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Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs

Eduard Hergenreider[1], Susanne Heydt[1], Karine Tréguer[1], Thomas Boettger[2], Anton J. G. Horrevoets[3], Andreas M. Zeiher[4], Margot P. Scheffer[5], Achilleas S. Frangakis[5], Xiaoke Yin[6], Manuel Mayr[6], Thomas Braun[2], Carmen Urbich[1], Reinier A. Boon[1,7] and Stefanie Dimmeler[1,7,8]

The shear-responsive transcription factor Krüppel-like factor 2 (KLF2) is a critical regulator of endothelial gene expression patterns induced by atheroprotective flow. As microRNAs (miRNAs) post-transcriptionally control gene expression in many pathogenic and physiological processes, we investigated the regulation of miRNAs by KLF2 in endothelial cells. KLF2 binds to the promoter and induces a significant upregulation of the miR-143/145 cluster. Interestingly, miR-143/145 has been shown to control smooth muscle cell (SMC) phenotypes; therefore, we investigated the possibility of transport of these miRNAs between endothelial cells and SMCs. Indeed, extracellular vesicles secreted by KLF2-transduced or shear-stress-stimulated HUVECs are enriched in miR-143/145 and control target gene expression in co-cultured SMCs. Extracellular vesicles derived from KLF2-expressing endothelial cells also reduced atherosclerotic lesion formation in the aorta of *ApoE*$^{-/-}$ mice. Combined, our results show that atheroprotective stimuli induce communication between endothelial cells and SMCs through an miRNA- and extracellular-vesicle-mediated mechanism and that this may comprise a promising strategy to combat atherosclerosis.

Atherosclerosis, the underlying cause of myocardial infarction and stroke, occurs predominantly in pre-disposed spots in the large arteries, despite the systemic nature of the classical risk factors for atherosclerosis. The focal nature of atherosclerosis is the result of differences in local blood flow patterns along the endothelium[1]. Endothelial cells that are exposed to laminar blood flow experience high shear stress and these parts of the vessel are protected from atherosclerosis formation, whereas turbulent blood flow, usually near bends and bifurcations, generates low endothelial shear stress, which facilitates atherosclerosis formation. In recent years, it has become clear that KLF2 plays a central role in mediating the atheroprotective endothelial phenotype generated by shear stress[2]. Constitutive and endothelial-restricted *KLF2*$^{-/-}$ mice die at embryonic day 10.5 owing to lack of vascular tone, bleeding and cardiac dysfunction secondary to vascular complications[3,4]. Interestingly, these *KLF2*$^{-/-}$ mice exhibit apparently normal endothelium, but dysfunctional and disorganized SMCs, indicating an important physiological link between endothelial KLF2 expression and the underlying SMC layer in the vasculature[5].

miRNAs are small (22 nucleotide long) single-stranded non-coding RNAs that are transcribed in the nucleus, processed by the enzymes Drosha (DROSHA) and Dicer (DICER1) and incorporated in RNA-induced silencing complexes, which mediate the translational inhibition or degradation of target messenger RNAs (refs 6,7). Many miRNAs have been identified to play key roles in (patho-)physiological processes, including atherosclerosis[8,9]. In particular, miR-126 was shown to provide an atheroprotective effect[10] and was reported to be upregulated by flow in a KLF2-dependent manner in zebrafish embryos[11]. The shear-sensitive miR-19a contributes to the anti-proliferative effect of shear flow[12], whereas miR-10a regulation is involved in the anti-inflammatory properties of shear stress[13]. miRNAs also regulate SMC functions, and, in particular, the cluster comprising miR-143 and miR-145 (miR-143/145) has been described to be of crucial importance for proper SMC function[14–16]. Here, we aim to identify miRNAs that are regulated by KLF2 in endothelial cells and might contribute to the vasculoprotective functions of KLF2.

RESULTS

KLF2 regulates expression of miRNAs, particularly miR-143/145

To assess which miRNAs are regulated by KLF2 in endothelial cells, we transduced human umbilical vein endothelial cells (HUVECs) with a lentiviral vector encoding KLF2, resulting in an increase of KLF2 expression levels similar to those found in HUVECs that are exposed to prolonged laminar flow[17] (Fig. 1a). We measured the expression level

[1]Institute of Cardiovascular Regeneration, Centre for Molecular Medicine, Goethe-University Hospital, 60590 Frankfurt, Germany. [2]Max-Planck-Institute for Heart and Lung Research, 61231 Bad Nauheim, Germany. [3]Department of Molecular Cell Biology and Immunology, VU University Medical Center, 1007 MB Amsterdam, The Netherlands. [4]Department of Internal Medicine III, Cardiology, Goethe-University Hospital, 60590 Frankfurt, Germany. [5]Institute for Biophysics, Goethe-University, 60438 Frankfurt, Germany. [6]Cardiovascular Division, King's College London, London SE5 9NU, UK. [7]These authors contributed equally to this work. [8]Correspondence should be addressed to S.D. (e-mail: dimmeler@em.uni-frankfurt.de)

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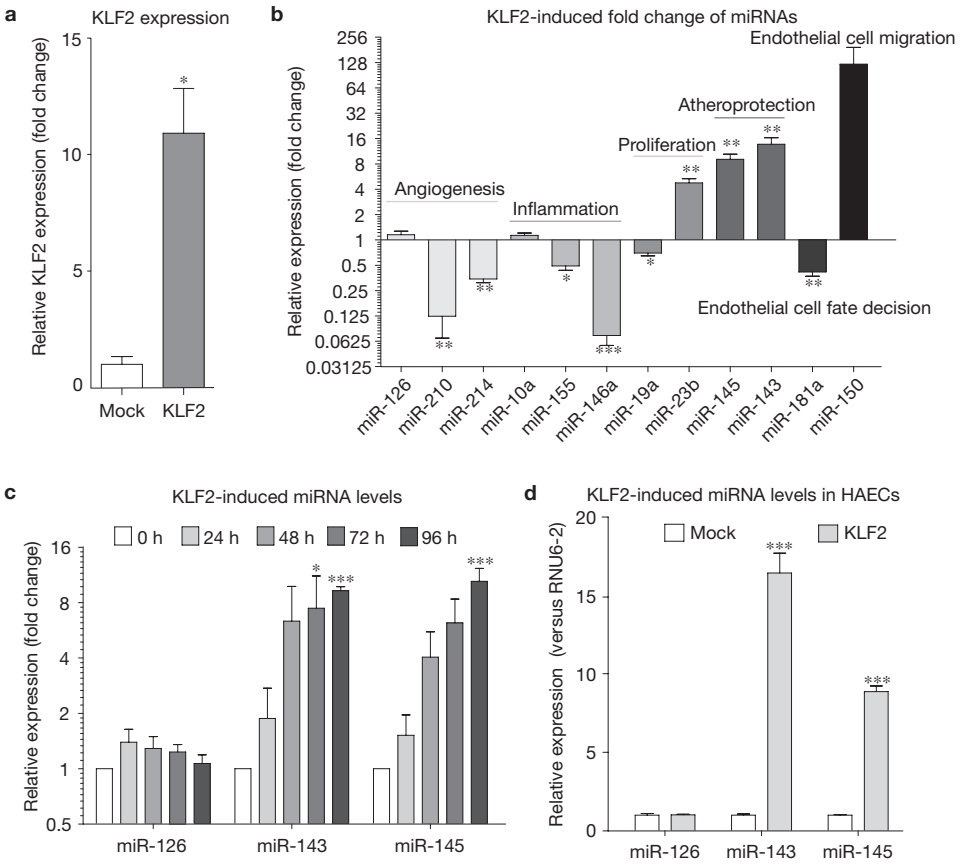

Figure 1 KLF2 regulates endothelial miRNA expression. (**a**,**b**) HUVECs were transduced with KLF2-overexpression or mock-control lentivirus, RNA was isolated after 4 days and the indicated miRNA and KLF2 expression levels were measured by real-time PCR. **a**, $n = 3$; **b**, miR-10a, $n = 6$; miR-143 and miR-145, $n = 5$; miR-19, miR-23b and miR-126, $n = 4$; all others, $n = 3$. (**c**) miR-126, -143 and -145 expression levels were measured by real-time PCR after the indicated periods of time after transduction with the KLF2-overexpression lentivirus ($n = 3$). (**d**) The KLF2-overexpression lentivirus was also used to transduce HAECs. RNA was isolated after 4 days and miR-126, miR-143 and miR-145 expression levels were measured by real-time PCR ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ when compared with mock-transduced cells. Error bars indicate s.e.m.

of a number of miRNAs with an established role in vascular biology by real-time PCR (Fig. 1b). Expression of the shear-responsive miRNA miR-23b, which has been reported to be upregulated after exposing endothelial cells to shear stress for 24 h (ref. 18), was also significantly induced by KLF2 (Fig. 1b). However, in contrast to previous studies in zebrafish embryos[11], the endothelial-enriched miRNA miR-126 was only transiently and not significantly regulated by KLF2 overexpression (Fig. 1b,c). KLF2 overexpression also did not change the expression level of miR-10a, which was reported to be downregulated in atherosusceptible regions of the aorta in pigs[13]. Furthermore, we found miR-19a, which has been reported to be rapidly induced by shear stress[12,18], to be downregulated by prolonged KLF2 overexpression, indicating that KLF2 regulates some but not all shear-regulated miRNAs.

Furthermore, a number of other miRNAs were significantly affected. KLF2 reduced the expression level of miR-210, which is involved in angiogenesis and the hypoxia response of endothelial cells[19], and miR-181a, which is known to play a role in endothelial cell fate decision[20]. Moreover, the expression level of miR-150, which regulates endothelial cell migration[21], was upregulated and that of miR-214, which is known to target eNOS expression[22], was significantly downregulated. As KLF2 is known to exert anti-inflammatory effects and inhibits NF-κB (ref. 23), we also determined the expression level of the inflammation-associated miR-146a (ref. 24) and miR-155 (ref. 25), which were both significantly downregulated by KLF2. Interestingly, in HUVECs and human aortic endothelial cells (HAECs), we found a significant and time-dependent upregulation of the expression of the atheroprotective miR-145 and its co-transcribed cluster member miR-143 (Fig. 1b–d), which are known for their atheroprotective function in SMCs (refs 14,16) but have not been studied in endothelial cells so far.

Physiological shear stress and statin treatment induce miR-143/145 through KLF2

To determine whether endogenous activation of KLF2 results in a comparable miRNA signature, we exposed HUVECs to prolonged laminar shear stress, which resulted in a robust induction of KLF2 expression (Fig. 2a). Prolonged shear stress significantly reduced the level of miR-210 expression, whereas that of miR-150 was increased, which is consistent with the regulation of these miRNAs by KLF2 (Fig. 2b). We also found that miR-10a expression was indeed induced by prolonged shear stress, which is consistent with the observation that it is reduced in atherosusceptible regions of the aorta in pigs[13]. As it was not regulated by KLF2 (Fig. 1b), the induction of miR-10a by flow seems to be KLF2 independent. Shear stress also increased the expression of the miR-143/145 cluster

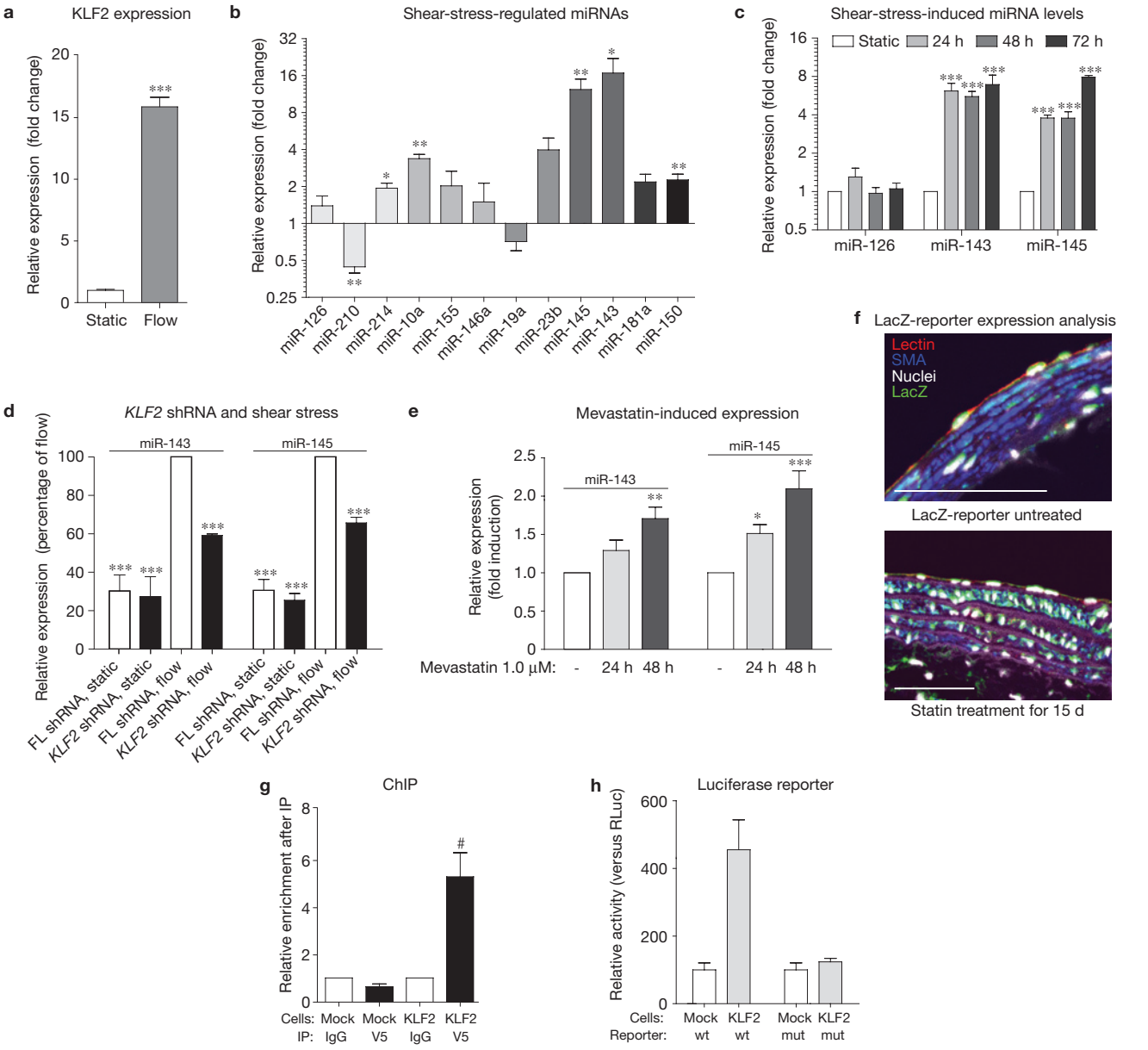

Figure 2 Shear stress and statins induce endothelial miR-143/145 expression through KLF2. (**a**,**b**) HUVECs were exposed to shear stress for 3 days. RNA isolation and real-time PCR were carried out to measure the expression levels of KLF2 as well as those of the indicated miRNAs. **a**, $n=4$; **b**, miR-126, miR-143 and miR-145, $n=7$; miR-150, $n=5$; miR-10a, miR-19a and miR-23b, $n=4$; all others, $n=3$. (**c**) Expression levels of miR-126, -143 and -145 in HUVECs that were exposed to shear stress for the indicated time periods ($n=3$ for each time point). (**d**) HUVECs were transduced with a lentiviral shRNA construct to silence KLF2 expression or with a control shRNA (designed to target firefly luciferase, FL shRNA) and exposed to laminar shear stress for 3 days or kept under static conditions. The miR-143 and miR-145 expression levels were measured by real-time PCR ($n=3$). (**e**) HUVECs were exposed to 1 μM mevastatin for 24 h (light grey), 48 h (dark grey) or left untreated (white), and miR-143 and miR-145 expression levels were measured by real-time PCR ($n=3$ for each time point). (**f**) Aorta sections of LacZ-reporter mice for miR-143/145 were analysed for LacZ expression by immunofluorescence microscopy. Scale bars, 50 μm. miR-143/145 reporter expression is visualized in green, endothelial cells in red, smooth muscle actin (SMA) in blue and nuclei in white. (**g**) HUVECs were transduced with lentivirus for V5-tagged KLF2 or control virus and immunoprecipitation (IP) was carried out with V5 antibody (black) or unspecific IgG (white) ($n=3$). Real-time PCR was carried out using primers specific for the region that contains the KLF-binding site on precipitated chromatin. (**h**) Luciferase reporter constructs containing either the wild-type (wt) or the mutated (mut) KLF-binding site were transfected into HUVECs that were transduced with the KLF2-overexpression virus or mock-control lentivirus 4 days before. Firefly luciferase activity was measured and normalized to *Renilla* luciferase (RLuc) using the Dual-Luciferase assay kit (Promega; $n=3$). $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $\#P=0.05$ when compared with static (**a**–**c**), FL siRNA, flow (**d**), untreated (**e**) or KLF2/IgG (**g**). Error bars indicate s.e.m.

to very similar levels to those observed with KLF2-transduced HUVECs, whereas the pro-inflammatory miR-146a and miR-155 showed a different expression pattern when comparing shear-stress-exposed HUVECs with KLF2 overexpression (Fig. 2b). As, among the KLF2-regulated miRNAs investigated, expression of the miR-143/145 cluster was most profoundly upregulated by KLF2 and shear stress, we further investigated the regulation and function of the miR-143/145 cluster in detail.

First, we confirmed its time-dependent expression after onset of shear stress (Fig. 2c), which yielded very similar results to those after KLF2 overexpression (Fig. 1c). Then, we found that the induction of miR-143/145 by shear stress is indeed KLF2 dependent by blocking KLF2 expression using *KLF2* short hairpin RNA (shRNA) lentivirus (Fig. 2d). Moreover, we investigated other activators of KLF2 such as HMG-CoA reductase inhibitors[26] (statins) and confirmed that mevastatin also significantly increased miR-143/145 expression in a time-dependent manner (Fig. 2e).

As, until now, miR-143/145 expression has been described only in SMCs (ref. 27,28), we assessed whether endothelial cells *in vivo* also express miR-143/145. Therefore, we analysed the expression of miR-143/145 using LacZ-reporter mice[16], which demonstrated that endothelial cells also express miR-143/145 *in vivo* (Fig. 2f). Statin treatment for 15 days further augmented endothelial miR-143/145 reporter gene expression in mice (Fig. 2f). To investigate whether KLF2 directly induces the transcription of the miR-143/145 cluster, we analysed the promoter of miR-143/145 *in silico* using rVISTA (ref. 29) and found one putative KLF-binding site in an evolutionarily conserved region ~6 kilobases upstream of the miR-143/145 cluster (Supplementary Fig. S1). Indeed, KLF2 binds to this part of the miR-143/145 promoter as demonstrated by chromatin immunoprecipitation (ChIP; Fig. 2g). Moreover, KLF2 and shear stress activate a miR-143/145-promoter driven luciferase reporter construct (Fig. 2h and Supplementary Fig. S2). Mutation of the conserved KLF2-binding site blocked the KLF2-dependent activation (Fig. 2h and Supplementary Fig. S2). Combined, these results show that overexpression or endogenous activation of KLF2 upregulates several miRNAs with known vasculoprotective effects, such as the miR-143/145 cluster.

KLF2 induces enrichment of miR-143/145 in extracellular vesicles

As endothelial-specific deletion of KLF2 leads to a profound impairment of SMC coverage *in vivo*[4] and the miR-143/145 cluster is known to modulate SMC phenotype, we reasoned that endothelial expression of KLF2 may influence SMC function in a paracrine manner. It has recently been shown that miRNAs can be transported by exosomes, ectosomes or apoptotic bodies[10,30,31]. We refer to this heterogeneous collection of vesicles, which are formed in distinct ways and vary in size, as extracellular vesicles[32,33]. To study the influence of KLF2 on the regulation of miRNAs in extracellular vesicles, we isolated vesicles from the supernatants of mock- and KLF2-transduced HUVECs. RNA was isolated from the vesicles and the remaining concentrated supernatant and miRNA expression levels were measured by real-time PCR (Fig. 3a). KLF2 transduction resulted in a ~30-fold enrichment of miR-143/145 in extracellular vesicles, whereas the remaining supernatant did not show an upregulation of miR-143/145 levels (Supplementary Fig. S3). As KLF2 overexpression induces cellular miR-143/145 by ~10-fold, the higher fold change in extracellular vesicles indicates a selective enrichment of miR-143/145 in vesicles induced by KLF2. Next, we examined whether shear-stress exposure, as the physiological stimulus for KLF2 expression, alters the miRNA composition of extracellular vesicles. Indeed, miR-143/145 expression was also highly enriched in microvesicles isolated from HUVECs that were exposed to shear stress, when compared with static controls (Fig. 3b).

To further characterize the miRNA-containing extracellular vesicles produced by KLF2-expressing HUVECs *in vitro*, we carried out electron microscopy on isolated vesicles (Fig. 3c,d). Quantitative analysis of electron micrographs showed that the diameter of the vesicles is heterogeneous, but most are between 60 and 130 nm (25th–75th percentile range) with a median size of 99 nm (Supplementary Fig. S4), indicating that most of the isolated vesicles comprise exosomes but not larger apoptotic bodies[33]. Next, extracellular vesicles were subjected to proteomic analysis by mass spectrometry (Supplementary Table S1), which showed that extracellular vesicles contain a number of proteins that were previously identified in microvesicles[34]. To distinguish between membrane-enclosed miRNAs, miRNAs that might be attached to the outside of these vesicles and miRNAs in protein complexes, we investigated the mode of protection or stabilization of miR-143/145 in the supernatant of HUVECs (Fig. 3e). We found that degradation of proteins alone (with proteinase K) before RNase treatment did not affect miR-143/145 levels (Fig. 3f). In contrast, treatment with phospholipid membrane disruptors such as Triton X-100, phospholipase or cyclodextrin before RNase treatment led to an almost complete degradation of miR-143/145, indicating that endothelial-derived miR-143/145 are preferentially released and stabilized by extracellular vesicles (Fig. 3f and Supplementary Fig. S5). Extracellular vesicles may derive from remnants of apoptotic cells ('apoptotic bodies') or actively exported exosomes. To determine which fraction of the vesicles is enriched for RNA, we carried out flow cytometry analysis using Annexin V as a marker for apoptotic bodies and the SYTO RNA-select dye as an indicator of RNA content. In both mock- and KLF2-transduced HUVECs, RNA was detected in both Annexin-V-positive and -negative vesicles (Fig. 3g). However, KLF2-transduction increased the number of Annexin-V-negative vesicles by twofold (Fig. 3g), indicating that KLF2-expressing HUVECs mainly release Annexin-V-negative vesicles containing RNA.

Extracellular vesicles transfer miRNAs from endothelial cells to SMCs

To determine whether endothelial-derived vesicles shed by KLF2-transduced cells can indeed control SMC functions in a paracrine manner, we employed a co-culture system of HUVECs with human aortic SMCs (HASMCs) in which the cells are separated by a membrane of 0.4 μm pore size to prevent direct cell contact or transfer of larger vesicles (Fig. 4a). First, we used HUVECs that were transduced with eGFP-overexpressing lentivirus or mock-control lentivirus and analysed the eGFP levels in SMCs after 3 days of co-culture by confocal microscopy. These studies showed that eGFP can indeed be transferred from the endothelial cells to SMCs during co-culture (Fig. 4a). Second, to delineate whether transfer of eGFP occurs in the form of protein or of mRNA encoding eGFP, we isolated RNA from the SMCs and carried out real-time PCR with primers specific for eGFP. Indeed, we were able to measure *eGFP* mRNA in SMCs in a time-dependent manner (Fig. 4b). Third, we transfected HUVECs with a miRNA that is naturally present only in *Caenorhabditis elegans* (cel-miR-39) and analysed cel-miR-39 expression levels in SMCs at different times after the start of the co-culture experiment (Fig. 4c) or after adding isolated vesicles to the SMCs (Fig. 4d), demonstrating that cel-miR-39 is transferred from HUVECs to SMCs. Consistently, pharmacological inhibition of sphingomyelinase, which was shown to inhibit exosome generation[35], attenuated the transfer of cel-miR-39 to SMCs, whereas inhibition of

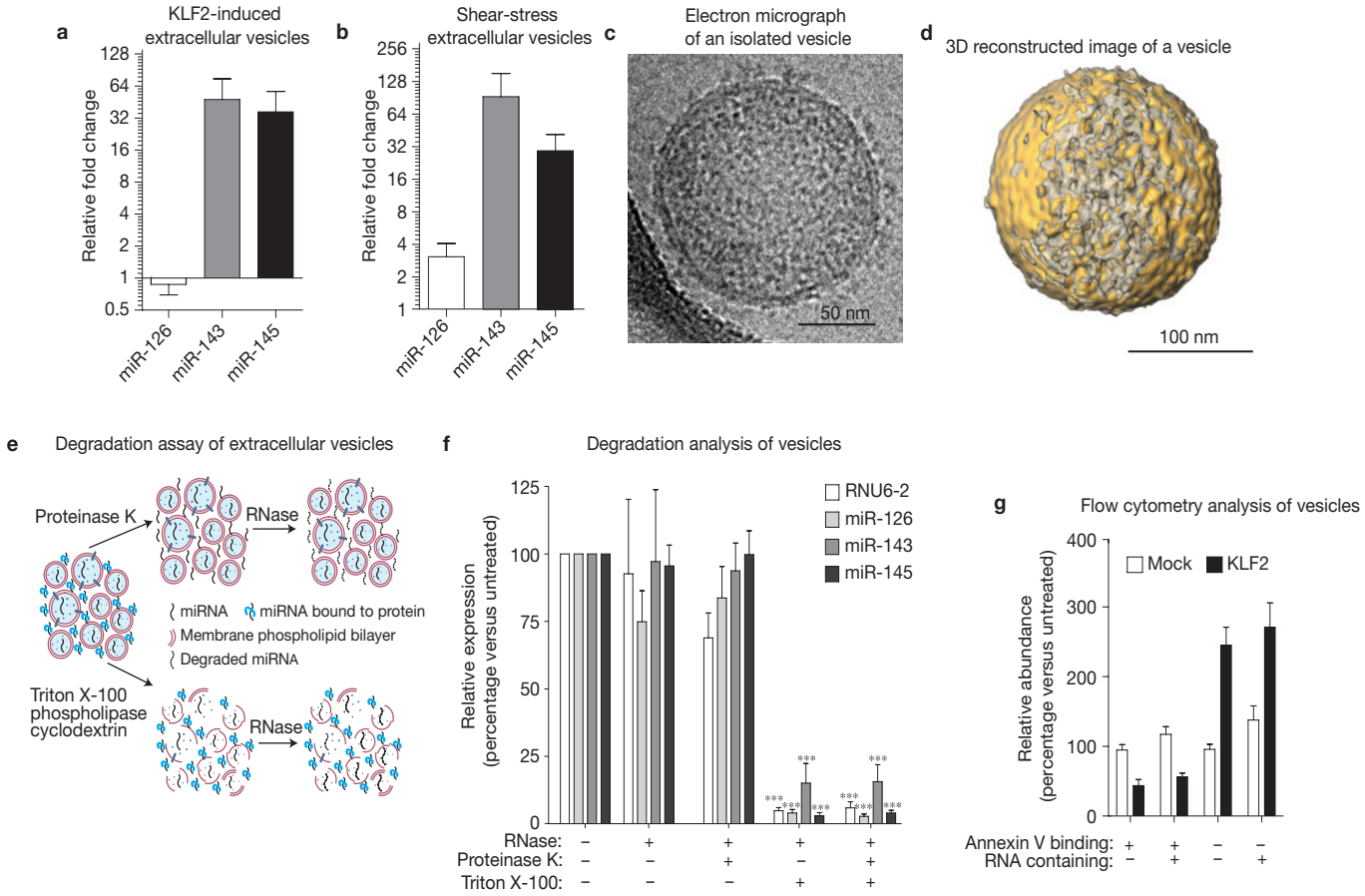

Figure 3 KLF2 and shear stress induce miR-143 and miR-145 content of extracellular vesicles. (**a**,**b**) Extracellular vesicles were isolated by centrifugation from supernatant of mock- and KLF2-transduced HUVECs (**a**; $n = 4$) or from HUVECs exposed to shear stress for 3 days and static controls (**b**; $n = 4$). (**c**) A 2D cryo-electron microscopy image of an isolated vesicle. The bilayer can be directly observed and the vesicle appears filled with electron-dense material. In the bottom left corner, the carbon support film can be seen. (**d**) A 3D isosurface rendering of a vesicle captured by cryo-electron tomography. Visualized in gold is the vesicle surface and in beige the content of the vesicle. (**e**) Schematic of the degradation assay used in **f**. (**f**) Extracellular vesicles of KLF2-transduced HUVECs were isolated and incubated with the indicated reagents for 45 min at 37 °C before isolating RNA and measuring the levels of the indicated miRNAs by real-time PCR ($n = 4$). (**g**) Flow cytometry analysis was carried out with isolated vesicles of untreated, mock- or KLF2-lentivirus-transduced HUVECs using Annexin V and SYTO RNA-select dye ($n = 5$). $^{***}P < 0.001$ when compared with untreated cells. Error bars indicate s.e.m.

apoptosis did not affect cel-miR-39 transfer (Fig. 4d), indicating that the transfer is mediated by actively formed vesicles, probably exosomes, rather than apoptotic bodies. These experiments demonstrate that mRNAs and miRNAs can be transferred from endothelial cells to SMCs.

Endothelial-derived miR-143/145-containing vesicles induce an atheroprotective SMC phenotype

SMCs normally contain high levels of miR-143/145, but these can be rapidly diminished by pro-atherogenic stimuli and injury[14,15]. To mimic this situation *in vitro*, we transfected SMCs with short interfering RNA (siRNA) against Drosha, a critical component of the miRNA biogenesis pathway, and used these SMCs in co-culture experiments with endothelial cells (Fig. 5a). Drosha knockdown led to a ~2-fold decrease in miR-143/145 levels in SMCs, which is similar to the atherosclerosis-mediated decrease *in vivo*[14]. Co-culture with mock-transduced endothelial cells did not affect the levels of miR-143/145 in SMCs, whereas co-culture with KLF2-transduced endothelial cells induced miR-143/145 levels almost to the levels as measured without Drosha knockdown (Fig. 5a). Then, we employed the co-culture system to study the effects of endothelial KLF2 on the expression of established miR-143/145-regulated genes such as *ELK1* (ref. 14), *KLF4* (ref. 36), *CAMK2d* (ref. 14), *SSH2* (ref. 36) and some predicted targets such as *PHACTR4* and *CFL1*. The presence of KLF2-transduced endothelial cells reduced the expression level of the established and predicted miR-143/145 target genes in SMCs, when compared with co-culture with control endothelial cells (Fig. 5b). To establish whether the miR-143/145 targets are indeed repressed by transfer of endothelial-cell-derived miR-143/145, we carried out the co-culture assay with lung endothelial cells isolated from either wild-type mice or *miR-143/145*$^{-/-}$ mice (Fig. 5c). Indeed, miR-143/145 targets were derepressed in the SMCs when miR-143/145-deficient endothelial cells were used for co-culture (Fig. 5c). These results illustrate that transfer of miR-143/145 from endothelial cells to SMCs is induced by endothelial KLF2 expression, which leads to an enhanced repression of miR-143/145 target genes and de-differentiation-associated gene expression.

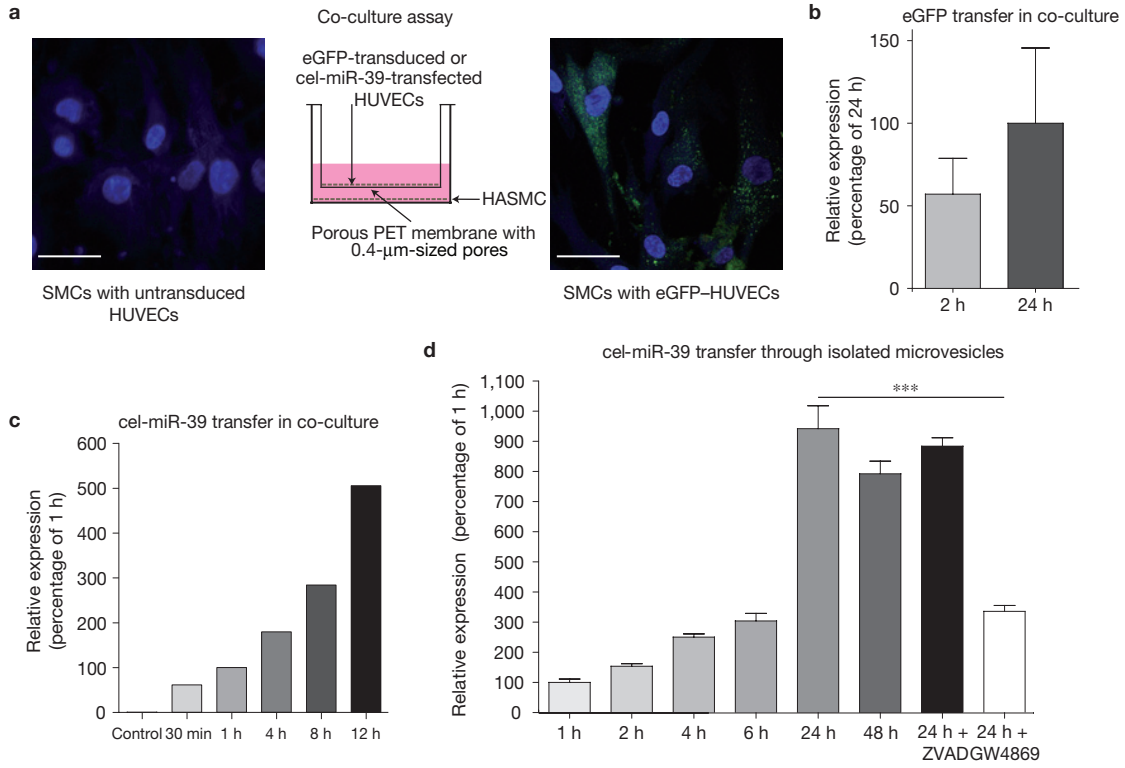

Figure 4 Endothelial cells transmit RNAs to SMCs *in vitro*. (**a**) An *in vitro* co-culture system was used where endothelial cells are seeded in the top compartment, which is separated by a porous membrane from SMCs that are cultured in the bottom compartment. HUVECs (top compartment) were transduced with mock- or eGFP-overexpression lentivirus and co-cultured with SMCs (bottom compartment). Scale bars, 50 μm. (**b**) eGFP expression in SMCs was analysed by confocal microscopy 24 h after initiation of co-culture or RNA was isolated and eGFP mRNA levels were measured by real-time PCR at 2 h and 24 h after the start of co-culture ($n = 3$). (**c**) HUVECs were transfected with cel-miR-39 or left untransfected and RNA was isolated from SMCs at different times after the start of the co-culture. cel-miR-39 levels in SMCs were measured by TaqMan-based real-time PCR (1 representative experiment out of 3 is shown). (**d**) SMCs were treated with vesicles isolated from cel-miR-39 transfected HUVECs for the indicated times 48 h, $n = 3$; all others, $n = 4$. HUVECs were also treated with either the apoptosis inhibitor Z-VAD-FMK (ZVAD) or the N-SMase inhibitor GW4869 after transfection with the cel-miR-39. $^{***}P < 0.001$. Error bars indicate s.e.m.

To determine whether extracellular vesicles that are released by KLF2-overexpressing endothelial cells can also protect against atherosclerosis formation, we injected vesicles from mock- or KLF2-transduced mouse endothelial cells intravenously twice weekly in $ApoE^{-/-}$ mice that were fed a high-fat diet over a period of six weeks. Mice were then killed and fatty lesions in the aorta were stained with Oil Red O (Fig. 5d). Quantification of lesion areas showed a significant reduction by ~2-fold for aortas of mice that were injected with vesicles from KLF2-overexpressing cells, whereas no change was observed for aortas of mice that were injected with vesicles from mock-transduced cells when compared with PBS-injected controls. *In vitro* inhibition of miR-143/145 expression in the vesicle-producing cells using locked nucleic acid (LNA)-modified antimirs abrogated the atheroprotective effect of the vesicles from KLF2-overexpressing cells (Fig. 5d). Thus, these results imply that endothelial vesicles generated from KLF2-expressing endothelial cells protect against atherosclerotic lesion formation in an experimental mouse model in a miR-143/145-dependent manner.

DISCUSSION

Taken together, these data demonstrate that KLF2 and atheroprotective flow regulate the expression of multiple miRNAs, most prominently miR-143 and miR-145. These miRNAs are enriched in extracellular vesicles and then transferred to SMCs, in which they reduce the level of expression of miR-143/145 targets to prevent SMC de-differentiation. It is well established that shear stress and its central transcriptional regulator KLF2 elicit atheroprotective properties to the endothelium by regulating the expression of atheroprotective genes[2]. The present study now provides further insights into the mechanisms underlying the endothelial protective effects by demonstrating that shear stress and KLF2 both regulate miRNAs known to modulate endothelial cell functions[22]. Whereas most of the miRNAs showed a similar response to shear stress and KLF2, some miRNAs were differentially expressed. This might be explained by the regulation of further signalling pathways that are affected by shear stress independently of KLF2 (ref. 37).

Our data also demonstrate that endothelial overexpression of KLF2 induces the expression and secretion of miRNAs that regulate SMC gene expression in co-culture systems. These findings may provide a rationale to explain the unexpected phenotype of endothelial-restricted KLF2-deficient mice, which demonstrate a severe impairment of SMC functions[4]. The cell-to-cell communication between endothelial cells and SMCs thereby seems to be mediated by phospholipid vesicles, which are enriched in miR-143/145. Several recent studies found that vesicle-mediated transfer of RNAs or miRNAs can mediate

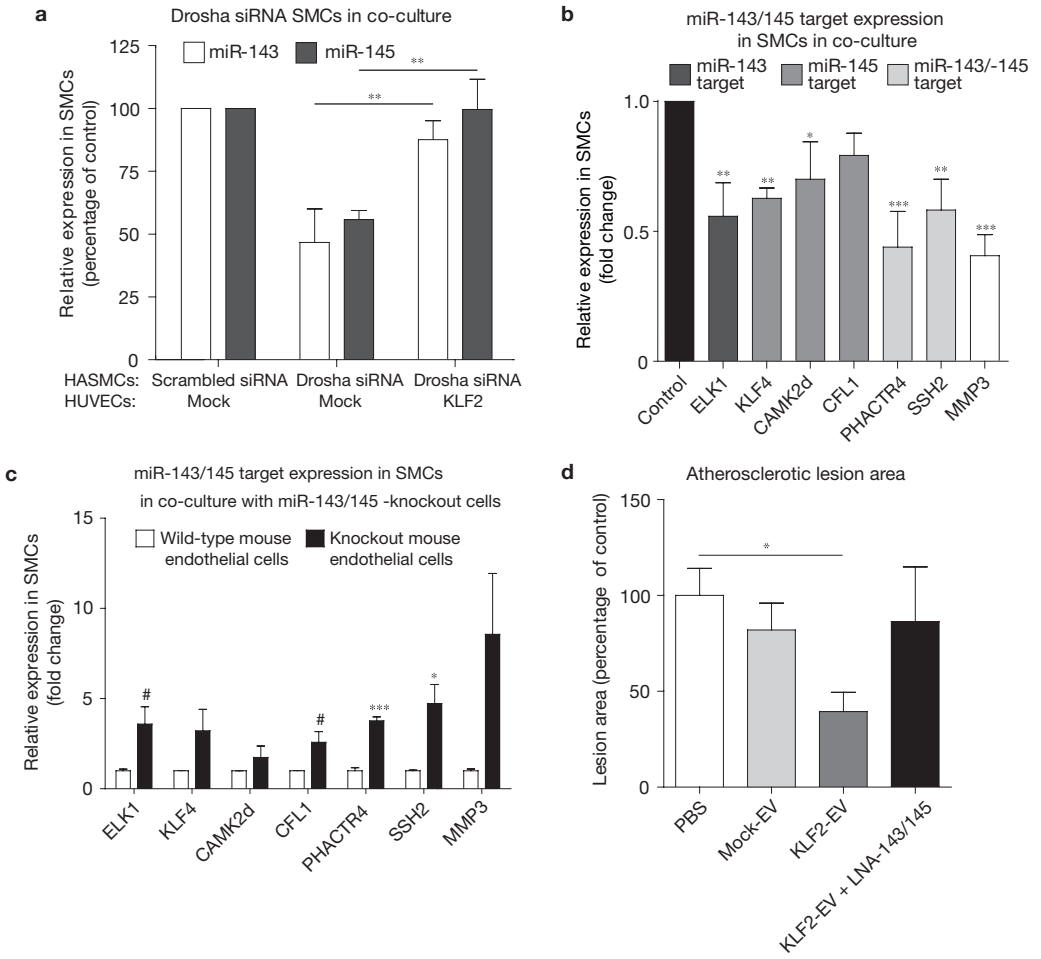

Figure 5 Endothelial cells transmit functional miR-143 and miR-145 to SMCs. (**a**) HUVECs were transduced with mock- or KLF2-overexpression lentivirus or left untreated and co-cultured with SMCs that were transfected with siRNA to silence Drosha expression or scrambled control siRNA. RNA was isolated from the SMCs at 24 h after the start of co-culture, and miR-143 (white) and miR-145 (grey) expression in SMCs was analysed by real-time PCR ($n=3$). (**b**) Mock- (black) or KLF2-transduced (grey and white) HUVECs were co-cultured with untreated SMCs for 24 h and the expression levels of ELK1 (miR-143 target), KLF4 and CAMK2d (miR-145 targets), CFL1, PHACTR4 and SSH2 (targets of both miR-143 and miR-145) and MMP3 (de-differentiation marker) were analysed by real-time PCR ($n=3$). (**c**) Mouse endothelial cells were isolated from wild-type or miR-143/145-deficient mice and were incubated with SMCs. miR-143/145 target mRNAs were measured by PCR ($n=3$). (**d**) $ApoE^{-/-}$ mice were fed a high-fat diet over a period of six weeks and injected with PBS, extracellular vesicles (EVs) from mock- or KLF2-transduced mouse endothelial cells, or extracellular vesicles from KLF2-transduced cells that were treated with LNAs to silence miR-143/145 expression twice a week. PBS, $n=6$; mock-EV, $n=7$; KLF2-EV, $n=11$; KLF2-EV + LNA, $n=5$. Mice were then killed and fatty lesions in the aorta were stained *en face* with Oil Red O and quantified. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ when compared with mock-transduced cells (**a**,**b**) or wild-type endothelial cells (**c**). Error bars indicate s.e.m.

signals between cells. Apoptotic bodies derived from endothelial cells were shown to contain miR-126, which controls endothelial cell signalling *in vitro* and provides atheroprotective effects *in vivo*[10]. Moreover, monocytes release miR-150 that is taken up by endothelial cells in which it regulates endothelial cell migration[21]. Other studies describe that stem cells can communicate through microvesicles to regulate differentiation[38,39]. So far, the exact nature of the vesicles, which carry miRNA, is not entirely known and may vary between the different studies. In our study, KLF2 increased the number of Annexin-V-negative vesicles and the release of miR-143/145-containing vesicles was not blocked by apoptosis inhibitors, indicating that the vesicles mediating the effect are distinct from the miR-126-enriched apoptotic bodies that were shown[10] to provide an atheroprotective effect in endothelial cells *in vitro* and *in vivo*. Consistently, extracellular vesicles derived from KLF2-transduced cells are not enriched in miR-126, but vesicle formation can be inhibited by blocking N-SMase, indicating that the miRNA-containing vesicles are mainly exosomes. Moreover, KLF2-transduced cells release vesicles with a selective enrichment of miRNAs, a finding that is not compatible with a passive general release of miRNAs during cell death. Selective packaging of miRNAs into vesicles may be crucial for the specificity of the biological functions of secreted miRNAs. Indeed, previous studies showed that the expression of miRNAs and RNAs in secreted vesicles does not necessarily reflect the intracellular expression of RNAs (refs 21,40). Therefore, we reason that extracellular vesicles from KLF2-transduced cells contain a specific combination of miRNAs, including miR-143/145, which mediate the biological properties. Indeed, vesicles from KLF2-transduced endothelial cells, but not from mock controls, reduce atherosclerotic lesion formation *in vivo*, in a miR-143/145-dependent manner. Therefore, KLF2-induced

vesicle-mediated transfer of miRNAs may provide a promising strategy to combat atherosclerosis. □

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturecellbiology

Note: Supplementary Information is available on the Nature Cell Biology website

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AUTHOR CONTRIBUTIONS

E.H. designed and carried out experiments, analysed data and wrote the manuscript. S.H. and K.T. carried out experiments and analysed data. X.Y. and M.M. carried out the proteomic analysis. M.P.S. and A.S.F. carried out electron microscopy. T. Braun, T. Boettger and A.J.G.H. provided essential materials. C.U. and A.M.Z. provided conceptual advice. R.A.B. and S.D. designed experiments, analysed data and wrote the manuscript. All authors have proofread the manuscript.

COMPETING FINANCIAL INTERESTS

E.H., R.A.B. and S.D. applied for a patent.

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METHODS

Mice. All animal experiments were conducted according to the principles of laboratory animal care as well as according to the German national laws. The studies have been approved by the local ethical committee (Regierungspräsidium Darmstadt, Hessen). LacZ-reporter mice with miR-143/145 deletion by insertion of an IRES–LacZ–Neo^{R} cassette were described previously[41]. *ApoE*$^{-/-}$ mice were purchased from Charles River and were kept on a high-fat diet for six weeks. During this time period, these mice were injected intravenously (tail vein) twice a week with PBS (200 µl), or extracellular vesicles derived from mock- or KLF2-transduced mouse endothelial cells or vesicles from KLF2-transduced mouse endothelial cells that were treated with LNA-143/145 (800 nM final concentration, Exiqon) suspended in 200 µl PBS.

Cell culture, transfection and luciferase assay. HUVECs were purchased from Lonza and cultured in EBM (Lonza) supplemented with 10% FBS and EGM-SingleQuots (Lonza). Confluent monolayers were grown and used for the experiments. HASMCs were purchased from Lonza and cultured in SmBM (Lonza) supplemented with 5% FBS and SmGM-2-SingleQuots (Lonza). HASMCs were passaged after reaching 80% confluency. Mouse endothelial cells were provided by E. Dejana (Department of Biomolecular Sciences and Biotecnologies, School of Sciences, University of Milan, Italy). These H5V mouse endothelial cells were cultured in DMEM (high glucose, no HEPES) supplemented with 10% FCS, 1% sodium pyruvate, 1% glutamine and 1% penicillin and streptomycin.

Lipofectamine RNAiMAX (Invitrogen) was used to transfect HASMCs with cel-miR-39 (10 nM or 100 nM final concentration) and with siRNA targeting Drosha[42] (60 nM final concentration) according to the manufacturer's instructions. GenJet *in vitro* DNA transfection kit was used to transfect HUVECs with a luciferase construct containing the cloned 1,669-base-pair (bp)-long region (from 750 bp upstream to 919 bp downstream of the putative KLF-binding site, using the primers: forward 5′-ACAAGGTACCTGTCCCCAGGCACAGCCCTT-3′, reverse 5′-ACAAAAGCTTTCGCCTGGACAACCTGGGCA-3′) of the miR-143/145 promoter (−6,750 to −5,081) in front of the luciferase gene as well as the mutated version of the KLF2-binding site at position −5,992 to −6,004 (from agacCCGCCCcca to agacATTAAAcca, using Stratagen's quick mutagenesis kit and the following primers: forward 5′-CATTTCCGAGCTGTTCCCAGACATTAAACCACTTCCCCCCTCACCCTCCC-3′, reverse 5′-GGGAGGGTGAGGGGGGAAGTGGTTTAATGTCTGGGAACAGC-TCGGAAATG-3′). Luciferase assays were carried out with the Dual-Luciferase reporter assay system (Promega).

Shear stress. Twenty-four hours after the initial plating, confluent HUVEC monolayers were exposed to a well-defined laminar flow with a shear stress of 20 dynes cm^{-2} for 72 h in µ-Slides I$^{0.4}$ Luer (ibidi) maintained and controlled by an ibidi perfusion system. Control cells were seeded into µ-Slides I with reservoirs of culture medium for long-term cultivation without flow.

Lentiviral particle generation and transduction. Long-term lentiviral overexpression of KLF2 was carried out as previously described[43].

Real-time PCR analysis. TaqMan miRNA assays (Applied Biosystems) were used to measure the levels of mature miRNAs on a StepOne-Plus machine (Applied Biosystems). RNU6-2 was used as a normalization control in all miRNA measurements. The mRNA levels were determined using real-time analysis with SYBR Green (Applied Biosystems). The ribosomal protein P0 was used as a normalization control for mRNA. Primers used: P0 forward 5′-TCGACAATGGCAGCATCTAC-3′, reverse 5′-ATCCGTCTCCACAGACAAGG-3′; KLF2 forward 5′-CAAGACCTACACCAAGAGTTCG-3′, reverse 5′-CATGTGCCG-TTTCATGTGC-3′; eGFP forward 5′-CTGTTCACCGGGGTGGTGCC-3′, reverse 5′-CAGCTTGCCGGTGGTGCAGA-3′; ELK1 forward 5′-CTGAGTCCCATTGCG-CCCCG-3′, reverse 5′-GAGCACCACGGGGGTCGAGA-3′; KLF4 forward 5′-CCGGAAAAGGACCGCCACCC-3′, reverse 5′-TCCACAGCCGTCCCAGTCACA-3′; CAMK2d forward 5′-AGGGGGCATTCTCAGTGGTGA-3′, reverse 5′-AGGGTGCTTCAAAAGACGGCAGA-3′; CFL1 forward 5′-TCGACGACCCCTAC-GCCACC-3′, reverse 5′-TAAGGGGCGCAGACTCGGGG-3′; PHACTR4 forward 5′-CCCTGCCAAGCAGCCCCCTA-3′, reverse 5′-GGTGGTAAGGGTGG-GGACGGT-3′; SSH2 forward 5′-ACGGAACAAGCATGCAGGCGA-3′, reverse 5′-TGAAACCACTACCATATAGCGTGTTCG-3′; MMP3 forward 5′-GGAGCCAGGCTTTCCCAAGCA-3′, reverse 5′-TGCATTTGGGTCAAACTCCA-ACTGTG-3′.

Extracellular vesicle isolation and flow cytometry. Vesicles were isolated using a multi-step centrifugation procedure as described previously[44]. Briefly, confluent monolayers of HUVECs were grown and the medium was refreshed. After cultivation for 72 h, the supernatant was collected and pre-cleared by centrifugation at 4,000*g* for 10 min at 4 °C to remove cell debris. To pellet the extracellular vesicles (size, <1 µm), the supernatant was centrifuged at 20,500*g* for 1 h at 4 °C. The supernatant was removed and discarded, and the pelleted vesicles were washed with ice-cold PBS and pelleted again by centrifugation at 20,500*g* for 1 h at 4 °C. Finally, the supernatant was removed and discarded and the pelleted vesicles were resuspended in 700 µl Qiazol (Qiagen) for RNA isolation using the miRNeasy kit (Qiagen) following the instructions provided by the manufacturer or in PBS for other experiments. For flow cytometry analysis, isolated extracellular vesicles were stained with an Annexin V antibody and RNA select SYTO green fluorescent stain (Molecular Probes) and analysed by FACS with Cell Quest software (BD Biosciences) on a FACS Canto II (BD Biosciences).

Confocal microscopy. Confocal microscopy was done on a Zeiss laser scanning microscope LSM-510 (Zeiss) using Plan-Neofluar objectives (×20/0.5 or ×40/1.3 Oil).

ChIP assay. The ChIP assay was done using anti-V5 tag antibody (ab9116, Abcam, diluted 1:200) and the Magna ChIP-G kit (Millipore) according to the manufacturer's instructions. Enrichment analysis was done using real-time PCR with the following primers for the putative KLF2-binding site: forward 5′-GGGGTGAGGGAGGTTCCAGGG-3′, reverse 5′-ATGGGAGAGGCTGCCGGAGG-3′.

Cryo-electron microscopy. A 3.5 µl aliquot of the sample was pipetted onto a Lacey grid (Plano) and plunge-frozen using a Vitrobot (FEI) with a 2° offset and 3.5 s blotting time. Gold particles of 10 nm in size and coated in protein A were used as fiducial markers. Images were recorded on a PolaraTecnai 300 kV transmission electron microscope (FEI) with a Gatan GIF 2002 post-column energy filter, and a 2k Multiscan CCD (charge-coupled device) camera (Gatan). The two-dimensional (2D) image shown was recorded at a nominal magnification of ×50,000 at a defocus of ∼−2 µm. Electron tomograms were recorded using the SerialEM software[45]. All data collection was carried out at 300 kV, with the energy filter operated in the zero-loss mode. Tilt series were recorded from −60° to +60° with an angular increment of 2° at a defocus of ∼−6 µm. The tilt series were obtained at a nominal magnification of ×22,500 (pixel size 6 Å), with a total dose of ∼100 electrons Å^{-2}. Three-dimensional reconstructions were obtained using weighted back projection. The software package Amira (Visage Imaging) was used for visualization of the tomographic data.

Proteomic analysis. Proteomic analysis of isolated extracellular vesicles was carried out by liquid chromatography with tandem mass spectrometry and difference in-gel electrophoresis as previously described[46].

Co-culture experiments. Well inserts for 6-well plates with a 0.4 µm pore-sized filter were purchased from Greiner and used following the manufacturer's instructions. HUVECs (200,000) were seeded into the well inserts and cultured in complete EBM 24 h before the co-culture experiments. HASMCs (100,000) were seeded into 6-well plates and cultured in complete SmBM 24 h before the co-culture experiment. Before starting the co-culture experiments both HUVECs and HASMCs were washed with PBS. All co-culture experiments were done in complete EBM. In the case of Drosha-siRNA-transfected HASMCs, the cells were transfected with a final concentration of 60 nM siRNA targeting Drosha the day before the co-culture experiment using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

Statistical analysis. All statistical calculations were carried out using Graphpad Prism 5. Student's *t*-tests were used when comparing two conditions and a one-way ANOVA with Bonferroni correction was used for multiple comparisons. Probability values of less than 0.05 were considered significant.

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Figure S1 KLF is predicted to bind in the miR-143/-145 promoter region. Illustration of the genomic region upstream of miR-143/145 and its evolutionary conservation. Examples of predicted conserved transcription factor binding sites are indicated below the conserved regions. The base genome is human.

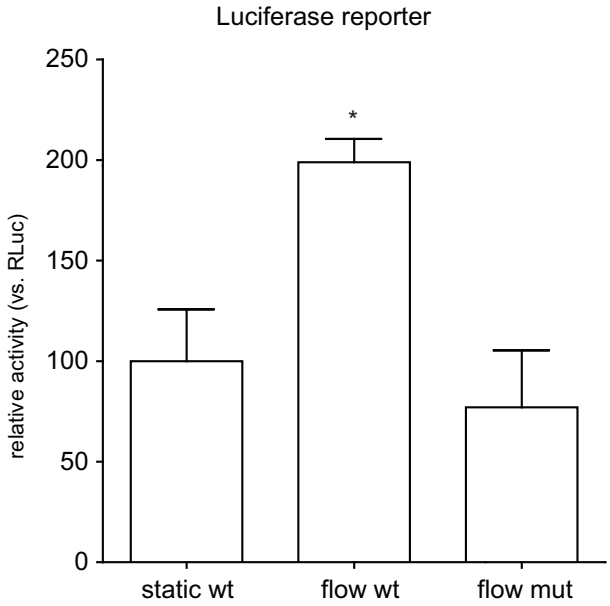

Figure S2 Activity of a luciferase reporter construct containing the miR-143/145 promoter region with the KLF2 binding site is induced by flow. Luciferase reporter constructs containing either the wild type or the mutated KLF-binding site were transfected into HUVECs that were exposed to 48h shear stress (20 dyne/cm2) or left under static conditions. Firefly luciferase activity was measured and normalized on renilla luciferase using the dual luciferase assay kit (Promega). N=3, *=P<0.05. Error bars indicate SEM.

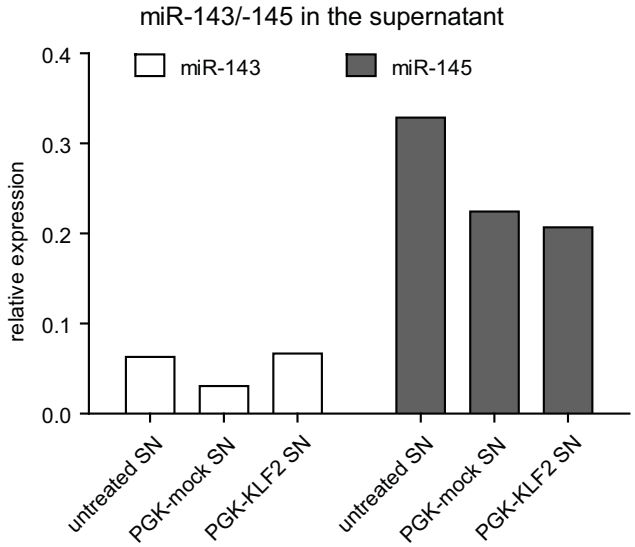

Figure S3 MiR-143/145 is not enriched in the supernatant of KLF2-transduced cells after removal of extracellular vesicles by centrifugation. Vesicles were isolated by ultracentrifugation from supernatants of mock- and KLF2-transduced HUVECs. RNA was isolated from the remaining supernatant after ultracentrifugation and miR-143 (white bars) and miR-145 (grey bars) were measured by real-time PCR. One representative experiment is shown.

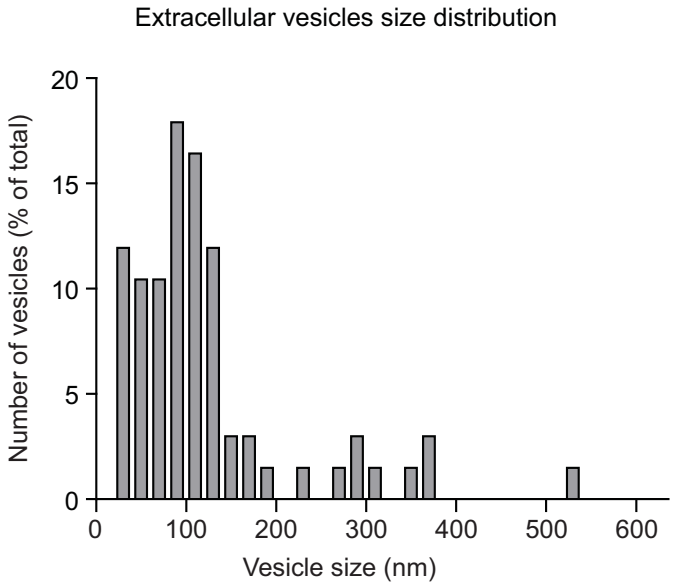

Figure S4 Analysis of size distribution of isolated extracellular vesicles. Vesicles of KLF2-transduced HUVECs were isolated and analysed using cryo-EM. The sizes of individual vesicles were measured and plotted in bins of 20nm.

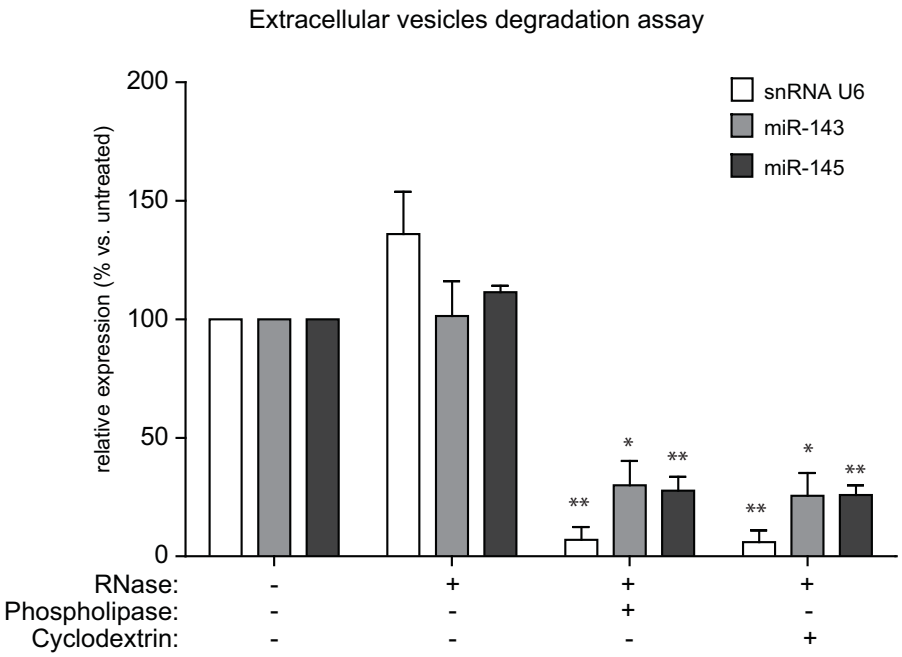

Figure S5 MiR-143/145 are protected from degradation by RNase by a phosphobilayer-based membrane. Vesicles of KLF2-transduced HUVECs were isolated and incubated with the indicated reagents before isolating RNA and measuring levels of the indicated miRNAs by real-time PCR. N=3, *=P<0.05, **=P<0.01. Error bars indicate SEM.