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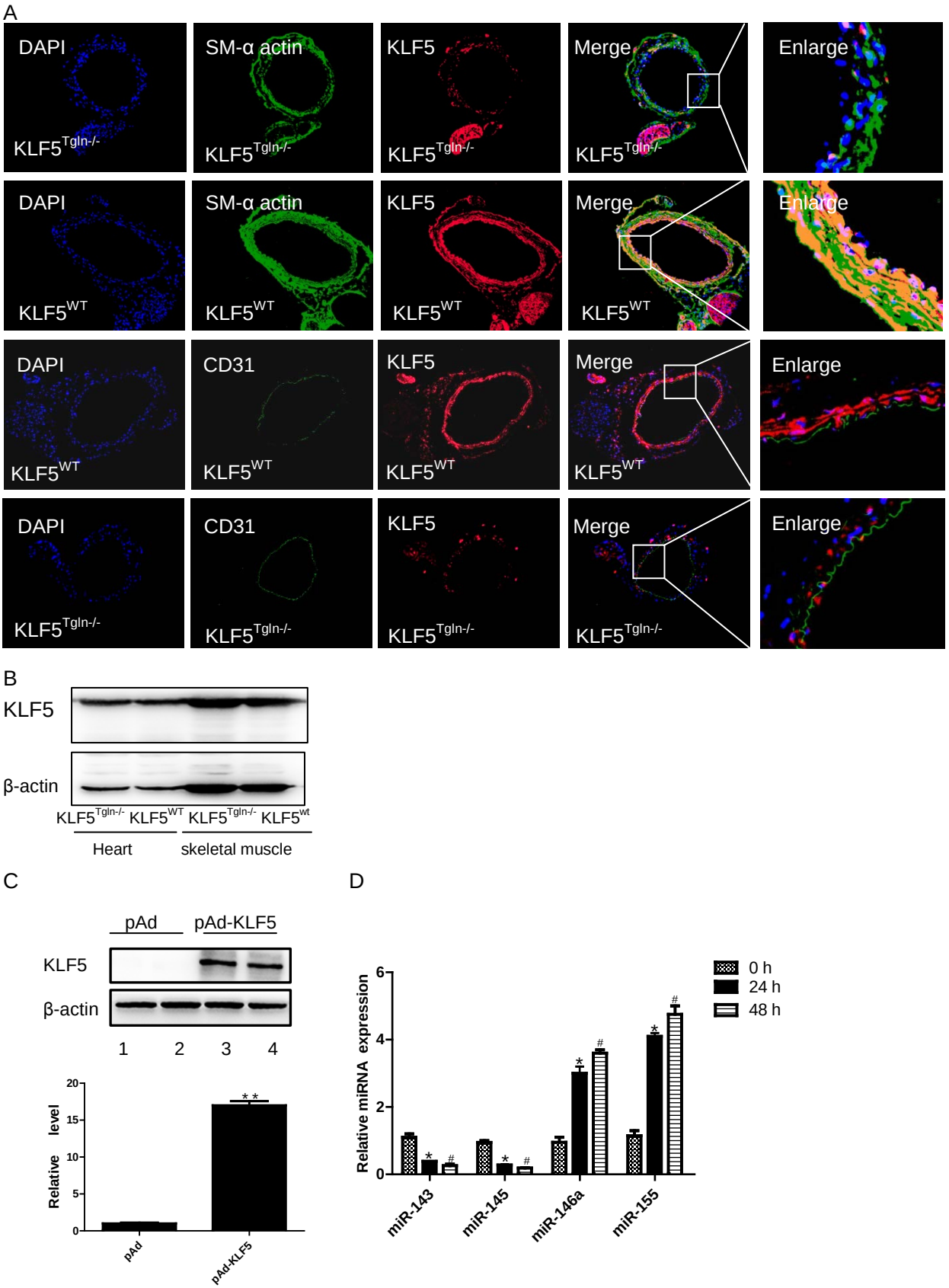
Supplemental Information

Exosome-Mediated miR-155 Transfer from Smooth Muscle Cells to Endothelial Cells Induces Endothelial Injury and Promotes Atherosclerosis

Bin Zheng, Wei-na Yin, Toru Suzuki, Xin-hua Zhang, Yu Zhang, Li-li Song, Li-shuang Jin, Hong Zhan, Hong Zhang, Jin-shui Li, and Jin-kun Wen

Supplemental material



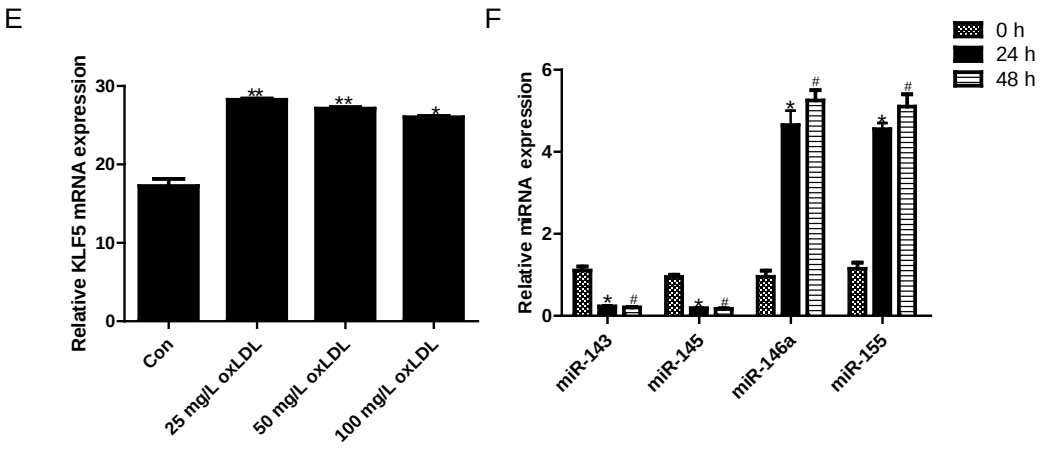
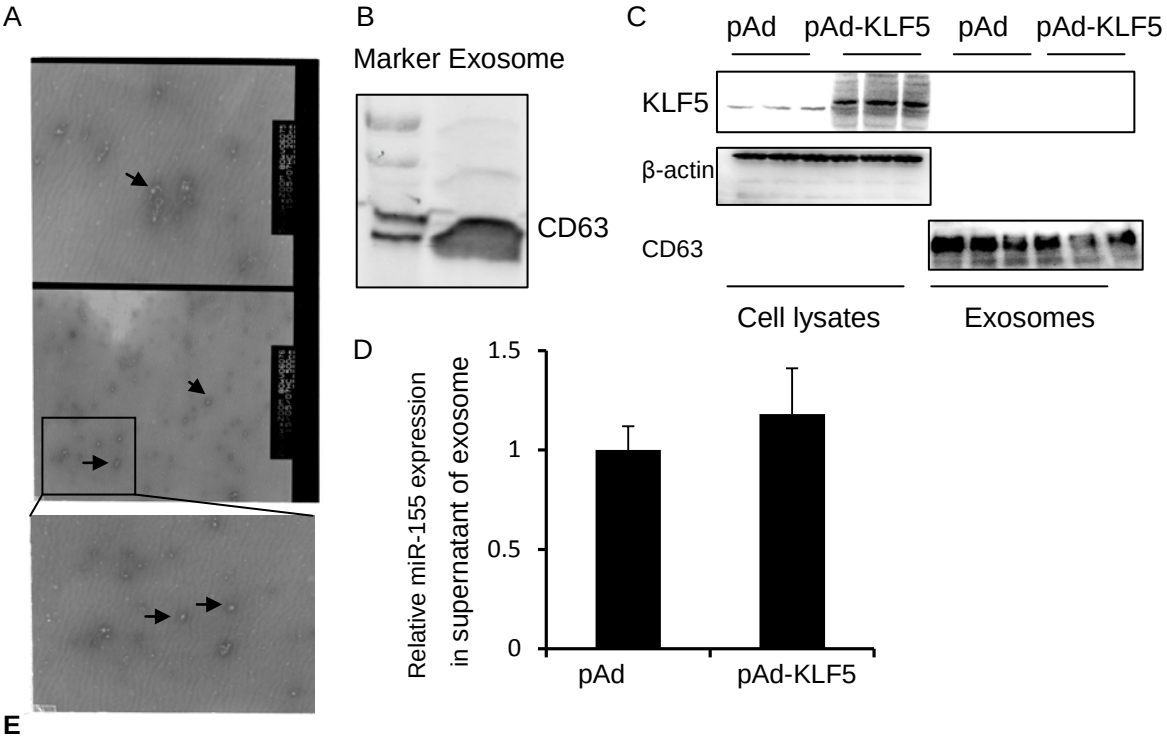


Figure 1. KLF5 regulates expression of miRNAs, particularly miR-155

A, Representative photographs of immunofluorescence staining with KLF5 staining, CD 31 and SM α-actin in carotid artery of $KLF5^{Tgln-/-}$ mice and $KLF5^{WT}$ mice. Magnification, ×100 (KLF5, SM α-actin staining and CD31 staining). B, KLF5 expression was detected by Western Blot (n=3) in different tissues of KLF5 in $KLF5^{Tgln-/-}$ (VSMC-specific knockout of KLF5) mice and $KLF5^{WT}$ mice. C, HASMCs were transfected with pAd or pAd-KLF5 for 48 h. KLF5 expression was detected by Western blotting (n=3). **P<0.01 vs pAd group. D, HASMCs were transfected with pAd or pAd-KLF5 for different times. miRNA expression was detected by real-time PCR (n=6). *P<0.05 vs 0 h group. $^{\#}$P<0.05 vs 0 h group. E, HASMCs were treated with oxLDL for 24 h. KLF5 mRNA expression was detected by Real-time PCR(n=6). *P<0.05 vs con group. **P<0.01 vs con group. F, HASMCs were treated with oxLDL for different times. miRNA expression was detected by real-time PCR (n=6). *P<0.05 vs 0 h group. $^{\#}$P<0.05 vs 0 h group.

Figure 2



E

-1789agcaatttcctgtttctattaaagtgcattatacaaacttgtcattctggggatgaaaggt**caccc**tagaattgcctatgg
cgaac(mutated)
gcaatttcttatagttcaacctagaatgagaaatgggaaattcagaaaggcattgtaggcatctgtaaccagcagagggac
gtgccccacctgg**gtggg**gaccatgcatccttgccacatgccccactgcacaacttccccagctcctcaacgtca-1552
gcaag(mutated)

F

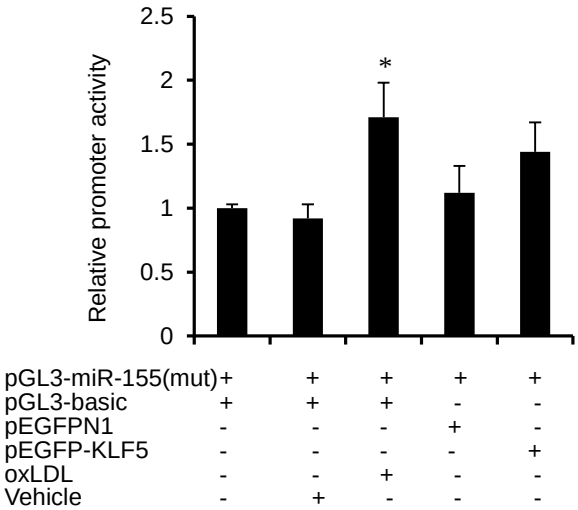


Figure 2. KLF5 induces enrichment of miR-155 in extracellular vesicles

A, Electron microscopy image of an isolated vesicle. B, Exosomes was extracted from the medium of VSMCs by ultracentrifuge. CD63 expression in exosomes was detected by Western Blotting (n=6). C, Exosomes were extracted from the medium of

VSMCs by ultracentrifuge. Cells were lysed and cell lysate was harvested. 20 μg protein was used to detected the expression of KLF5 and CD63 in exosomes by Western Blotting (n=3). D, miR-155 levels were detected by real-time PCR (n=6) in the remaining supernatant of exosomes from the medium of VSMCs. *P<0.05 vs pAd group. E, Part of ~2500 bp upstream sequence of pri-miR-155. KLF5 binding site was marked with underline (green). Mutated sites of KLF5 binding site was marked with underline (red). F, Reporter gene assay using miR-155 mutation promoter (n=6). VSMCs were transfected with indicated vectors for 48 h, luciferase activities were measured with the Dual-Luciferase Reporter System (Promega). Results are means±S.E.M. for six separate transfection assays with duplicate plates. *P <0.05 vs vehicle group.

Figure 3

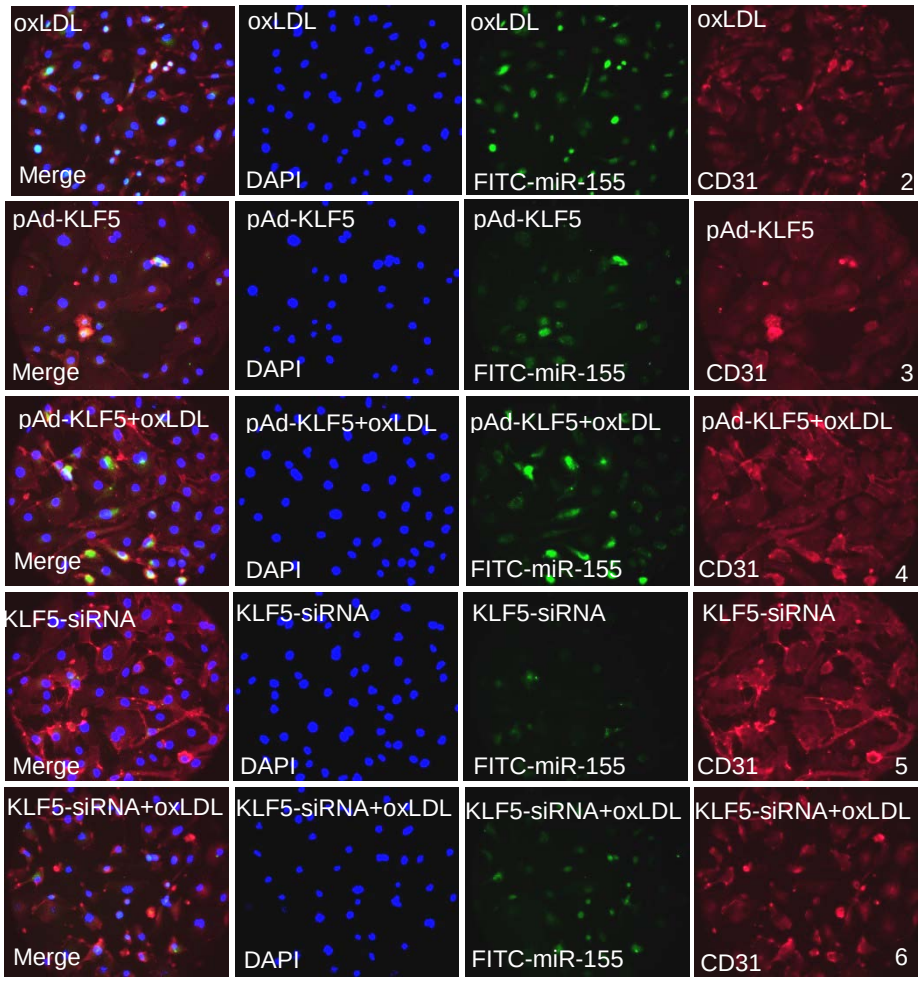
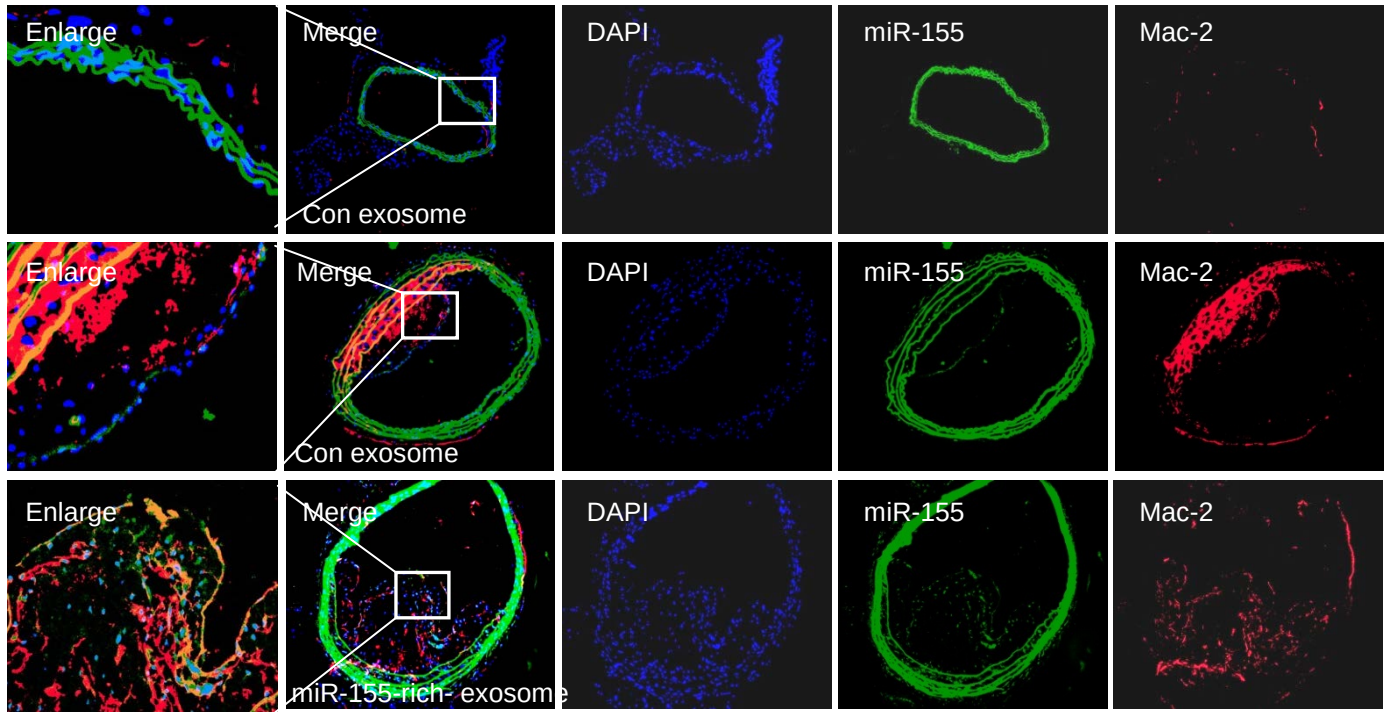


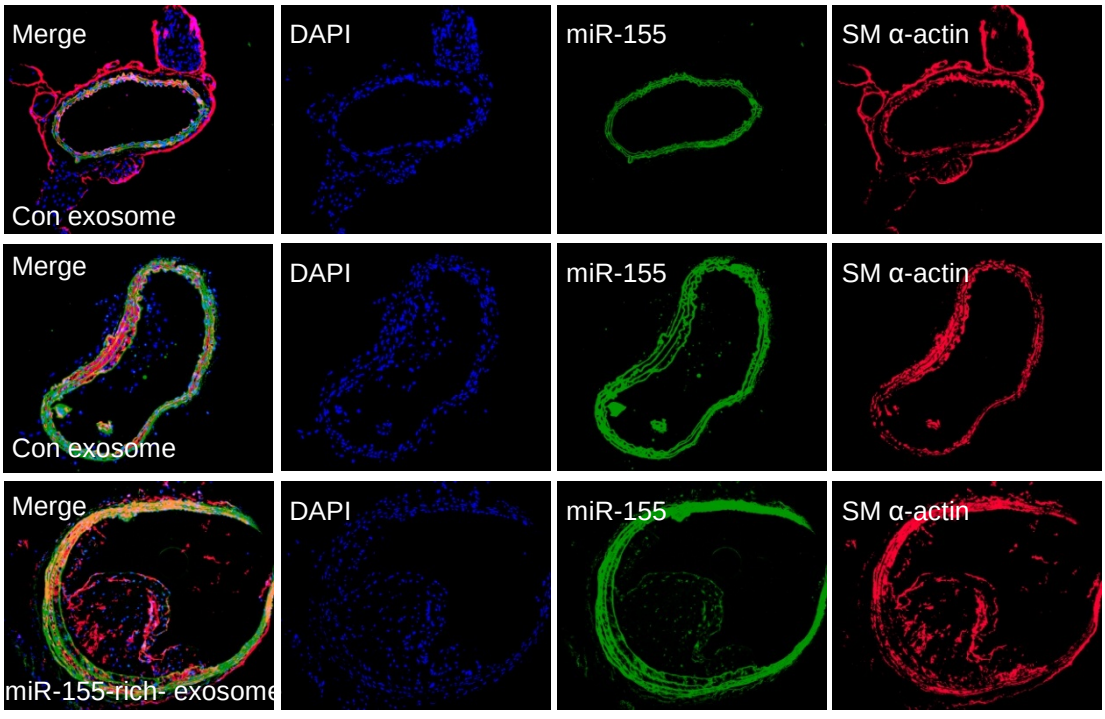
Figure 3 KLF5 affects the transmission of miR-155 between HASMCs and endothelial cells

An in vitro co-culture system was used where HASMCs are seeded in the top compartment, which is separated by a porous membrane from endothelial cells that are cultured in the bottom compartment. HASMCs (top compartment) were transfected with FITC-miR-155 together with indicated virus or siRNA and treated with or without oxLDL and co-cultured with endothelial cells (bottom compartment). FITC-miR-155 (green) and CD 31 expression (red) in endothelial cells was analyzed by confocal microscopy.

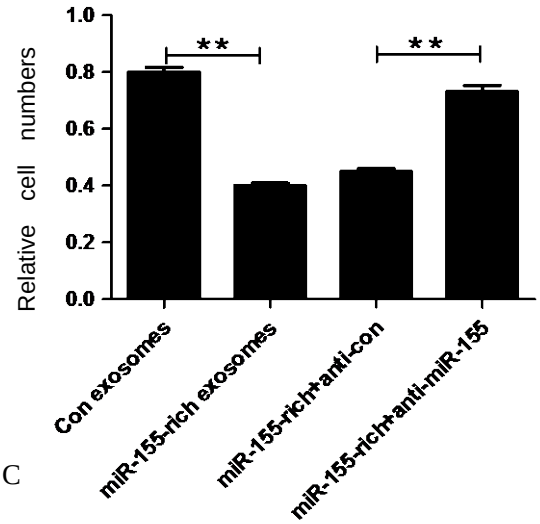
Figure 4

A

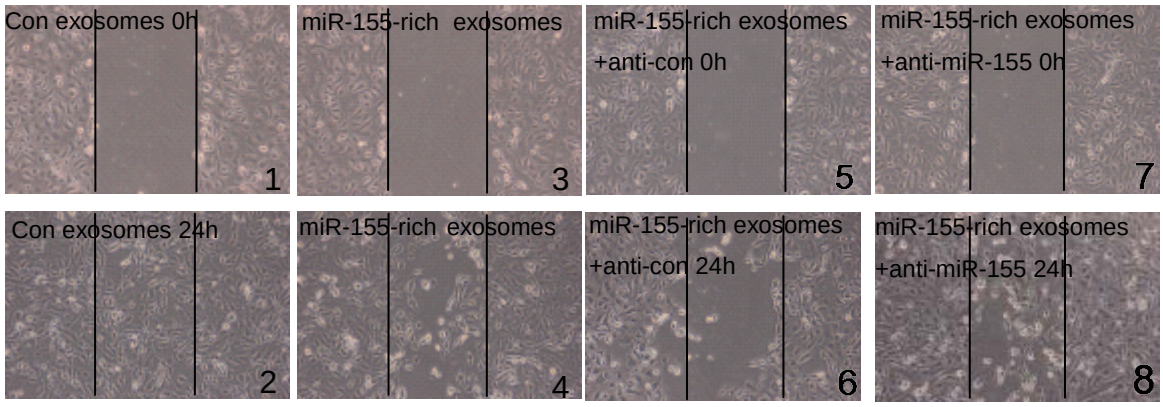

Normal feeding
HFD feeding
HFD feeding
Enlarge
Merge
DAPI
miR-155
Mac-2
Con exosome
miR-155-rich- exosome

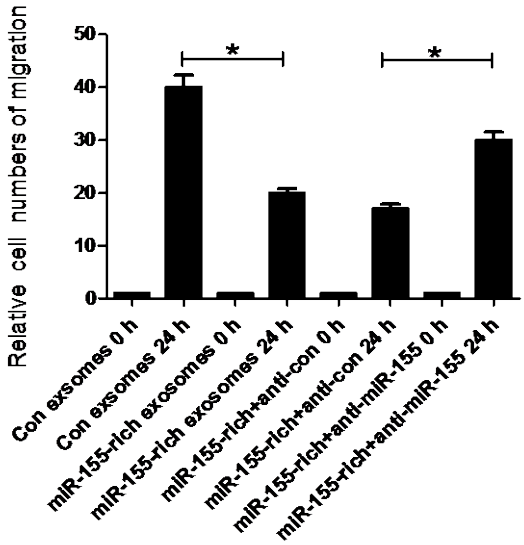

Normal feeding
HFD feeding
HFD feeding
Merge
DAPI
miR-155
SM α-actin
Con exosome
miR-155-rich- exosome

B

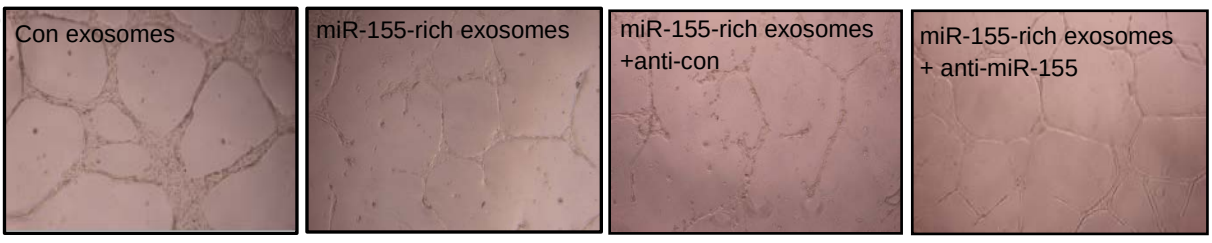

Relative cell numbers
1.0
0.8
0.6
0.4
0.2
0.0
**
**
Con exosomes
miR-155-rich exosomes
miR-155-rich+anti-con
miR-155-rich+anti-miR-155

C


Con exosomes 0h
1
miR-155-rich exosomes
3
miR-155-rich exosomes
+anti-con 0h
5
miR-155-rich exosomes
+anti-miR-155 0h
7
Con exosomes 24h
2
miR-155-rich exosomes
4
miR-155-rich exosomes
+anti-con 24h
6
miR-155-rich exosomes
+anti-miR-155 24h
8


Relative cell numbers of migration
50
40
30
20
10
0
*
*
Con exsomes 0 h
Con exsomes 24 h
miR-155-rich exosomes 0 h
miR-155-rich exosomes 24 h
miR-155-rich+anti-con 0 h
miR-155-rich+anti-con 24 h
miR-155-rich+anti-miR-155 0 h
miR-155-rich+anti-miR-155 24 h

D


Con exosomes
miR-155-rich exosomes
miR-155-rich exosomes
+anti-con
miR-155-rich exosomes
+ anti-miR-155

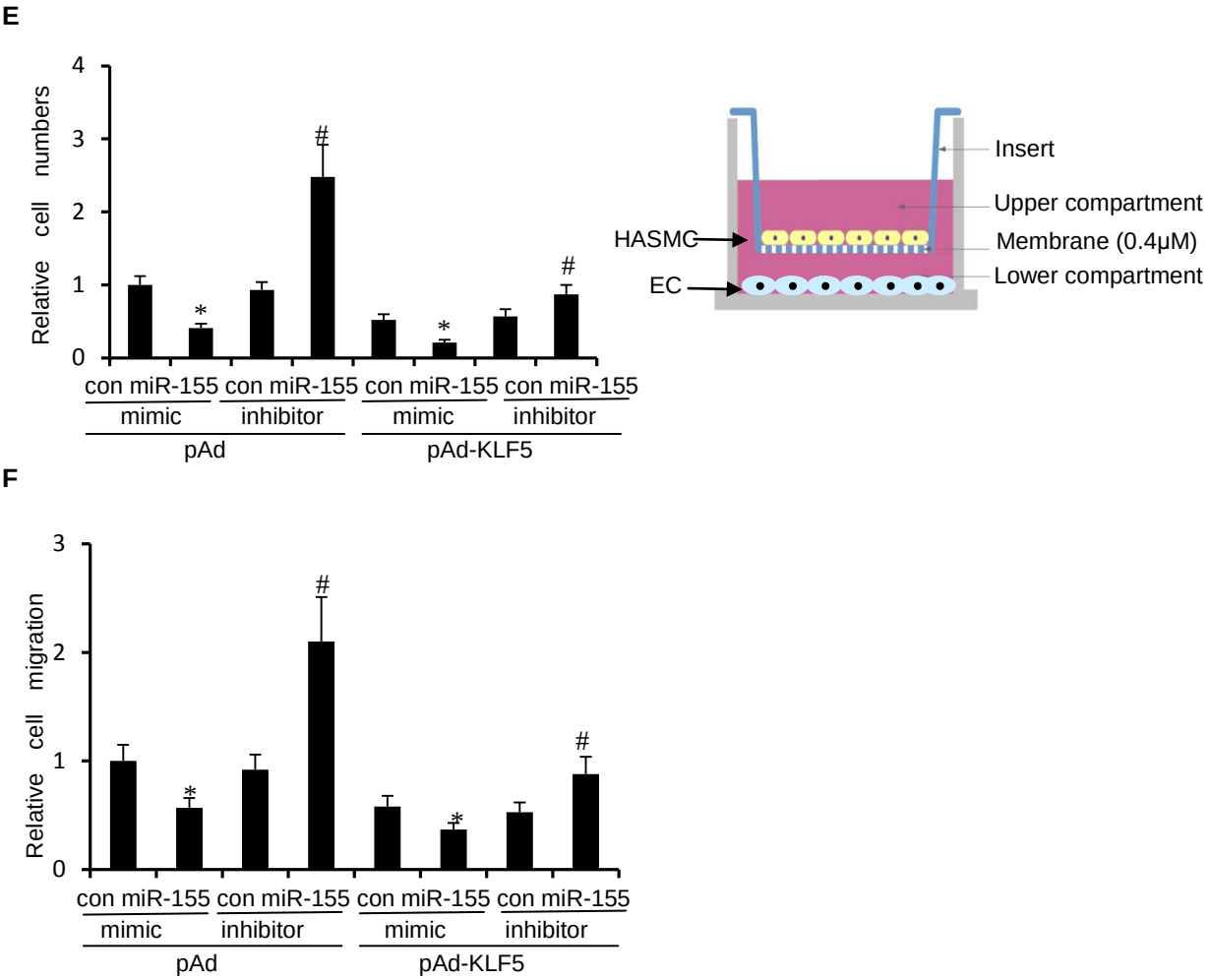


Figure 4. Vascular-derived miR-155 is transported to endothelial cells, leading to cellular dysfunction

A, Exosomes secreted by pAd- or pAd-KLF5-transduced HASMCs were intravenously injected into the tail vein of apoE$^{-/-}$ mice fed with HFD (n = 6) once a week. Representative photographs of miR-155 in situ hybridization (green) and mac-2 staining (red), SM α-actin staining (red), respectively. B, MTS assay was used to detect the proliferation of endothelial cells. **P <0.01 vs con exosome or miR-155-rich+anti-con group. C, Wound-healing assay was used to detect the migration of endothelial cells. *P <0.05 vs con exosome or miR-155-rich+anti-con group. D, Tube formation assay was used to detect the function of endothelial cells. E, An in vitro co-culture system was used where HASMCs were seeded in the top compartment, which is separated by a porous membrane from endothelial cells that are cultured in the bottom compartment. HASMCs (top compartment) were transfected with indicated mimic or inhibitor together with indicated virus and co-cultured with endothelial cells (bottom compartment). MTS assay was used to detect the proliferation of endothelial cells. *P <0.05 vs con mimic group or con mimic+pAd-KLF5 group, respectively. $^{\#}$P <0.05 vs anti-con group or anti-con+pAd-KLF5 group, respectively. F, Wound-healing assay was used to detect the migration of endothelial cells. *P <0.05 vs con mimic group or con

mimic+pAd-KLF5 group, respectively. $^{\#}P < 0.05$ vs anti-con group or anti-con+pAd-KLF5 group, respectively.

Figure 5

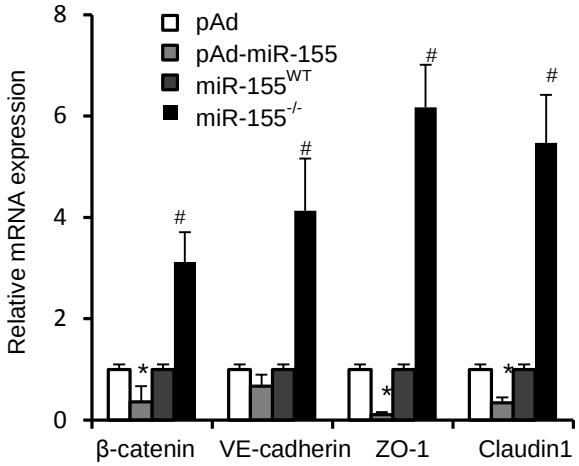
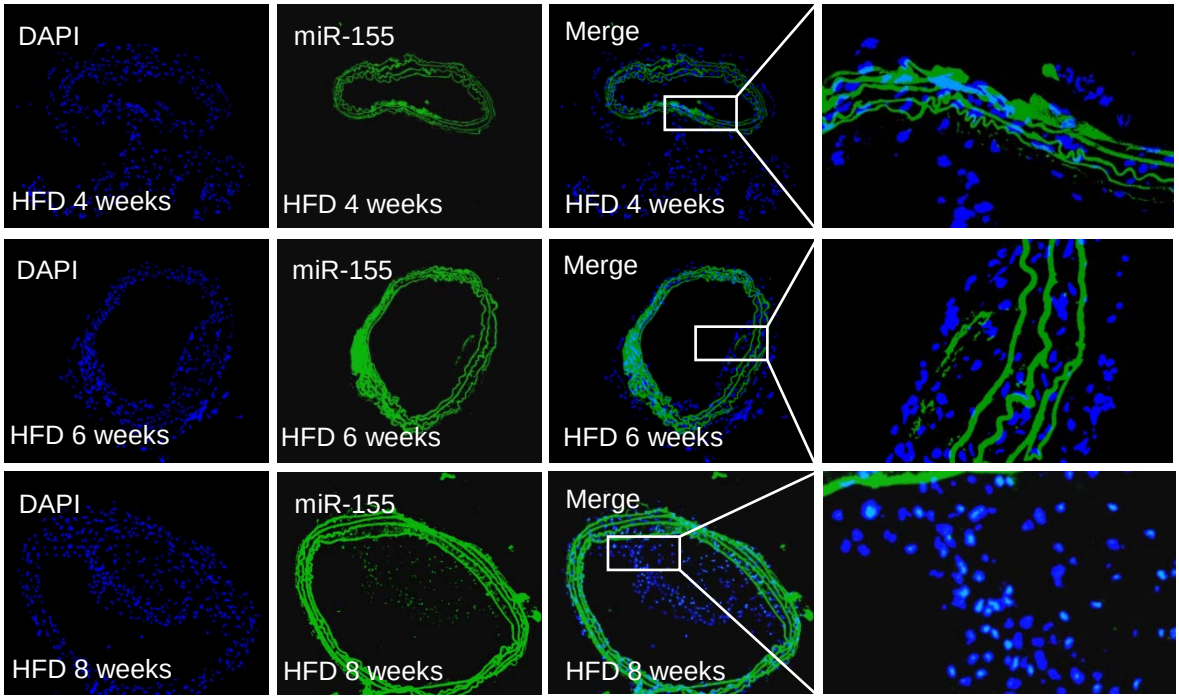


Figure 5. miR-155 regulates endothelial barrier function

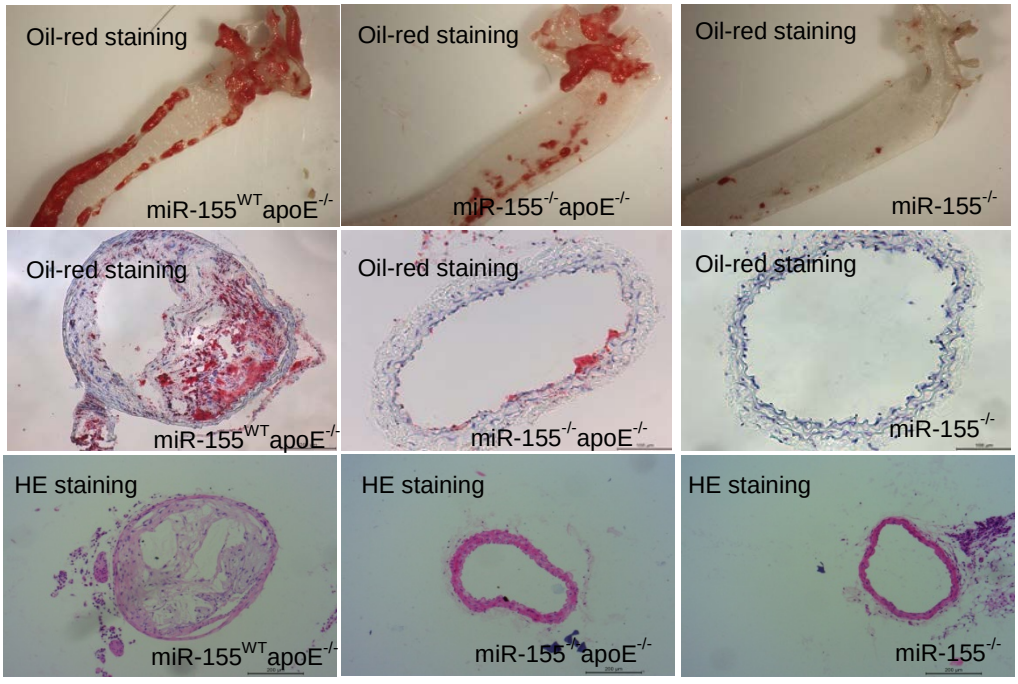
pAd or pAd-KLF5 were intravenously injected into the tail vein of C57BL/6 mice (n = 6) once a week. Total miRNA from aortic tissue of miR-155^{WT} or miR-155$^{-/-}$ mice (n=6) were prepared and indicated TJ protein mRNA were detected by real-time PCR. *p< 0.05 vs pAd group; $^{\#}$p< 0.05 vs miR-155^{WT} group.

Figure 6

A


DAPI
HFD 4 weeks
miR-155
HFD 4 weeks
Merge
HFD 4 weeks
DAPI
HFD 6 weeks
miR-155
HFD 6 weeks
Merge
HFD 6 weeks
DAPI
HFD 8 weeks
miR-155
HFD 8 weeks
Merge
HFD 8 weeks

B


Oil-red staining
miR-155WTapoE-/-
Oil-red staining
miR-155-/-apoE-/-
Oil-red staining
miR-155-/-
Oil-red staining
miR-155WTapoE-/-
Oil-red staining
miR-155-/-apoE-/-
Oil-red staining
miR-155-/-
HE staining
miR-155WTapoE-/-
HE staining
miR-155-/-apoE-/-
HE staining
miR-155-/-

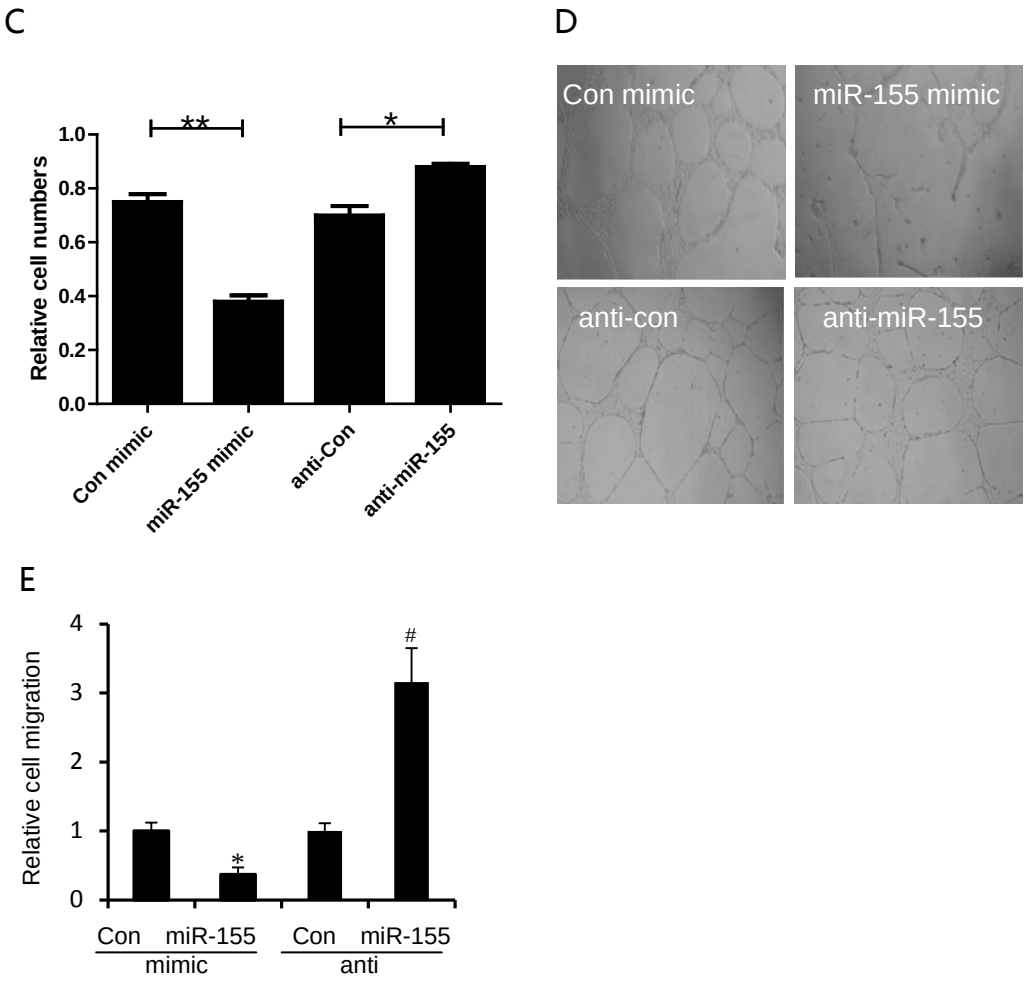


Figure 6. miR-155 inhibition suppresses atherogenesis

A, Representative photographs of staining of miR-155 by FISH in carotid artery of apoE$^{-/-}$ mice fed with HFD for 4 weeks, 6 weeks or 8 weeks, respectively. Magnification, ×100. B, Representative photographs of en face oil red O-stained thoracic aortas and HE-stained sections of the aortic root of apoE$^{-/-}$ or apoE$^{-/-}$miR-155$^{-/-}$ mice. C，Endothelial cells were transfected with miR-155 mimics or miR-155 inhibitor (anti-miR-155) for 24 h, and the proliferation of endothelial cells was then detected by MTS assay. **P<0.05 vs con mimic group. *P<0.05 vs anti-con group. D, Endothelial cells were transfected with miR-155 mimics or miR-155 inhibitor (anti-miR-155) for 24 h, and tube formation assay was performed. E, Endothelial cells were transfected with miR-155 mimics or miR-155 inhibitor (anti-miR-155) for 24 h, and the migration of endothelial cells was then detected by wound-healing assay. *P<0.05 vs con mimic group. $^{\#}$P<0.05 vs anti-con group.

Figure 7

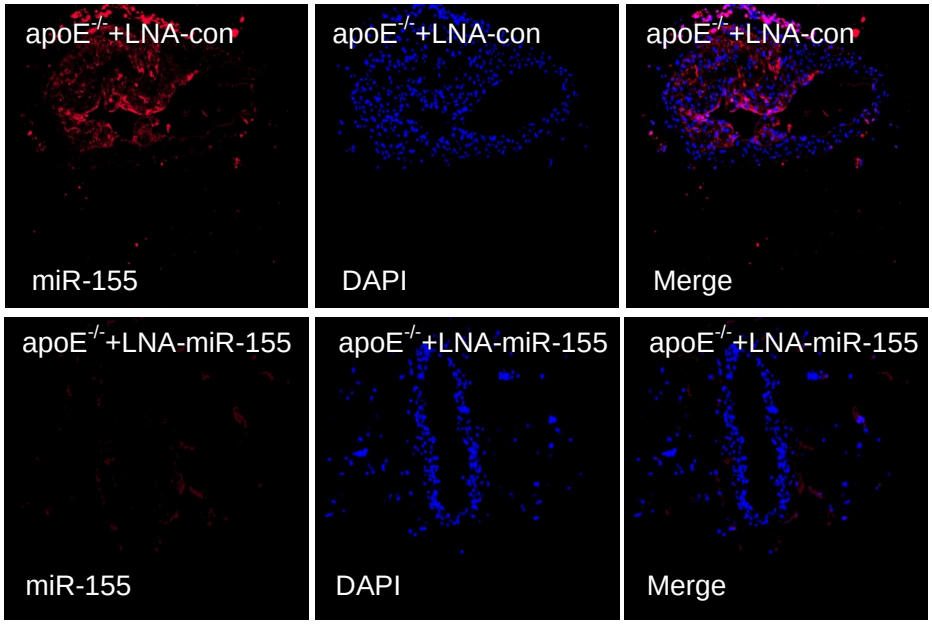


Figure 7 Inhibition of miR-155 prevents atherogenesis
Immunofluorence staining of miR-155(red) and DAPI (blue) in apoE$^{-/-}$ mice feed with HFD for 8 weeks.

Table I. miRNAs detected in HASMCs transfected with pAd or pAd-KLF5 displaying a variation lower than 2 between replicates

Upregulated miRNAs
hsa-miR-31-5p； hsa-miR-943； hsa-miR-24-3p； hsa-miR-625-5p； hsa-miR-4305； hsa-miR-3178； hsa-miR-1290； hsa-miR-4443； has-miR-29a-5p； hsa-miR-4290； hsa-miR-638； hsa-miR-20b-3p； hsv1-miR-H7-3p； hsa-miR-4791； hsa-miR-1304-5p； hsa-miR-935； hsa-miR-1285-3p； hsa-miR-4292； hsa-miR-421； hsa-miR-4449； hsa-miR-665； hsa-miR-1273e； hsa-miR-410-3p； hsa-miR-502-3p； hsa-miR-1469； hsa-miR-4732-5p； hsa-miR-1827； hsa-miR-1207-3p； hsa-miR-4644； ebv-miR-BART8-3p； hsa-miR-324-3p； hsa-miR-3195； hsa-miR-4530； hsa-miR-454-3p； hsa-miR-622； hsa-miR-493-3p； hsa-miR-4497； hsa-miR-652-3p； hsa-miR-214-3p； hsa-miR-199b-5p； hsa-miR-106a-5p； hsa-miR-4765； hsa-miR-1246；

Table II. miRNAs detected in HASMCs-derived exosomes transfected with pAd or pAd-KLF5 displaying a variation lower than 1.5 between replicates

Upregulated miRNAs
hsa-miR-381-3p； hsa-miR-4317； hsa-miRPlus-A1073； hsa-miR-26b-5p； hsa-miR-155-5p； hsa-miR-381-5p； hsa-miR-181d-5p； hsa-miR-154-3p； hsa-miR-190a-5p； hsa-miR-335-5p； hsa-miR-1185-1-3p； hsa-miR-320c； hsa-miR-885-5p； hsa-miR-376a-5p； hsa-miR-214-5p； hsa-miR-193a-3p； hsa-miR-543； hsa-miR-500a-5p/hsa-miR-500b-5p； hsa-miR-377-3p； hsa-miR-1207-3p； hsa-miR-26a-5p； hsa-miR-140-3p； hsa-miR-320a； hsa-miR-654-3p； hsa-miR-548t-5p； hsa-miR-5681b； hsv2-miR-H20； hsa-miR-34a-5p； hsa-miR-145-5p； hsa-miR-493-3p； hsa-miR-337-5p； hsa-miR-5571-5p； hsa-miR-432-5p； hsa-miR-143-3p； hsa-miR-154-5p； hsa-miR-551b-3p；