has been well documented both for in vitro models such as murine skin isografts 6 , endothelial cells 9 and fibroblasts 10 , as well as in vivo for mouse, rat and porcine myocardial ischemic models 7, 11 . Importantly, evidence exists that SWT may also improve cardiac function in human patients suffering from severe coronary disease 12 , and that SWT may be routinely and successfully applicable in a clinical setting 13 . Furthermore, a recent review and meta-analysis on SWT anti-anginal efficacy in humans confirms a beneficial effect (14; covering a total of 1,189 patients in 39 relevant studies).

In contrast to the relatively well-documented effects of SWT, very little is known regarding the underlying mechanisms at the cellular and molecular level, and only recently we began to shed light on this matter. Specifically, we found that SWT causes the release of RNA which activates Toll-Like receptor 3 (TLR3) signaling and thereby induces angiogenesis and arteriogenesis in different tissues 15, 16 . The involvement of TLR3 in the SW response was substantiated in vitro by showing that the supernatant of SW-treated HUVECs evoked TLR3 signaling-relayed expression of IFN-β1 in target cells, which was abolished by siRNA-mediated silencing of the receptor 16 . Furthermore, RNAse treatment of the supernatant prevented TLR3 activation. A mouse hind limb ischemia model confirmed this mechanism in vivo, with angiogenesis and improved blood perfusion evoked in wild type animals treated with SWT, whereas TLR3- deficient mice remained totally unresponsive and

resembled untreated controls [16] . The enhanced release of double-stranded RNA was by itself not dependent on TLR3 and, importantly, appeared to not be the consequence of SW induced cell damage. We thus previously suggested that RNA release might occur via exosomes [11], the importance of which as transmitters of intercellular and tissue level communication has received increased recognition over recent years [17-19] . Exosomes are nano-sized lipid bilayer vesicles with a size of 30 to 100nm. They are derived from intraluminal vesicles of multivesicular bodies (MVB) and released into the extracellular space upon fusion of MVBs with the cell membrane [17] . In contrast to these exosomes, microvesicles that are larger in size and produced by plasma membrane shedding show mainly unfavorable effects on the cardiovascular system [20] .

In the present study, we sought to test the hypothesis that SWT causes the release of exosomes. Furthermore, we wanted to substantiate the pro-angiogenic impact of the released factors, to obtain more specific insight into the nature of the cargo released with the presumed exosomes, and to finally test the efficacy of an in vivo treatment with these exosomes for supporting regeneration and recovery of ischemic myocardium.

5. Methods

5.1. Cell culture and exosome isolation After obtaining written informed consent of patients, umbilical cords were obtained from Caesarean sections for isolation of human umbilical vein endothelial cells (HUVECs). Permission was given from the ethics committee of Innsbruck Medical University (no. UN4435) and complied to the Declaration of Helsinki. Isolation of endothelial cells was performed as described in detail previously [16] .

5.2. Induction of myocardial infarction

Experiments were approved by the Austrian animal care and use committee (BMWF66.011/0124-WF/V/3b/2016). The investigation conformed to the 'Guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health (NIH Publication No. 85-23, 1996, revised 2011; available from: www. nap.edu/catalog/5140.html). Euthanasia was performed by cervical dislocation in anesthesia. Anesthesia was performed via an intraperitoneal injection of ketamine hydrochloride (Ketanest®, Graeub, Switzerland, 80mg/kg body weight) and xylazine hydrochloride (Xylasol® , aniMedica, Germany, 5mg/kg body weight). 12 to 15 weeks old male C57/BL6 mice (Charles River, Sulzfeld, Germany) and 12 to 15 weeks old male severe combined immunodeficient (SCID)-beige mice (NOD.CB17- Prkdcscid/J, Charles River) were used. A lateral thoracotomy was performed and the left anterior descending artery (LAD) was ligated using 7-0 polypropylene sutures (Ethicon, Somerville, NJ). Myocardial infarction was confirmed by blanching of the myocardium. Immediately after ligation immunodeficient (SCID)-beige mice (n=10) were injected with exosomes from 2 million HUVECs suspended in 300μl PBS intramyocardially into the periinfarction zone. C57BL/6 mice were treated with 80ng Miridian hsa-miR-19a-3p (UGUGCAAAUCUAUGCAAAACUGA), Miridian hsa-let-7i-5p (UGAGGUAGUAGUUUGUGCUGUU) (both Thermo Scientific) and scrambled miRNA (Qiagen, Venlo, Netherlends) suspended in Dharma FECT 1 Transfection Reagent (GE Dharmacon Inc., Lafayette, USA).

5.3. Echocardiography

Echocardiography was performed as described previously [11] .

5.4. Nanoparticle tracking analysis (NTA)

In order to determine the quantity and dimensions of released exosomes, cell culture supernatants and mouse plasma samples were analyzed on a Zetaview PMX 110 V3.0 particle analyzer (Particle Metrix GmbH, Meerbusch, Germany).

5.5. Flow cytometry For characterization of EV, an EV capture assay with antibody-coated magnetic beads was used. Samples were incubated with anti-CD63, CD9 or CD81-coated magnetic beads (ExoCap, JSR Life Sciences, Leuven, Belgium) overnight, and washed by aspiration on a magnet (Hydro Speed, Tecan; Männedorf, Switzerland) upon adding Bio-Plex® Wash Buffer (Bio-Rad Laboratories, Hercules, CA). Secondary CD63-Alexa647 (BD Bioscience, Franklin Lakes, NJ), CD9-Alexa647 (Exbio, Prague, Czech Republic), CD81-Alexa647 (Santa Cruz Biotechnology, Dallas, TX) antibody in PBS was added, and incubated for 2 hours at RT while shaking. Beads were washed with washing buffer, and resuspended in PBS. Mean fluorescent intensity of the samples was measured by FACS (BC CytoFLEX flow cytometer, Beckman Coulter, Brea, CA).

5.6. miRNA array and miRNA transfection Inflammatory Response and Autoimmunity miScript miRNA PCR Array (Qiagen, Venlo, Netherlands) was used for miRNA screening of exosome content and used as suggested by the manufacturer. Transfection of HUVECs was performed using Dharmafect transfection reagent 4 (Thermo Scientific, Waltham, MA).

5.7. Gene expression profiling

Total-RNA was isolated from three samples of differently treated HUVEC cells and was submitted to transcriptome analysis for the purpose of gene-expression profiling as described in the supplements.

5.8. Statistical Analysis

All graphs are presented as scatter/dot plots. Experiments with a time course are represented as line graphs to depict time-dependent changes. All results are expressed as mean+SEM. Statistical comparisons between two groups were performed by Student's t or Mann–Whitney test as appropriate. Multiple groups were analyzed by one-way ANOVA with Tukey post hoc analysis to determine statistical significance. Probability values <0.05 were considered statistically significant.

For further details regarding materials and methods, see the online Data Supplement.

6. Results

6.1. SWT causes exosome release in vitro and in vivo

In the first series of experiments we evaluated if SW treatment induces the release of EVs from endothelial cells and determined their characteristics. HUVECs were cultured in exosome-free conditions and subjected to SW treatment, followed by isolation of EVs released into the conditioned medium. As measured by nanoparticle tracking analysis, SW treatment induced a rapid, significant increase in the number of particles in the CM as compared to those from untreated controls after 1 and 4 hours (Fig. 1A). As they represent the relevant cell type in myocardial ischemia and numbers of released exosomes are reduced in elder patients, we confirmed our results in primary human coronary artery endothelial cells derived from elder donors (CAECs) and found the same rapid release of particles after mechanical stimulation (Fig. 1B). This finding was supported by quantitative electron microscopy of HUVECs 4 hours after SW treatment and untreated controls under steady state conditions. Both cell populations displayed normal, healthy subcellular architecture. Corresponding to the nanoparticle counts, SW-treated cells contained twice as many multivesicular bodies (MVB), the source of exosomes, as the controls (Fig.1C). In line with this, we

furthermore observed that expression of neutral sphingomyelinase 2 (nSMase2), an essential regulator of exosome secretion, was higher in SW-treated cells than in untreated controls (Fig. 1D). Furthermore, nanoparticle tracking also allowed estimation of the particle size, which was approximately 100nm for EVs from both the controls and treated cells and remained constant over time, corroborating their largely uniform nature (Fig. 1E). This size corresponds to that considered typical for exosomes and, in line, transmission electron microscopic analysis supported this conclusion, with the particles displaying the exosome-typical spherical to cup-shaped morphology 21 (Fig. 1E). Furthermore, applying flow cyometry, we also detected expression of exosomal marker proteins CD 9, CD 63 and CD 81 on the isolated EVs (Fig. 1F-H).

We next set out to evaluate if SW treatment might also induce in vivo the formation of MVB, the source of subsequent exosome release, using a hind limb ischemia model. For this purpose, mouse hind limbs were subjected to ischemia and received SW treatment immediately thereafter or were left untreated (controls). After 6h muscle was excised and immediately fixed for electron microscopy. Since direct detection of exosomes released into the blood is not possible in tissue samples, we used the same direct approach as for HUVECs. Thus, MVB were quantified in the cytoplasm of endothelial cells 17, 20. As depicted in the representative images in and the corresponding bar graph in Fig. 1I, SW treatment induced a significant increase in the number of MVB after SWT.

6.2. Exosomes released in response to SWT are highly angiogenic in vitro
Having established that SWT stimulated the formation of MVB and/or release of exosomes in HUVECs and muscle endothelial cells, we investigated the exosomes' angiogenic impact. To this end, we exposed HUVECs to SWT, collected the supernatants, and isolated the exosomes released from the cells (Fig. 2A). Adding these exosomes to previously untreated cells elicited several angiogenic responses. Thus, we observed significantly increased tube formation in vitro, as reflected by enhanced numbers of segments, branches and nodes in the presence of exosomes from SW-treated cells as compared to controls (Figs. 2B, C). In addition, exosomes from SW-treated cells stimulated cellular proliferation to levels significantly exceeding that of untreated cells (Fig. 2D). This was associated with a quick and transient activation, and hence phosphorylation, of protein kinases AKT and ERK (Fig. S1), both of which are involved in signaling pathways eliciting cardioprotective responses 2, 22.

6.3. Inhibition of exosome release abolishes SW effect
The dependence of these documented effects of SWT on the actual release of exosomes was corroborated in experiments using the nSMase2-inhibitor GW 4869. As shown in Fig. 2E, the SWT-induced release of vesicles into the supernatant was completely abolished in HUVECs pre-treated with GW 4869. Similarly, enhanced tube formation elicited by direct SWT of the cells was fully suppressed by the inhibitor (Fig. 2F, G), and the same inhibitory action was observed in relation to the proliferative stimulation exerted by SWT (Fig. 2H). This indicates that the angiogenic shock wave effect is exosome-dependent.

6.4. Released exosomes are highly angiogenic in vivo
Assessment of in vivo angiogenic effects of exosomes released in response to SWT was performed applying a Matrigel plug assay in nude mice, as schematically shown in Fig. 3A. Perfusion of these plugs, evaluated 5 days post-injection, was significantly elevated in the presence of exosomes from SW-treated cells as compared to exosomes from controls (Fig. 3A, B). This was also reflected in enhanced numbers of arterioles and capillaries in these plugs, as evidenced by stronger immunostaining with the capillary marker CD31 and the combined expression of CD31 and α-SMA characteristic for smooth muscle cells of arterioles and when quantified microscopically after 5 days (Figs. 3C). In vivo imaging revealed that

vessels formed upon exosome injection were fully functional (Video 1).

6.5. Released exosomes induce angiogenesis and improve cardiac function after myocardial infarction

Next, the efficacy of exosomes released from SW-treated cells was tested in an in vivo myocardial infarction model. Mice suffering from myocardial infarction due to LAD ligation were injected immediately after injury with exosomes released either from control or SW-treated HUVECs into the periinfarction zone of the heart. Animals were then left to recover for 4 weeks, as depicted in the scheme in Fig. 3D. Echocardiographic examination indicated that myocardial infarction reduced LV ejection fraction from approximately 60% in sham-treated animals to about 25% one day after the ischemic episode, independent of whether control or SWT exosomes were injected. However, whereas LV ejection fraction showed a further steady decrease in the control animals over the next 4 weeks, it was maintained at a significantly higher level in animals that had received exosomes from SW-treated cells (Fig. 3E). A better preservation of ventricular function was also suggested by transthoracic echocardiography documenting improved contractility of the left ventricular anterior wall in these mice as compared to controls (Fig. 3E). Furthermore, quantification of post-infarctional fibrosis in the mouse hearts revealed a significantly higher area of fibrotic tissue in the LV of animals with control exosomes than with SW exosomes (Fig. 3F). Finally, corroborating the angiogenic effect of SW-treated exosomes, in the hearts of animals treated with those exosomes the expression of VEGF and VEGFR2 was significantly elevated compared to animals treated with control exosomes (Fig. 3G).

6.6. Released exosomes contain angiogenic miRNAs

The degree of angiogenesis in the Matrigel plugs was clearly related to whether the samples had been loaded with exosomes from SW-treated or control cells. However, this effect could be due either to the increased number of exosomes released from SW-treated cells or due to the nature of cargo transmitted by the cells, i.e. the RNA species contained. To clarify whether SW exosomes trigger different gene expression than control exosomes, we treated endothelial cells with SW or control exosomes and performed RNAseq analysis thereafter. Indeed, treatment with SW exosomes resulted in a different gene expression profile than treatment with control exosomes (Fig. 4A). We found 1.5 fold upregulation of 215 genes, whereas 161 genes were downregulated (Fig. 4B). Genes with most significant regulation are depicted in Fig. 4C. Differential gene expression profile strengthened our assumption that SW exosomes carry a different cargo than control exosomes. We thus set out to characterize this cargo, determining the sequences and relative expression levels of cargo miRNAs by means of a sequencing array. This analysis revealed a pool of 29 miRNAs expressed to similar levels in both types of exosomes (Fig. 4D, middle panel). In addition, we detected a similar number of miRNAs that appeared to be significantly higher expressed in control exosomes (Fig 4D, left panel), the most pronounced differences were observed for hsa-miR-17-5b, hsa-miR-520e, and hsa-miR-21-5p. At the same time, another population of 31 miRNAs was significantly more abundant in SWT exosomes (Fig 4D, right panel), with highest expression of miR-7i, miR19a, mir-19b, and miR-301b. Separate testing of enhanced expression levels by qPCR confirmed these findings obtained with the array for miR-7i, miR-19a, mir-19b, but not for miR301b (Fig. 4, E-H). Out of the remaining three miRNAs, we chose to study hsa-miR-19a-3p in depth, as it has been described previously as angiogenic in breast cancer[23] [24], [25] .

6.7. Exosome cargo miR-19a-3p is angiogenic in vitro

Therefore, we next tested whether any of the miRNAs highly overexpressed in SWT exosomes would by itself be angiogenic, but failed to detect such activities for miR-7i and miR-19b (data not shown). Conversely, we could find indications for angiogenic effects for miR-19a-3p. Scrambled miRNA (scr) decreased tube formation, an effect likely due to the transfection reagents. In comparison to scr, miR-19a-3p significantly increased tube formation (Fig. 5A, B). In addition, miR-19a-3p transfection stimulated the proliferation of HUVECs to a similar degree as FCS used as a positive control (Fig. 5C). This might indicate that the angiogenic effect of miR-19a-3p surpassed the apparently toxic effect of the exposure to the transfection reagents. To further evaluate this, we developed an antagomir, specific against miR-19a-3p and tested its impact on SWT-induced angiogenesis. As shown in Figs. 5D and E, this antagomir blocked both the outgrowth of segments and branches and fully suppressed the development of nodes elicited by the SW treatment. Importantly, scrambled miRNA had no significant effect in this context, supporting the specificity of the antagomir effect. In line with this, the increase in cell proliferation induced by SWT was not affected by scrambled miRNA, but was totally blocked by the antagomir (Fig. 5F). To confirm potential implications in vivo, we performed an animal model of hind limb ischemia and performed SWT in combination with prior scr or antagomir injection. As anticipated, angiogenic SW effects were abolished by injection of antagomir against miR-19a-3p but not by scr (Fig. S2A). In line, improved perfusion of subcutaneously injected matrigel plugs was abolished by anti-miR-19a-3p (Fig. S2B). Thrombospondin 1 is a potent inhibitor of angiogenesis and has been described to be regulated by miR-19a-3p 26. We therefore transfected HUVECs with miR-19a-3p and analyzed TSP-1 expression. TSP-1 mRNA as well as protein levels were downregulated with concomitant upregulation of VEGFR2 (Fig. S3A-C). We found similar results in vivo: injection of miR-19a3p into ischemic myocardium resulted in downregulation of TSP-1 (Fig. S3D) and increased numbers of capillaries (Fig. S3E). This suggests that miR-19a-3p induced inhibition of TSP-1 mRNA translation facilitates angiogenic effects.

6.8. Exosome cargo miR-19a-3p is angiogenic in vivo

Finally, we tested miR-19a-3p in an acute myocardial infarction model in C57BL/6 mice by injecting them with miR-19a-3p or scrambled miRNA suspended in transfection reagent after induction of MI (Fig. 5G). One day after infarction, LV ejection fraction was reduced to 35% in these mice and was maintained at this level in the controls for 14 days before it displayed a further drop to below 25% at 28 days (Fig. 5H). In contrast, LV ejection fraction remained largely unaltered over the 4-week period in the mice injected with miR-19a-3p, suggesting a protective effect on left ventricular function after myocardial infarction. In line, ventricular anterior wall contractility was much better in these mice as compared to controls, although not significant (Fig. 5H).

7. Discussion

Over the past decade, it has been reported that ischemia causes the release of exosomes from cells in culture 1, 27 , but also elevates plasma exosome concentrations in vivo 2 . In recent years it has become clear that exosomes can relay signals eliciting repair and regeneration responses of damaged tissues 2, 3. Inspired by these observations, we hypothesized that these vesicles would be a suitable vehicle to transport RNA and/or RNA/protein complexes we have found to be released in response to shock wave therapy 11, 16, triggering the angiogenic effects we have amply documented as a result of this treatment 13. Indeed, we could clearly demonstrate that SW treatment can stimulate EV release in HUVECs. The basic characteristics of these EVs in terms of size, microscopic structure and expression of surface markers qualify them as exosomes. Further evidence was that SW treated HUVECs showed elevated expression of an essential regulator of exosome secretion, nSMase2. In addition, we showed that the number of MVBs, the source of exosomes, is increased after SWT in endothelial cells in ischemic murine muscle, which further

supports our hypotheses. We then examined if exosomes were indeed the secreted entities that would transmit the angiogenic effects previously described for the supernatant from SW treated cells 11. In line with this notion, exosomes isolated from such supernatants induced significantly enhanced tube-formation and proliferation in in vitro assays, accompanied with the rapid activation of signaling pathways typical for angiogenic activity. Importantly, when adding the nSMASe inhibitor GW 4869, indirectly blocking exosome generation, not only the release of exosomes was inhibited, but this compound also fully blocked enhanced tube formation and proliferation observed after direct SW treatment of the cells in its absence. These findings further corroborate the role of the exosomes, and concurrently render an involvement of microvesicles shed from the cell surface unlikely. In line, the mechanical stimulation associated with induction of cardiac pressure overload in mice has previously been described to cause the release of exosomes into the plasma 28 . Similarly, remote ischemic conditioning which is associated with shear stress causes an increase in plasma exosome number 2 .

Experiments aimed at establishing the in vivo efficacy of exosomes collected from SW treated cells confirmed our in vitro findings. Thus, mice injected with Matrigel plugs containing SW exosomes displayed significantly enhanced blood flow in the vicinity of these plugs, as well as increased numbers of capillaries and arterioles, supporting their angiogenic capacity.

Following myocardial infarction, SW exosomes improved cardiac contractility and a better preservation of LV ejection fraction, reduced the share of post-infarctional fibrosis in ventricular tissue, and caused enhanced expression of angiogenic factors such as VEGF and VEGFR2.

A closer look into the type of miRNA contained in the exosomes released from SW treated cells indicated that a number of miRNAs were contained at expression levels significantly different from that in control exosomes. In vivo, acute myocardial infarction as well as chronic cardiomyopathy were described to be associated with specific miRNA species contained in exosomes in the serum of patients 29. Further, cardioprotective miR-214, which can be secreted via exosomes from endothelial cells 30, was found elevated in the post-ischemic mouse heart 31 and in plasma of coronary artery disease patients 32 . Other studies have implied numerous other miRNA species in the cardiac response to ischemia-reperfusion 33 , and the specific involvement of exosomal miRNA has recently been reviewed by Emanueli et al. 34 . Here, we present evidence that miR-19a-3p, one of the four most prominently enriched miRNA species in SW exosomes, was of pivotal importance in the transduction of the angiogenic effects. Furthermore, an antagomir against miR-19a-3p was anti-angiogenic and antiproliferative. Importantly, the in vivo delivery of miR-19a-3p to the myocardial infarction mouse model helped to better preserve LV ejection fraction compared to controls and to maintain ventricular contractility. Given that miR-19a-3p has been previously implied in enhanced tumor neoangiogenesis 26 and, more generally, in an oncogenic context 35-37, it appears plausible that its beneficial impact in the mouse model was also related to its in vivo angiogenic activity. miR19a-3p has been described as component of miR-17-92 cluster, a well-characterized set of miRNAs. It has been shown to promote angiogenesis and cell proliferation via inhibition of the antiangiogenic factor TSP-1 and connective tissue growth factor, but also via regulation of the PI3K/AKT pathway38. Whether its beneficial effects on postinfarctional remodeling are mainly due to angiogenic effects, remains unknown and needs to be addressed in further studies. Based on the observation that its angiogenic effect seems to be mediated by inhibition of the anti-angiogenic regulator thrombospondin 1 26, we thus propose a simplified mechanistic model for the beneficial effect of SWT as outlined in Figure 6. There is evidence for causal relationship of VEGF and the miR-17-92 cluster39. We found upregulation of VEGFR2 after transfection with miR-19a-3p. Whether VEGFR2 is a direct target of miR-19a-3p needs to be addressed in future studies.

Limitations.

Nanoparticle tracking analysis (NTA) showed high varying results regarding exosome release after SWT. However, to verify NTA results we therefore confirmed exosome release after SWT via transmission electron microscopy. Overall, our study provides evidence that exosome release can be stimulated by shock wave therapy in the post-ischemic heart, and that one of the miRNA species contained in these exosomes as cargo, miR-19a-3p, is of particular importance in mediating the angiogenic and proliferative effects of SWT. As shock waves have been in clinical use for many years in other indications and no severe adverse effects have been observed so far, it will be translated very efficiently and develop a powerful tool for the regeneration of ischemic myocardium.

10. Conflict of interest JH and MG are shareholders of Heart Regeneration Technologies GmbH, an Innsbruck Medical University spin-off aiming to promote cardiac shockwave therapy (www.heartregeneration.com). All other authors have nothing to disclose.

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Figure Legends

Figure 1. Release of exosomes after shock-wave treatment (A) Particle release from HUVECs under control conditions (CTR) and after SW treatment (SWT). Data are means ± SEM. *$p < 0.05$. n=3-6. (B) Confirmation of results in primary human coronary artery endothelial cells (CAECs). Data are means ± SEM. ****$p< 0.05$. n=5-6. (E) Transmission electron microscopic image of particles released from HUVECs and size estimation of these particles assessed over time. Scale bar=100nm. n=3-5. (F-H) FACS analysis of released particles (bead assay) revealed presence of the typical exosomal marker proteins CD 9, CD 63 and CD 81. Data are means ± SEM. n=6. (I) Thin section electron micrographs of ischemic murine muscle tissue after 6h with no further treatment (CTR) or following SWT. Characteristic multivesicular bodies (MVB: arrows), the source of exosomes, are visible in the endothelium; caveolae are marked by arrow-heads. Scale bars=200nm. Quantification of MVB (different maturation stages pooled) per area ($10\mu m2$) examined in SWT and control murine muscle endothelium 6h post ischemia. Data are means ± SEM. **$p < 0.01$. n=3. Statistical comparisons between two groups : Student's t test, multiple groups : one-way ANOVA with Tukey post hoc analysis

Figure 2. In vitro angiogenic effect of exosomes from SW treated cells (A) Schematic presentation of exosome preparation. (B) Representative images of the tube formation assay conducted with cells exposed to exosomes from controls (CTR) or from SW treated HUVECs (SWT). Scale bar=100µm. (C) Quantitative read-out of the tube formation assay expressed as the number of nodes formed per unit area. Exposure of cells to PBS or VEGF served as a negative and a positive control, respectively. Data are means ± SEM. **$p < 0.01$. n=4-6. (D) Effect of control and SWT-exosomes on cell proliferation. Exposure to EGM2 served as a positive control. Data are means ± SEM. ****$p < 0.0001$. n=4. (E) Quantification of particles released from control and SW treated HUVECs in presence of nSMase2-inhibitor GW 4869. Data are means ± SEM, with individual data points displayed in addition. n=6. (F) Representative image of tube formation assay evaluated in SW treated cells in the

absence or presence of GW 4869. Scale bar=100μm. (G) Quantification of tube formation as the number of nodes formed per unit area, with cells exposed to FCS serving as a positive control. Data are means ± SEM. *p < 0.05. n=4-7. (H) Effect of GW 4869 on cell proliferation in controls and SW treated cells. Means ± SEM. *p < 0.05. n=3. Statistical comparisons between two groups : Student's t test, multiple groups : one-way ANOVA with Tukey post hoc analysis
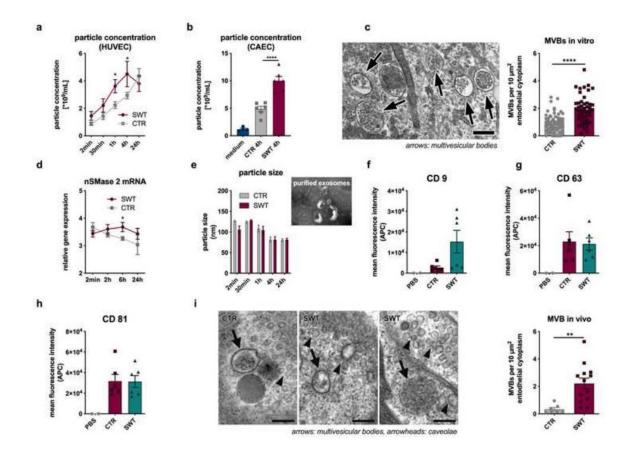
Figure 3. In vivo angiogenic effect of exosomes from SW treated cells (A) Schematic depiction of in vivo treatment of mice with Matrigel plugs containing exosomes isolated from controls and SW treated cells and representative pictures from Laser Doppler perfusion imaging in mice injected with exosomes from controls and SW treated cells (plugs marked with arrow). (B) Quantification of blood flow observed through these plugs 5 days postinjection. Data are means ± SEM. **p < 0.01. n=5-6. (C) IF staining of endothelial cell marker CD31 typifying capillaries and of CD31 and smooth muscle marker α-SMA typical expressed in capillaries, and quantification of the number of blood vessels observed per HPF in plugs containing control and SWT exosomes. Data are means ± SEM. ***p < 0.001. ****p < 0.0001. n=5. Scale bar=50μm. (D) Experimental scheme for testing efficacy of exosomes in a myocardial infarction model. (E) Echocardiography of mice that had received control or SWT exosomes immediately after myocardial infarction showing improved contractility in the SWT group, and quantitative comparison of LV ejection fraction in both groups over 4 weeks posttreatment. Data are means ± SEM. *p < 0.05. n=5. (F) Representative images of hearts displaying post-infarction fibrosis, as assessed by Masson's trichome staining 28 days after treatment, in mice treated with control and SWT exosomes, and quantitative comparison of fibrotic tissue in both groups. Means ± SEM. *p < 0.05. n=5. Scale bar=500μm. (G) Relative gene expression levels of VEGF and VEGFR2 in hearts of post-infarction mice that had received control or SWT exosomes 28 days after treatment. Means ± SEM. *p < 0.05. **p < 0.05. n=4-5. Statistical comparisons between two groups: Student's t test, multiple groups : one-way ANOVA with Tukey post hoc analysis
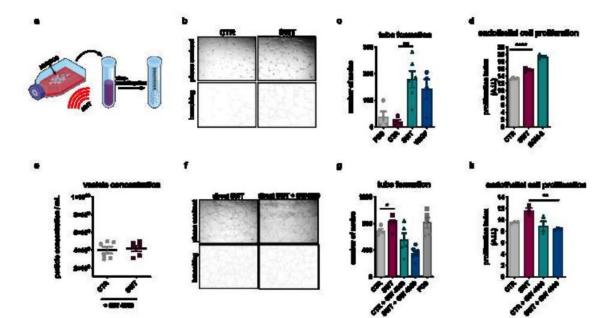
Figure 4. Characterization of exosome cargo (A) RNAseq revealed a different gene expression profile of endothelial cells treated with SW exosomes compared to treatment with control exosomes. (B) Transcriptome analysis revealed >1.5 fold upregulation of 215 genes, whereas 161 genes were downregulated. (C) The heatmap depicts genes with the most significant regulation upon treatment with SW exosomes. (D) Relative expression levels, determined with a sequencing array, of miRNA species contained in exosomes released from controls and SW treated cells, with relative sizes of spheres corresponding to relative expression levels. (E), (F), (G), (H) Quantitative PCR detection of relative expression levels of the miRNAs with the most pronounced differences indicated by array analysis. Data are means ± SEM . *p < 0.05; **p < 0.01. n=5. Student's ttest.
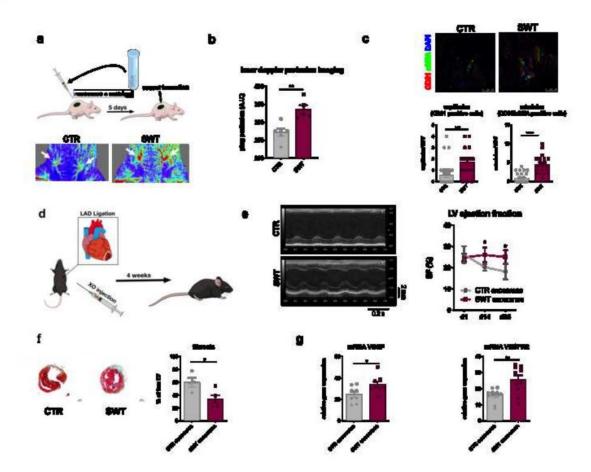
Figure 5. In vitro and in vivo angiogenic impact of miR-19a-3p (A) Representative examples of the tube formation assay conducted with cells exposed to scrambled miRNA (scr) or miR-19a-3p. Scale bar=100μm. (B) Tube formation expressed as the number of nodes formed per unit area in scr or miR-19a-3p exposed cells and in the presence of PBS or FCS, serving as negative and positive controls, respectively. Data are means ± SEM. *p < 0.05. n=6. (C) Effect of miR-19a-3p on cell proliferation quantified via CSFE proliferation assay. Means ± SEM. ***p < 0.001. n=4. (D) Representative images showing the effect of SWT on tube formation in cells treated with antagomir specific against miR-19a-3p or with scrambled miRNA. Scale bar=100μm. (E) Quantitative assessment of tube formation after SWT with antagomir against miR-19a-3p or with scrambled miRNA present. PBS and EGM-2 are the negative and positive controls, respectively. Means ± SEM. *p < 0.05. n=4-6. (F) Cell proliferation under conditions outlined in (E). Means ± SEM. ****p < 0.0001. n=4. (G) Experimental scheme for testing the in vivo impact of miR-19a-3p in the mouse infarction model. (H) Echocardiography of infarcted hearts from mice treated with scr miRNA or miR-19a-3p, and

quantitative comparison of LV ejection fraction in both groups over 4 weeks post-treatment. Data are means ± SEM. n=6-10. Statistical comparisons between two groups : Student's t test, multiple groups : one-way ANOVA with Tukey post hoc analysis

Figure 6: Suggested mechanism of SWT induced angiogenic effects SWT stimulates nSMAse2-catalyzed and GW 4869-sensitive formation of ceramide, the accumulation of which is known to trigger budding of exosomes into multivesicular bodies (MVBs) (35). Upon fusion with the cell membrane these MVBs release exosomes into the extracellular space which are then taken up at the surface of receiving cells, either by fusion with the target membrane or by endocytosis (36). The exosomes can then release their cargo, part of which is miR-19a-3p. The latter can act as an inhibitor of thrombospondin 1 (TSP-1) (31), a known suppressor of angiogenesis and proliferation, which in effect causes a net stimulation of these processes.
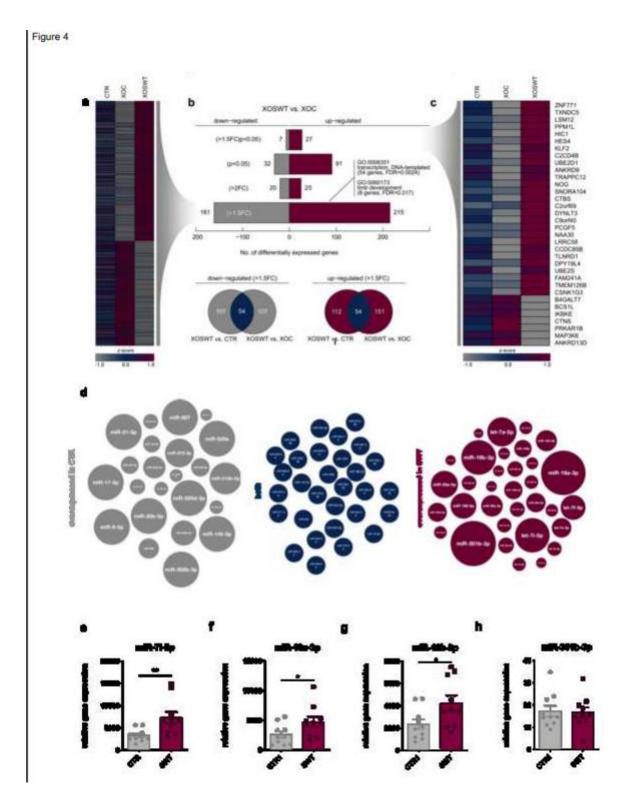
Figure 1

a — particle concentration (HUVEC)
b — particle concentration (CAEC)
c — MVBs in vitro; *arrows: multivesicular bodies*
d — nSMase 2 mRNA
e — particle size; purified exosomes
f — CD 9
g — CD 63
h — CD 81
i — MVB in vivo; *arrows: multivesicular bodies, arrowheads: caveolae*

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6