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(miRVasc)

Role of miRNAs in vascular diseases

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Final Report

BACKGROUND

Abdominal aortic aneurysm (AAA) is a dilation of the aorta characterized by a risk of arterial wall rupture at advanced stages leading to clinical complications. AAA is defined as an abdominal aortic diameter of at least 30mm, although the risk of rupture only becomes significant at larger diameters. While the only option for patients with AAA >55 mm is surgical repair of the aorta, for patients with smaller AAAs there is currently no treatment. The growth of AAA is discontinuous, and alternate periods of stability with other of acute expansion and occasionally rupture, suggesting that other molecular and/or imaging parameters in addition to AAA size should be evaluated in the follow-up of small AAA patients. While noninvasive imaging techniques are being investigated to improve characterization of the size and morphology of the aorta, the other field of growing interest is the search of potential prognostic biomarkers that may be identified in blood. Although several strategies for blood-based biomarker discovery have shown promise (1, 2), the development of clinically validated biomarkers remains an unmet challenge. New approaches that can complement and improve on current strategies for disease detection and follow-up are urgently needed.

The pathogenesis of AAA is complex and incompletely understood, but is though to represent a dynamic remodelling process of the aortic wall, that involves inflammatory cell recruitment, vascular smooth muscle cells (VSMCs) apoptosis, extracellular matrix degradation and increased oxidative stress (3_5). Is it well documented that genetic factors play an important role in AAA. In this sense, recent twin study estimated the heritability of AAA to be as high as 70% (6). However, genome wide association studies used in an attempt to find genes implicated in the inherited risk of AAA, identified regions distant from known genes. One possible explanation for the importance of loci identified within gen deserts is via transcription of noncoding RNAs (7).

MicroRNAs (miRNAs) are small, 22 nt sequences of noncoding RNA able to modulate the expression of specific mRNA targets. There are estimates that over 1500 miRNAs are encoded in the human genome and computational analysis estimated that they modulate more that 60% of total genes (8). miRNAs play important roles in a wide range of physiologic and pathologic processes including cell differentiation, proliferation and apoptosis (9, 10).

miRNAs can be relased by cells within exosomes or others microvesicles such as apoptotic bodies

altering the mRNAs expression in the recipient cells, acting as cellular communication mediators. miRNAs can be also detected in serum or plasma samples in circulating exosomes (11,12), but also in HDL particles (13) and in lipid-free protein complexes such as AGO2-miRNAs (14) in a remarkably stable form, supporting the potential usefulness of miRNA as blood biomarkers. Being able to predict who is at risk of an acute cardiovascular event, is at present one of the major challenges of cardiovascular medicine. While noninvasive imaging techniques are being investigated to improve characterization of the size and morphology of the vessels (atherosclerotic plaques/AAA), the other field of growing interest is the search of potential prognostic biomarkers that may be identified in blood. Although several strategies for blood-based biomarker discovery have shown promise (13, 14), the development of clinically validated cardiovascular biomarkers remains an unmet challenge. New approaches that can complement and improve on current strategies for disease detection are urgently needed.

In the last years many studies demonstrated that miRNAs had distinct expression profile and played crucial roles in cardiovascular diseases. In some of them, miRNAs were postulated as biomarkers for different vascular pathologies, such as acute myocardial infarctation (15_17), coronary artery disease (18,19) or hearth failure (20,21). Other studies postulated miRNAs as powerful mediators of the processes associated with cardiovascular pathology. Thus miRNAs have been shown to control key aspects of development and pathophysiology (22).

miRNAs have numerous targets often within the same functional pathway (23).Thus the regulation of miRNAs expression or function can have an enormous impact on cellular processes. In contrast to classical drugs, which act on specific cellular targets, their effects on individual genes can be modest, however, miRNAs alter cellular responses via a coordinated effect on multiple targets. All these properties make miRNAs attractive candidates for therapeutic manipulation. In this sense, recent studies have explored the use of miRNAs (24, 25) or antagomiRs (26, 27) as tools for treating cardiovascular disorders. In regard to aneurysm, in the last few years we and other have focused our effort to understand the biological roles of miRNAs in this pathology. However this is still a field poorly investigated.

RESULTS

miRNA pattern in plasma samples of AAA patients compared to healthy control subjects

The miRNA pattern of expression was investigated using miRNA microarrays chips (V2) from Agilent. The analysis was performed using 4 pools each from AAA patients and healthy aged- and sex-matched controls. Each pool contained 1 mL serum of 7 different persons. 64 miRNAs were identified to be differently expressed between the two groups, 33 of them showed a different between groups up to 2 fold (Table 1, ANNEX 1). Among them, the expression levels of 13 miRNAs were increased, whereas the levels of 20 miRNAs were decreased in the patients. Two of upregulated miRNAs, miR-32* and miR-595, presented the highest expression, indicating that their different profile could not be related to individual differences.

miR-32* and miR-595 circulating levels are up-regulated in AAA patients

Circulating miRNAs are thought to be present in within microvesicles (endosomes) or apoptotic bodies that protect them from degradation. Therefore, we isolated total RNA from exosomes from serum of n=24 patients with AAA patients and n=23 healthy controls in order to validate the results obtained in the array hybridizations. Some of the samples were also present in the microarray experiment, but we introduced new subjects to avoid possible individual differences. Real-time PCR results on candidate miRNAs (miR-32* and miR-595) were found to be consistent with the expression profile obtained by miRNA array (Fig. 1, ANNEX 1) .

miR-32* and miR-595 are up-regulated in AAA tissue samples

To evaluated weather the circulating levels are representative of tissue alterations levels, we analysed miR-32* and miR-595 expression levels in AAA tissue and control healthy vascular wall by QPCR. We observed that both miRNAs were elevated in abdominal aortic tissue samples from AAA patients in comparison with control vascular tissue (Fig. 2, ANNEX 1), indicating that vascular tissue could secrete functional miRNAs to bloodstream contributing to the miRNA serum profile

alteration. The fact that their expressions were altered in aneurysmal tissues made us to hypothesize that these miRNAs could have an important role in the processes occurring during pathological wall remodelling.

miR-32* and miR-595 increase staurosporine-induced apoptosis in HEK cells

We explored the possible contribution of these two miRNAs to aneurysm formation. Because loss of cell contributes to aneurismal disease and is one of the most striking histological features associated to AAA, we sought the potential role of our miRNAs in this process. Poly (ADP-ribose) polymerase (PARP) has been implicated in the initiation and progression of the apoptosis cascade is commonly used as a marker of apoptosis To study the role of miR-32* and miR-595 in cell viability, cleaved PARP was analyzed in staurosporine-treated HEK cells tranfected with these miRNAs. Both miR-32* and miR-595 increase the amount of cleaved PARP induced by staurosporine in a statistically significant manner (Fig.3, A; ANNEX 1). However, miR-32* appeared to be more effective in inducing staurosporine-mediated apoptosis. To further investigate the proapoptotic activity of miR-32* and miR-595, cells were transfected for 18h and then depleted for 24 h without additional staurosporine treatment. Both miRNAs were able to induce apoptosis in HEK depleted cells, but miR-595 induce a bigger response than miR-32* (Fig. 3, B; ANNEX 1).

miR-32* and miR-595 also induce apoptosis in hVSMCs in culture

We studied the effect of cell viability in hVSMCs. For that purpose, hVSMCs were transfected with premiR-595, premiR-32* or control pre-miR mimics for 48h, depleted for 6 h, and then incubated with medium containing elastase 10nM for 18 h more. Westernblot to detect cleaved PARP and cell death ELISA from Roche were performed. Both methods revealed that these miRNAs induce cell death in a similar manner (Fig. 4). These results suggest that the proapototic activity of miR-595 and miR-32* not only affect cancer cell lines, but also vascular cells. Since apoptosis of VSMCS is one of key processes involved in the pathogenesis of AAA, miR-595 and miR-32* could be considered as mediators of aortic damage in AAA.

miR-32* and miR-595 regulate ApoH expression

Apoptosis related protein target scan using ontologizer (27) (http://compbio.charite.de/index.php/ontologizer2.revealed a large number of potential proapoptotic target for these two miRNAs. Some of them were predicted to be target by both miR-595 and miR-32*, one of these proteins, Apolipoprotein H (APOH, B2-Glycoprotein I), have been previously related to vascular biology. Apo H is an abundant plasma glycoprotein that protects endothelial cells (28), macrophages and VSMCs (29) from cell death. We performed luciferase reporter assays to test whether ApoH is directly regulated by miR-595 and miR-32*. HEK cells were transfected with pMIR reporter plasmids containing ApoH 3_-UTR binding sites (pMIR-ApoH) and treated with premiR-595, premiR-32* mimics or premiR negative control. Treatment with miR-595 and miR-32* inhibited firefly luciferase activities of reporter plasmid by 50%, confirming that miR-595 and miR-32* directly target ApoH 3_-UTR (Fig. 5, A; ANNEX 1).

To study ApoH expression regulation in vascular context, we analysed ApoH expression by RT-PCR and western blot after premiR-595, premiR-32* mimics or premiR negative control tranfection in hSMCs. Both miRNAs were able to modulated Apo H mRNA expression(Fig. 5, B; ANNEX 1).

miR-32* modulates ATF-1 expression

We tried to elucidate the higher proapoptotic effect of miR-32* compared with miR-595 in staurosporine treatedHEK cells. One possibility could be that miR-32* decreases a cancer cell survival factor modulated by staurosporine. Staurosporine is a Protein Kinase C inhibitor whose exact mechanism of inducing cell death is unknown, but appears to be at least partially Ca2+-dependent (30, 31). Activating transcription factor 1 (ATF-1) can activate transcription in response to calcium elevation (32) and acts as a survival factor in cancer cells (33_35). Since ATF-1 possesses two binding sites in the 3'-UTR for miR-32* (Fig. 6, A; ANNEX 1), we explore the possible contribution of miR-32* to ATF-1 regulation. In order to investigate the e_ect of miR-32* in ATF-1 expression, HEK cells were transfected with pMIR reporter plasmids containing ATF-1 3'-UTRa and treated with premiR-595, premiR- 32* or premiR negative control. In the luciferase reporter assays premiR-32* reduced control reporter activity by 50% compared with cells treated with premiR

negative control or premiR-595, indicating that miR-32* directly regulates ATF1 mRNA expression (Fig. 6, B; ANNEX 1).

We also analysed the effect of miR-32* on ATF-1 expression in hSMCs. The cells tranfecction with miR-32* mimics reduced ATF-1 expression at both protein and RNA levels (Fig 6, C and D; ANNEX 1).

DISCUSSION

We have observed that miRNA-32* and miRNA-595 are upregulated in serum from AAA patients in comparison with control healthy subjects. Furthermore, miRNA-32* and miRNA-595 administration induces apoptosis in cultured HEK cells. The involvement of these miRNAs in apoptosis could be partially explain by their regulatory effect on the antiapoptotic mediators ApoH and ATF.

Abdominal aortic aneurysm (AAA)is a common aortic disease in the elderly associated with a high mortality. In contrast to other types of vascular disease which involve mainly the intima, aneurysm involves the elactic medial with a signicant loss of SMCs. Thus, although pathogenesis of the disease is not completely elucidated, apoptosis appears to represent one of the main involved processes. SMCs are the major sources of extracellular matrix proteins, and therefore, it has been postulated that depletion of SMCs may contributes to aneurysm development not only by reducing cellularity, but also by eliminating a cell population capable of directing connective tissue repair (3, 36). Thus, accumulating evidence indicates that SMCs apoptosis is an early event occurring in arterial wall alteration during aneurysm development (3, 36-40), and also a underlying mechanism of aneurysm rupture (41_44). In this context, the upregulation of apoptosis-related miRNAs in serum from AAA patients observed in this study, could be related to the apoptotic process occurring into the arteria wall. In addition, some studies has revealed that blockage of apoptosis remarkably reduces both the incidence and severity of induced aneurysm in animal models (45, 46). Several studies have revealed that miRNAs derived from different tissues are present in serum or plasma. Thus, the incresed expression of miRNA-595 and miRna-32* in cells underlying apoptosis could explain the origin of the circulating miRNAs in AAA patients. Analysis of exosomes-10 derived miRNAs by QPCR revealed that miR-595 and miR-32* are transfered by exosomes in

serum. Recent studies support that microvesicles or apoptotic bodies-mediated transfer of microRNAs could be a mechanism of comunication between cells (47_49). Thus, miR-595 and miR-32*expression and release within apoptotic bodies by apoptotic SMCs could induce death of the neighboring cells or indeed in other vascular areas through their release into the blood stream. In vitro experiments that explore miRNAS loading into exosomes and transfer to SMCs will be needed to corroborate this hypothesis.

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ANNEX 1: Figures

miRNA name	p-value	Correlated p-value	FCAbs	Regulation (Patients vs Healthy subjects)
hsa-miR-411*	0,004	0,021	4,64	up
hsa-miR-554	0,002	0,015	4,27	down
hsa-miR-125b*	0,003	0,018	3,93	down
hsa-miR-758	0,001	0,048	3,88	down
hsa-miR-342-3p	3,33e-5	0,022	3,33	down
hsa-miR-568	0,005	0,015	4,27	down
hcmv-miR-UL148D	8,79e-4	0,001	3,02	down
hsa-miR-29a*	0,001	0,013	2,98	up
hsa-let-7b	0,013	0,0454	2,97	up
ebv-miR-BART12	0,002	0,014	2,95	up
hsa-miR-192	2,43e-7	1,74e-5	2,86	down
hsa-miR-891a	1,61e-7	1,74e-5	2,77	down
hsa-miR-32*	**0,002**	**0,016**	**2,74**	**up**
hsa-miR-550*	0,004	0,021	2,74	down
ebv-miR-BART7	7,05e-4	0,001	2,69	down
hsa-miR-92b	3,75e-6	1,81e-4	2,55	down
hsa-miR-212	2,70e-7	1,74e-5	2,52	down
hsa-miR-30c	0,007	0,029	2,51	down
hsa-miR-200c*	4,41e-5	0,001	2,48	down
hsa-miR-133a	**0,014**	**0,045**	**2,45**	**down**
hsa-miR-23a*	0,012	0,043	2,41	up
hsa-miR-150	5,55e-4	0,009	2,31	down
hsa-miR-18b	0,002	0,015	2,17	up
ebv-miR-BART16	0,001	0,013	2,98	up
kshv-miR-K12-10b	3,55e-4	0,007	2,17	down
hsa-miR-148a*	0,008	0,034	2,16	down
hsa-miR-574-5p	0,009	0,035	2,08	up
hsa-miR-548b-3p	0,004	0,021	2,07	up
ebv-miR-BART2-5p	0,004	0,021	2,02	down
hsa-miR-595	**0,003**	**0,019**	**1,99**	**up**
ebv-miR-206	0,004	0,020	1,99	up
hsa-miR-640	0,015	0,048	1,97	down
hsa-miR-181b	0,006	0,028	1,97	up

Table 1. Summary of the dysregulated miRNAs in serum samples from AAA patients vs Healthy subjects.

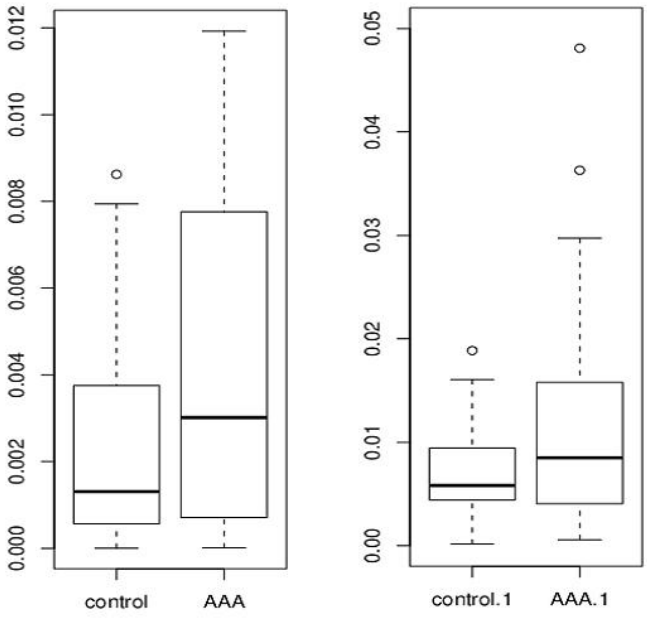

Figure 1: Circulating miRNAs in patients with AAA vs healthy voluntareers. Levels of miR-32* and miR-595 in serum-derived exosomes from healthy subjects (Control, n=23) and AAA patients (AAA, n=24) assessed by -QPCR. Both miRNAs are highly expressed in AAA patients. miR-32* and miR-595 values were normalized to miR-328 and U6 expression. Values shown are means±SEM, *P< 0:05 vs. control healthy subjects.

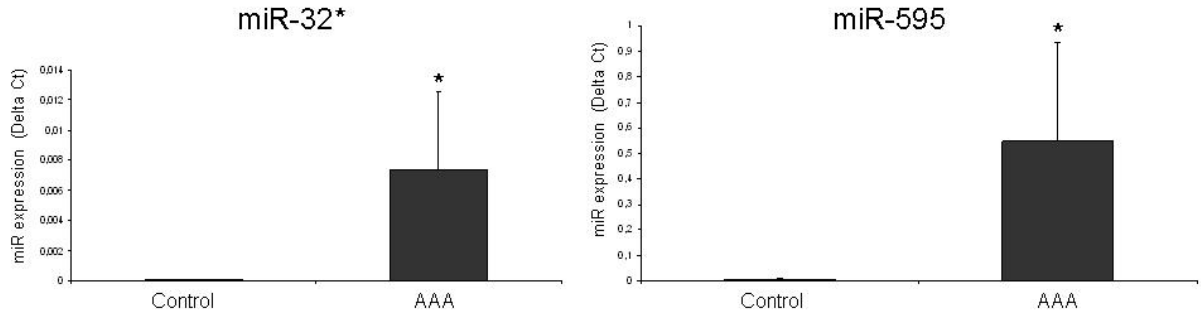

Fig 2. miR-32* and miR-595 expression in vascular tissue. miRNAs expression were analysed by RT-QPCR in 6 abdominal aortic aneurysmal tissues vs 6 control healthy vascular tissues. Values were normalised to U6 expression. Values shown are means±SEM, *P< 0:05 vs. control healthy subjects.

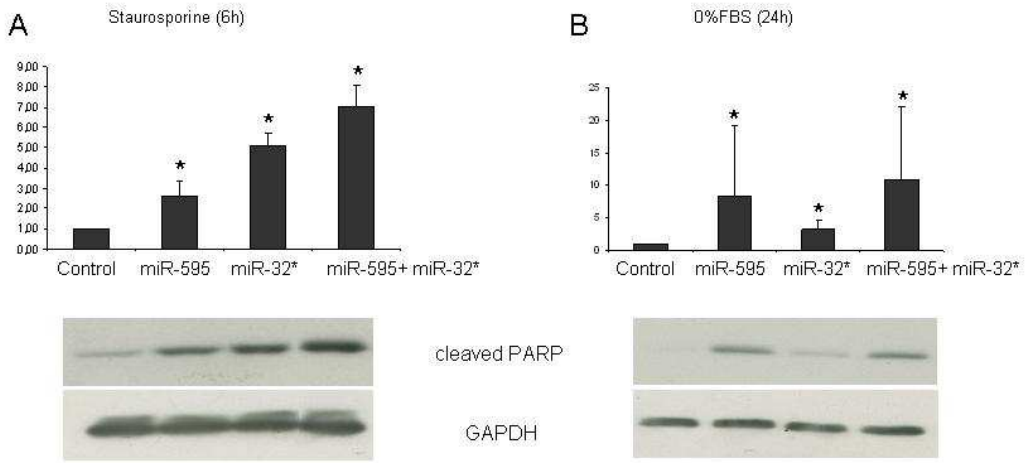

Figure 3: miR-32* and miR-595 induce apoptosis in HEK cells. Cultured HEK-293 cells were treated with pre-miR32*, premiR-595 or pre-miR negative control for 18 h. The cells were then treated with Staurosporine (0.25 _M) for 6 h (A) or with 0%FBS Medium for 24 h (B). Values were normalized to GADPH expression, and results are expressed as multiples of control values. Values shown are means ± SD of three independent experiments. *P< 0:05 vs. control.

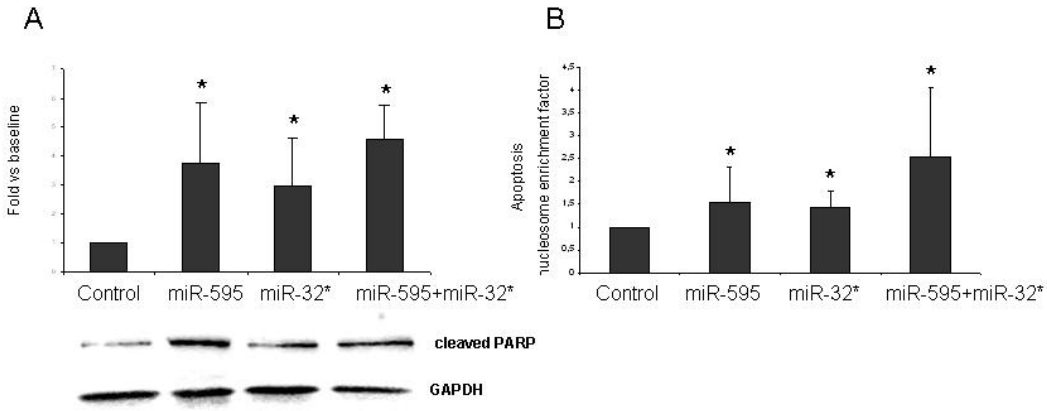

Figure 4: miR-32* and miR-595 induce apoptosis in hVSMCs in culture. VSMCs were treated with preMIR-32*, preMIR-595 mimics or pre-MIR negative control for 48 h, then the cells were depleted for 6 h, and after them they were treated with Elastase 10nM for 18 h more. Data are the mean of three independent experiments. Values were normalized to GADPH expression, and results are expressed as multiples of control values. Values shown are means ± SD of three independent experiments. *P< 0:05 vs. control.

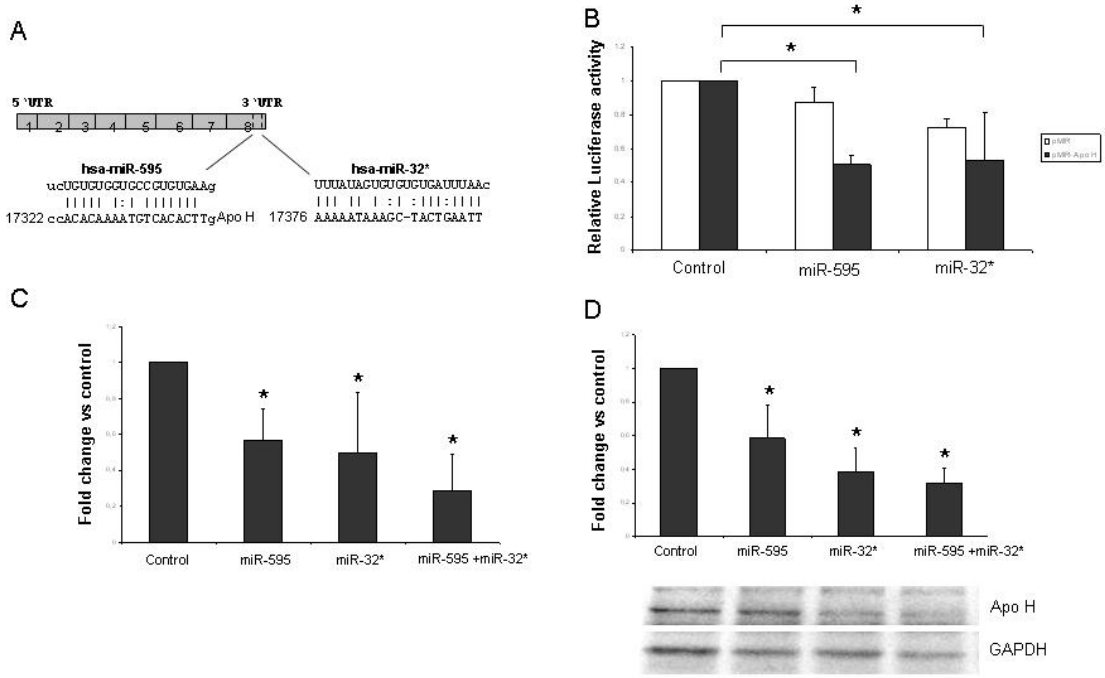

Figure 5: miR-32* and miR-595 target ApoH. Apo H is a 17.5 kb gene with 8 exones. (A)The exon 8 codes for the C-terminus and 3'-UTR which contains both miR-32* and miR-595 potentially target sequences. The Apo H 3'UTR containing the putative miR-32* and miR-595 binding sites was cloned downstream of a firefyl uciferase gene of the pMIR-REPORT vector to construct the pMIR-APOH vector. (B) pMIR-APOH was transfected in HEK-293 cells with either premiR-595, premiR-32* or premiR- negative control Luciferase reporter assay values shown are means_SD of three independent experiments performed in triplicate. Renilla luciferase activity was measured following a 24 h transfection. Both, miR-32* and miR-595 reduce luciferase activity. Values represent means _SD of triplicate analyzes .Three independent experiments were performed in triplicate with similar results **P< 0:01 vs pMIR-Apo H transfected with pre-miR negative control. These miRNAs also reduced Apo H mRNA (C) and protein (D) expression levels. Values shown are means ± SD of three independent experiments. *P< 0:05 vs. control.

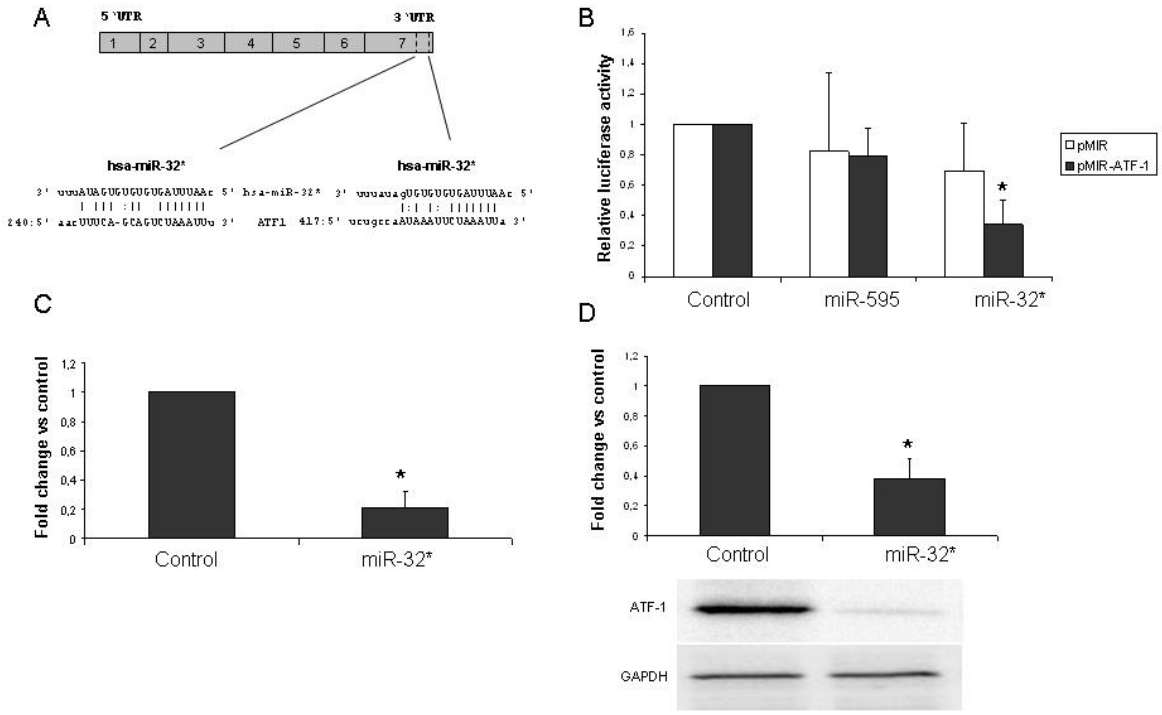

Figure 6: miR-32* directly regulates ATF1 expression. (A) Schematic representation of miR-32* target binding sites in the 3'UTR ApoH mRNA. B Fragment containing of the 3-'UTR of ATF-1 mRNA with the two putative miR-32* binding sequences was cloned into a luciferase reporter construct (pMIR-REPORT) to generate the pMIR-ATF1 vector. **(B)** pMIR-ATF1 was transfected into HEK-293 cells with either premiR-32*, premiR-595 or premiR negative control. Empty pMIR-report vector was used as control. Renilla luciferase activity was measured following a 24-h transfection. miR-32* but not miR-595 inhibited luciferase activity. Three independent experiments were performed in triplicate with similar results.*P< 0:01 vs pre-miR negative control. miR-32* tranfecction in hSMCs reduced dramatically mRNA (C) and protein (D) ATF-1 expression levels. Values shown are means ± SD of three independent experiments. *P< 0:05 vs. control.