MiR-205 is a novel biomarker for hereditary hemorrhagic telangiectasia with antiangiogenic function

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Abstract

Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant disorder characterized by arteriovenous malformations and hemorrhage. This vascular disease results mainly from mutations in 2 genes involved in the TGF-β pathway (ENG and ACVRL1) that are exclusively expressed by endothelial cells. The present study identified miR-27a and miR-205 as two plasma circulating miRNAs differentially expressed in HHT patients. The plasma level of miR-27a is increased while plasma level of miR-205 is reduced in both HHT1 and HHT2 patients compared to healthy controls. The role of miR-205 in endothelial cells was further investigated. Our data indicate that miR-205 expression displaces the TGF-β balance towards the anti-angiogenic side by targeting Smad1 and Smad4. In line, expression of miR-205 in endothelial cells reduces proliferation, migration and tube formation. This study not only suggests that detection of circulating miRNA (miR-27a and miR-205) could help for the screening of HHT patients but also provides a functional link between the deregulated expression of miR-205 and the HHT phenotype

Keywords

Hereditary hemorrhagic telangiectasia, microRNA, TGF-beta

Introduction

Hereditary hemorrhagic telangiectasia (HHT) or Rendu–Osler–Weber syndrome is an autosomal dominant vascular disorder characterized by recurrent nose bleeding telangiectases in mucosa and internal arteriovenous malformation (AMV- direct arterio-venous connections lacking intervening capillaries). AVMs that occur in the lungs, brain, or gastrointestinal tract can cause life-threatening complications secondary to either hemorrhage or the shunting of blood through abnormal blood vessels. No cure for HHT has been found and there is a current need to find new effective drug treatments for the disease. HHT is diagnosed on clinical grounds. To be considered as an HHT patient, a person must have three or more of the following diagnostic criteria: spontaneous-recurrent epistaxis, mucocutaneoustelangiectases, internal AVMs, and/or a first degree relative with HHT. The diagnosis is declared possible or suspected when two criteria are present and unlikely when there are fewer than two [1]. Since HHT is a clinically heterogeneous disorder, with symptoms differing even among the members of a same family, it is difficult to establish a proper diagnosis.

There are two main types of HHT. Type 1 is due to mutations in the *endoglin* gene, encoding an accessory endothelial transforming growth factor-β (TGF-β) receptor. Type 2 is caused by mutations in *ALK1* (*ACVRL1*; activin receptor-like kinase 1) gene, encoding a TGF-β type I receptor expressed in endothelial cells. In both, HHT1 and HHT2, the TGF-β signaling pathway of endothelial cells is affected [2]. TGFβ superfamily ligands mediate their effects through heteromeric complexes of type I and type II receptors. Upon ligand binding to type II receptor, the latter is activated and phosphorylates the TGF-β type I receptor, which in turn phosphorylates specific R (receptor-regulated)-Smads that can then interact with the Co (common-mediator)-Smad4 and translocates to the nucleus to regulate transcription of specific target genes, usually interacting with other transcription factors [3,4]. In endothelial cells, TGF-β1 can activate two distinct type I receptor/Smad signaling pathways with opposite effects on angiogenesis [5]. TGF-β1, in most cell types, predominantly signals via the type I receptor ALK5 leading to the activation of Smad2/3 and transcription of the corresponding target genes, such as PAI-1 (plasminogen activator inhibitor-1) which in endothelial cells is associated with cell quiescence. In addition in endothelial cells, TGFβ1 can also signal via ACVRL1/ALK1 which leads to phosphorylation of R-Smad1/5/8 with the consequent activation of targets distinct from ALK5, such as ID1 (inhibitor of DNA binding 1) which is associated with cell migration, proliferation and angiogenesis.

MicroRNAs (miRNAs) are short (+/- 22 nucleotides long) non-coding small RNAs that repress gene expression post-transcriptionally by targeting the 3'-untranslated regions (3'UTRs) of specific mRNAs [6]. MiRNAs play a major role in vascular biology [7-9] and "angio-miRs" (miRNAs that stimulate or repress angiogenesis) have opened up a novel aspect for therapeutics associated with deregulated angiogenesis [10] and vascular diseases [11]. Recently, blood-based miRNA detection, measuring miRNAs in the plasma, protected from endogenous RNase activity included in exosomes or in association with proteins, has emerged as a promising diagnostic tool.

In this study, a total of 24 HHT patients (HHT1 and HHT2) were screened to find a circulating miRNA signature that could help identify HHT patients. We observed that levels of miR-27a are significantly higher while levels of miR-205 are significantly lower in plasma from HHT patients compared to healthy controls. The role of miR-27 in promoting angiogenesis was previously described [12] but the role of miR-205 in endothelial cells was unknown so far. We found that miR-205 reduces EC proliferation, migration and tube formation. In addition, we demonstrated that the expression of miR-205 affects TGF-β

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pathway, by targeting Smad1 and Smad4. This study is the first to establish a link between miRNA expression and the HHT disorder and paves the way for developing better diagnostic tools for HHT patient screening.

Results

Altered circulating levels of miR-27a and miR-205 in HHT patient plasma

In order to identify specific biomarkers of HHT, we performed a miRNA expression profiling, using miRNA PCR panels (Exiqon) of plasma samples from HHT patients (2 HHT1 and 2 HHT2) and 4 healthy controls (data not shown). This pilot study suggests that miRNAs belonging to the miR-23a-27a-24-2 cluster were more abundant in the plasma from HHT patients while miR-205 was less abundant in plasma from HHT patients compared to healthy controls. MiR-27a and miR-205 were chosen for validation, by qRT-PCR, in the plasma samples from a cohort of 24 HHT patients (11 HHT1 and 13 HHT2) and 16 healthy controls. Our data showed that miR-27a level was significantly elevated in HHT patient plasma samples (p=0.021) (Fig. 1A). Conversely, miR-205 level was significantly lower in plasma samples of HHT patients compared to healthy controls (p=0.006) (Fig. 1B). We did not observe any significant differences between HHT1 and HHT2 patients for miR-27a, and miR-205. Receiver operating characteristic (ROC) curve analysis, a plot of the true positive rate against the false positive rate for the different possible cutoff values of a diagnostic test, showed that circulating miR-27a and miR-205 levels were of significant diagnostic value for HHT patients. Mir-27a and miR-205 yield an area under the curve (AUC) of 0.76 (Fig. 1C) and 0.79 respectively (Fig. 1D). Taken together miR-27a and miR-205 (ratio miR-27a/miR-205) yield an AUC of 0.87 (Fig. 1E).

MiR-205 impairs angiogenesis

Since endothelial cells are the primary cellular targets of HHT, we investigated the effect of miR-205 on endothelial cell function by overexpressing miR-205 and evaluating the impact of this ectopic expression on the endothelial proliferation, migration and tube formation. Over-expression obtained by transfection of pre-miR-205 (Fig. 2A) significantly decreased the proliferation of HUVECs compared to cells transfected with a pre-miR-control (Fig. 2B). In order to investigate whether miR-205 affects endothelial cell migration, another crucial step of angiogenesis, we performed a wound closure assay, which showed that increasing miR-205 level reduced endothelial cell capacity to close the scratch (Fig. 2C). The role of miR-205 as a modulator of endothelial cell organization into tubes was next evaluated. When seeded onto Matrigel^TM, HUVECs develop into a capillary-like vessel network. Increasing levels of miR-205 in HUVECs reduced vascular network formation, as revealed by a lower number in tube branching (Fig. 2D-E).

MiR-205 alters TGF-beta signaling in endothelial cells

TGF-β signaling is the main pathway affected in HHT endothelial cells. As endothelial cells express two types of TGF-β type I receptors, ALK5 and ALK1/ACVRL1, the influence of miR-205 on both pathways was first investigated by measuring, by qRT-PCR, the RNA expression level of two downstream targets of each pathways: ID-1 whose expression is under the control of the TGF- β/ALK1/Smad1/5/8 pathway and PAI-1 whose expression is under the control of the TGF- β/ALK5/Smad2/3 pathway [5,13,14].

Over-expression of miR-205 by pre-miR-205 transfection in HUVECs resulted in a significant reduction of ID-1 (Fig. 3A) and a significant increase of PAI-1 mRNA levels (Fig. 3B). These results suggest that both TGF-beta pathways are affected by miR-205 expression in opposite directions: the TGF- β/ALK1/Smad1/5/8 pathway, whose target is ID1, is repressed, while the TGF- β/ALK5/Smad2/3 pathway, targeting PAI-1, is stimulated. To further confirm these results, we analyzed the impact of miR-205 over-expression in assay

reporters of the activation of the ALK1/Smad1/5/8 (BRE-Luc reporter) and of the ALK5/Smad2/3 pathway (CAGA-Luc reporter) [15]. Consistent with the results described above, over-expression of miR-205 led to a lower activation of the BRE-Luc reporter (Fig. 3C) while the CAGA-Luc reporter activity was increased (Fig. 3C). These data confirm that miR-205 affects both TGF-beta pathways, in an opposite manner, displacing the TGF-β balance in EC towards the anti-angiogenic side.

MiR-205 targets Smad1 and Smad4

In order to unravel the mechanism by which miR-205 affects the TGF-beta pathway, we decided to investigate the regulation of two predicted targets of miR-205, namely Smad1 and Smad4 (Targetscan, Fig. 4A). These two proteins play a key role in the TGF-beta pathway. Over-expression of miR-205 did not significantly affect Smad1 (Fig 4B) and Smad4 (Fig. 4C) at the mRNA level. However, we observed a drastic reduction of Smad1 (Fig. 4D) and Smad4 (Fig. 4E) at the protein level by miR-205 over-expression. To confirm that Smad 1 and 4 are directly regulated by miR-205, we constructed luciferase reporter vectors encoding segments of the 3'UTR of Smad1 (human) and Smad4 (human) (WT Smad1 3'UTR and WT Smad4 3'UTR), as well as a control vectors containing 4 mismatches in the predicted miR-205 binding site (Mut Smad1 3'UTR and Mut Smad4 3'UTR). Cotransfection of the Smad1 3'UTR plasmid with pre-miR205 in HEK293T cells resulted in a significant decrease in luciferase activity compared to cells cotransfected with the pre-miR-control. Over-expression of miR-205 did not decrease the luciferase activity in cells transfected with the mutated 3'UTR target sequence (Fig. 4F). Similar results were obtained with the 3'UTR of Smad4 (Fig. 4G) indicating that both Smad1 and Smad4 are direct targets of miR-205.

Discussion

MiRNAs are present in all biological fluids mainly protected inside exosomes, small vesicles of endocytic origin. These newly discovered accessibility and stability of the circulating miRNAs have opened the way to new opportunities to screen and monitor a large variety of diseases non invasively [16-19]. Currently, molecular diagnosis of HHT involves sequencing of *ACVRL1/ALK1* and *ENG* coding regions, large deletion/duplication analysis, and if no mutation is identified, analysis of Smad4 coding region. Still, approximately 15% of HHT cases have no mutations found in the coding regions of these three genes. Our study identified the miR-27a and miR-205 as new circulating biomarkers, which could help to a better screening of HHT patients. Our data show an elevated level of miR-27a and a reduced level of miR-205 circulating in plasma samples from HHT patients, compared to healthy controls. Interestingly, this observation is valid for both HHT1 and HHT2 patients suggesting that the regulation occurring at the level of miR-27a/205 is a common feature independently of the causal mutation of the disease. Further studies will be required to confirm these data including HHT patients with mutations in other genes than *ENG* and *ACVRL1/ALK1*.

It is well established that HHT syndrome is due to alterations in angiogenesis and microvessel density [20]. A further study on Blood Outgrowth Endothelial Cells (BOECs) from HHT patients compared to those from control donors, reveals an HHT gene fingerprinting where a set of genes tightly related with angiogenesis was identified [21]. The increased level of miR-27 (proangiogenic) [22,12] and the decreased level of miR205 (antiangiogenic) are thus consistent with the altered angiogenesis observed in HHT patients due to endoglin or ALK1 haploinsufficiency and will open a new window for future therapies. Other plasma soluble components were previously identified as biomarkers of HHT as sEng and Ang-2 [23], but their lower levels in plasma seemed to be a result of the haploinsuficiency in endoglin or ALK1 on endothelial cells. Since we show here that miR-205, which is expressed in EC, impairs angiogenesis, we could speculate that miR-205 plays an endogenous role in modulating EC function. Consequently, miR-205 might thus not only be a marker of HHT but also a putative therapeutic target. Endoglin and ALK1 are predominantly expressed in endothelial cells and therefore these cells are the primary cellular targets of the disease. We therefore hypothesize than the reduced level of circulating miR-205 reflects a lower miR-205 expression by the endothelial cells. However other cell targets such as smooth muscle, hematopoietic and endothelial precursor cells are also important to promote the lesion secondarily to an endothelial cell defect. Our study does not rule out that these cells could participate in the secretion of the deregulated miRNAs in the circulation. In addition, it has been recently proposed that the transfer of microRNA by exosomes could be a new mechanism of communication between cells [24,25]. Therefore, one can speculate that the reduced level of miR-205 in the plasma of HHT patients, even if primary caused by endothelial cells, could also affect the phenotype of other target cells. In addition, the mechanisms or miR-205 regulation in EC remains to be elucidated. One possible mechanism would be that miR-205 is regulated by the TGF-β through the ALK1/Smad1/5/8 pathway. As this pathway is affected in the HHT patients, the resulting levels of miR-205 would be decreased.

The HHT mutations so far reported involve components of a common signal transduction pathway that is altered in HHT [26]. Endoglin is a co-receptor for multiple receptor complexes of the TGF-ß superfamily [27]. Endoglin interacts in endothelial cells with ALK-1, an endothelial-specific type I receptor that can associate with TGF-ß type II receptor (TGFßRII). Interestingly, TGFβRII in endothelial cells, can associate with two different TGF-ß type I receptors: TGFßRI (also known as ALK-5), or ALK-1[5]. ALK-1 and

ALK-5 activate different Smad pathways, resulting in opposing endothelial cell responses in terms of pro- or anti-angiogenic gene expression [28,13,14,29]. TGF-β signaling through ALK1/Smad1/5/8 is pro-angiogenic, eliciting proliferation, migration and the transcription activation of target genes such as ID1. While signaling through ALK5/Smad2/3 activates target genes involved in inhibition of proliferation and migration, and matrix deposition, such as PAI-1, processes associated with the endothelial cell quiescence. Moreover, a fine tuning on signaling and communication of both pathways is necessary. Thus, ALK5 contributes to a TGFβ-dependent recruitment of ALK1 to the receptor complex and is required for optimal ALK1 activation. Moreover, ENG recruitment to the TGF-βR2–ALK1–ALK5 receptor complex promotes signaling via the Smad1/5/8 pathway and this indirectly inhibits Smad2/3 signaling via the ALK5/Smad2/3 pathway [5,13].

In the present work, we have described for the first time, that the miR-205 is downregulated in HHT patients and plays an anti-angiogenic role in endothelial cells. Indeed, miR-205 expression inhibits proliferation, migration and tube formations. We also show that miR-205, by its ability to target Smad1 and Smad4, plays in EC an important role in the angiogenesis regulation by the TGF-β signaling pathway. These two targets are indeed involved in the proangiogenic signaling pathway TGF-β/ALK1/Smad1/5 controlling the transcription of ID-1. Our data also show that concomitantly to the reduction of the proangiogenic pathway, miR-205 expression would favor the antiangiogenic TGF-β/ALK5/Smad2/3 pathway (Fig. 5). MiR-205 was first described as a blocker of the EMT transition [30]. Interestingly, miR-205 has also been reported as an inhibitor of the VEGF expression in tumor cells [31]. These data are important in the context of HHT since VEGF levels is elevated in the circulation of HHT patients. We can therefore suspect that the reduced level of miR-205 could participate to the over-production of VEGF. This excess of VEGF combined with the decrease of miR-205 and the increase of miR-27a in HHT patients would promote an excessive and abnormal angiogenesis.

Leaving aside the arteriovenous malformations which may be present in brain, liver, and lung, the rupture of which would lead to fatal haemorrhages in HHT patients, the more common and disabilitating problem is epistaxis. So far, attempts to treat epistaxis with pharmacological products have followed different strategies: 1) increasing the stability of the coagulation process with antifibrinolytics [21], (2) increasing the transcription of ENG and ALK1 promoters by SERMs like Raloxifene [32], the only orphan drug for the HHT so far designated, or (3) suppressing of excessive and abnormal angiogenesis through antiangiogenic agents such as Bevacizumab (recombinant full-length humanized antibody active against VEGF-A). However, the currently available drugs can result in serious adverse events: frequent and unpredictable side effects for antiangiogenic strategies include thrombosis, hemorrhage, decreased wound healing, and organ perforation. Therefore, alternative therapeutic options are required and therapies based in the correction of the decreased levels of miR205 in HHT appear promising.

Material and Methods

Cell culture and reagents

Isolation and cultivation of human umbilical vein ECs (HUVEC, passages 6 to 11) were described previously [33].

Patients

Peripheral venous blood samples were collected from all donors with EDTA as anticoagulant. Blood samples were centrifuged at 15,000 g for 15 min to obtain plasma, and samples were stored at -80ºC until analysis. A total of 24 HHT patient samples were evaluated; eleven of them were HHT1 and the remaining 13 patients were HHT2. miRNAs plasma levels were also assayed in 16 control subjects to establish the normal range. All HHT patients included in the present study were clinically diagnosed following the Curaçao criteria and their genetic mutations were identified by sequencing. Genetic characteristics and clinical manifestations are summarized in table 1.

Transfection of pre-miR-205 in HUVEC

Pre-miR 205 and pre-miR-control (50 nM, Ambion) was transfected into HUVECs with Dharmafect-4 (Dharmacon Research Inc) according to the manufacturer's instructions. After transfection for 24 h, cells were washed and kept for additional 48 h in serum-free medium. Functional assays were performed as previously described below and in [34,35].

Cell proliferation.

Pre-miR-transfected cells were plated in 96-well culture plates at a density of 10,000 cells per well in 100 µl of EGM-2 (Lonza) supplemented 0.5 % serum, respectively and incubated for 72 h. Proliferation was analyzed 24 h later by measuring BrdU incorporation using the Cell Proliferation ELISA, BrdU (Colorimetric) (Roche).

Scratch wound migration assay

Pre-miR-transfected cells were plated in 48-well culture plates at a density of 50000 cells per well in 300 µl of EBM-2 (Lonza) and 0.5 % serum and incubated for 24 h to reach confluence. Using a tip, a wound was made in the monolayer (at time 0). The cells were then washed with PBS and incubated with EBM-2 medium containing 10 ng/ml recombinant bFGF (Promega Corp.) and 50 ng/ml recombinant VEGFa (RELIATech GmbH) for 16 h. The distance between the two sides of the wound was measured with a graduated ocular lens coupled with an Olympus CKX41 microscope (Olympus). The distance between the two sides of the wound after 8 h of migration was subtracted from the distance at time 0 and represented on a graph.

Capillary network formation on a Matrigel matrix

Pre-miR -transfected cells were plated in 96-well culture plates pre-coated with 35 µl Matrigel per well (BD Biosciences) at a density of 10000 cells per well in 100 µl of EGM-2 (Lonza). The cells were incubated for 16 h. Pictures were taken with an Olympus microscope (Olympus). Quantitative analysis of network structure was performed with ImageJ software (http://rsbweb.nih.gov/ij/) by counting the number of intersections in the network and measuring the total length of the structures.

Luciferase Assay (3'UTR reporter assays)

HEK293T cells were transfected with 30 pmol of pre-miR-Control or pre-miR-205 with DharmaFECT-4 (Dharmacon Research Inc). The next day, cells were transfected with 25 ng of the psiCHECK2 vector (Promega) encoding segments of the 3'UTR of the human Smad1 (149 pb spanning the miR-205 seed match sequence) and human Smad4 (415pb spanning the miR-205 seed match sequence) or their mutated 3'UTR (4 mutations in the miR-205 predicted target site) with JET-PEI (Polyplus transfection) following manufacturer's instructions. Wild-type region of 3'UTR human Smad1 and Smad4 mRNA binding seed sequence of miR-205 are respectively 5'-UGAAGAA-3'and 5'-AUGAAGGA-3'. Mutated region of 3'UTR human Smad1 and Smad4 mRNA binding seed sequence of miR-205 are respectively 5'-AGUACCAU-3' and 5'-AGCAUGCA-3'. Forty-eight hours later, luciferase assay was performed using Dual-Luciferase Reporter Assay System (Promega). *Renilla* luciferase activity was normalized to Firefly luciferase.

Luciferase Assay (promoter reporter assays).

HUVEC cells were transfected with 30 pmol of pre-miR-Control or pre-miR-205 with DharmaFECT-4 (Dharmacon Research Inc). The next day, cells were transfected with 25 ng of the Bre-luC [15] and CAGA-Luc [36] vectors kindly provided by Dr P ten Dijke (Leiden University Medical Center, The Netherlands) with JET-PEI (Polyplus transfection) following manufacturer's instructions. Forty-eight hours later, luciferase assay was performed using Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase.

RNA extraction, miRNA expression analysis by TaqMan MicroRNA Assay and Quantitative Real-time PCR Analysis

Total RNAs were extracted with the miRNeasy kit (Qiagen). Taqman methods were used to assess miRNA expression. Briefly, 10 ng RNA was reverse transcribed to cDNA with the Taqman microRNA Reverse Transcription kit and the Taqman microRNA assay stem loop primers (Applied Biosystems). Resulting cDNAs were used for quantitative real-time PCR using Taqman microRNA assay and Taqman universal PCR master mix reagents (Applied Biosystems). Thermal cycling was performed on an Applied Biosystem 7900 HT detection system (Applied Biosysems). The relative miRNA levels were normalized to two internal controls, RNU-44 or RNU-48. For the Plasma samples, the relative miRNA levels were normalized to two spikes-in miRNAs: cel-miR-39 and cel-miR-238 (Applied Biosystems).

For mRNA expression analysis, RNAs were extracted with the miRNeasy kit (Qiagen) according to the manufacturer's protocol. cDNA synthesis was performed with 1 μg total RNA and the iScript cDNA Synthesis Kit (Biorad) according to the manufacturer's instructions. Resulting cDNAs (20 ng) were used for quantitative real-time PCR using the SYBR green method (Roche Applied Sciences). Thermal cycling was performed on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). For all reactions, no-template controls were run, and random RNA preparations were also subjected to sham reverse transcription to check for the absence of genomic DNA amplification. Quantitative real-time PCR was performed with SYBR green method (Bioline). Thermal cycling was performed on an Applied Biosystem 7900 HT detection system (Applied Biosysems). The relative transcript level of each gene was normalized to the housekeeping genes cyclophilin-A (PPIA), beta-2 microglobulin (B2M). Primers (IDT-DNA) were designed using Primer Express software and selected to span exon-exon junctions to avoid detection of genomic DNA (primer sequences are provided below).

hsa Smad1 for   CTCATGTCATTTACTGCCGTGT
hsa Smad1 rev   TATTCGCTGTGTCTTGGAACC
hsa Smad4 for   CTCATGTGATCTATGCCCGTC
hsa Smad4 rev   AGGTGATACAACTCGTTCGTAGT
hsa PAI1 for   ACGTGGTTTTCTCACCCTATGG
hsa PAI1 rev   CATGCCCTTGTCATCAATCTTG
hsa Id1 for   CTGCTCTACGACATGAACGG
hsa Id1 rev   GAAGGTCCCTGATGTAGTCGAT
hsa B2M for   GAGTATGCCTGCCGTGT
hsa B2M rev   AATCCAAATGCGGCATCT

Preparation of cell extracts

Cells were washed twice with cold PBS and scraped into lysis buffer (25 mM HEPES (pH 7.9), 150 mM NaCl, 0.5% Triton, 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride) on ice. Insoluble cell debris was removed by centrifugation at 10,000 g for 15 min. Aliquots of protein-containing supernatant were stored at -80°C. Protein concentrations were determined by the Bradford method, with the Bio-Rad protein assay reagent (Bio-Rad Laboratories).

Western Blot Analysis

Soluble cell lysate (30 μg) was resolved by SDS-PAGE (12%) and transferred to a polyvinylidene fluoride membrane (Milipore Corp.). Blots were blocked overnight and probed with primary antibodies: anti-Smad1 (D59F7, Cell Signaling Technology), anti-Smad4 (sc-7966, Santa Cruz Biotechnology), and anti-beta-tubulin (ab6046, Abcam). After washes with Tris-buffered saline containing 0.1% Tween 20, antigen-antibody complexes were detected with peroxidase-conjugated secondary antibody (Cell Signaling Technology) and an enhanced fluoro-chemiluminescent system (ECL; Pierce Biotechnology).

Statistics

All values were expressed as means ± SD (or SEM for patient data). Comparisons between different conditions were assessed using the Student t-test.). A *P* value of <0.05 was considered statistically significant. The plasma level of the different miRNA was used as the input to plot Receive Characteristics Curves (ROC). ROC curves are displayed as the true positive rate (Sensitivity) versus 1 minus the false positive rate (Sensibility). The area under the ROC curve (AUC) is a measure of discrimination accuracy.

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Conflict of interest

The authors declare that thy have no conflict of interest.

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Figure legends
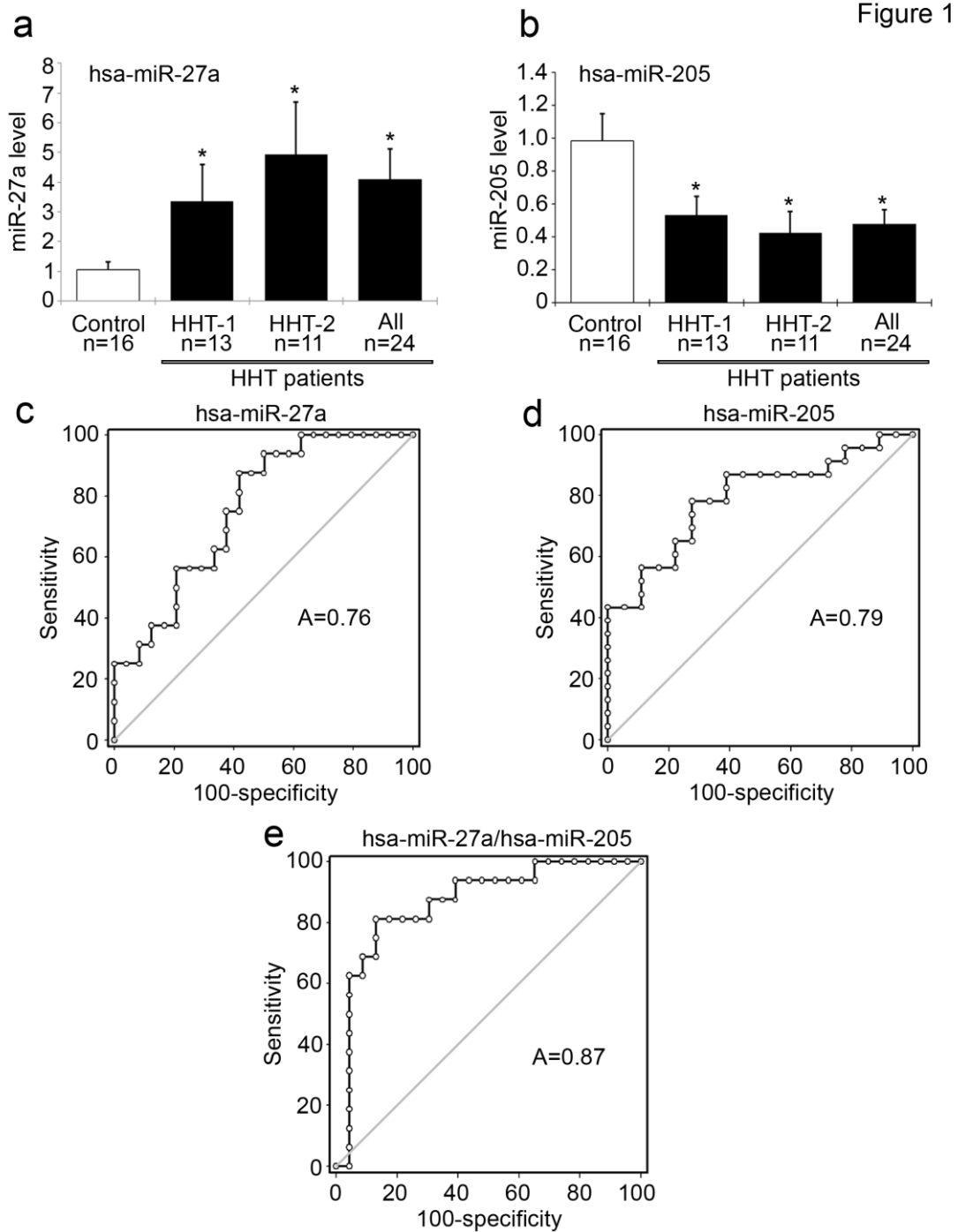
Fig. 1 Plasmatic miR-27a and miR-205 level in HHT patients

Levels of miR-27a (**a**) and miR-205 (**b**) were measured in plasma samples of a total of 24 HHT patients (HHT1 (n=11) and HHT2 (n=13)) and in 16 healthy controls by TaqMan qRT-PCR. Data are expressed as relative miRNA levels normalized to two spikes-in *c.elegans* miRNAs and are expressed as means ± SEM. * P<0.05 *vs*. healthy patients. ROC curve representing the sensitivity and the specificity of miR-27a (**c**) and miR-205 (**d**) to discriminate between healthy controls (n=16) and HHT patients (n=24). (E) ROC curve obtained by using the ration between miR-27a and miR-205 to discriminate between control (n=16) and HHT patients (n=24).

Figure 2
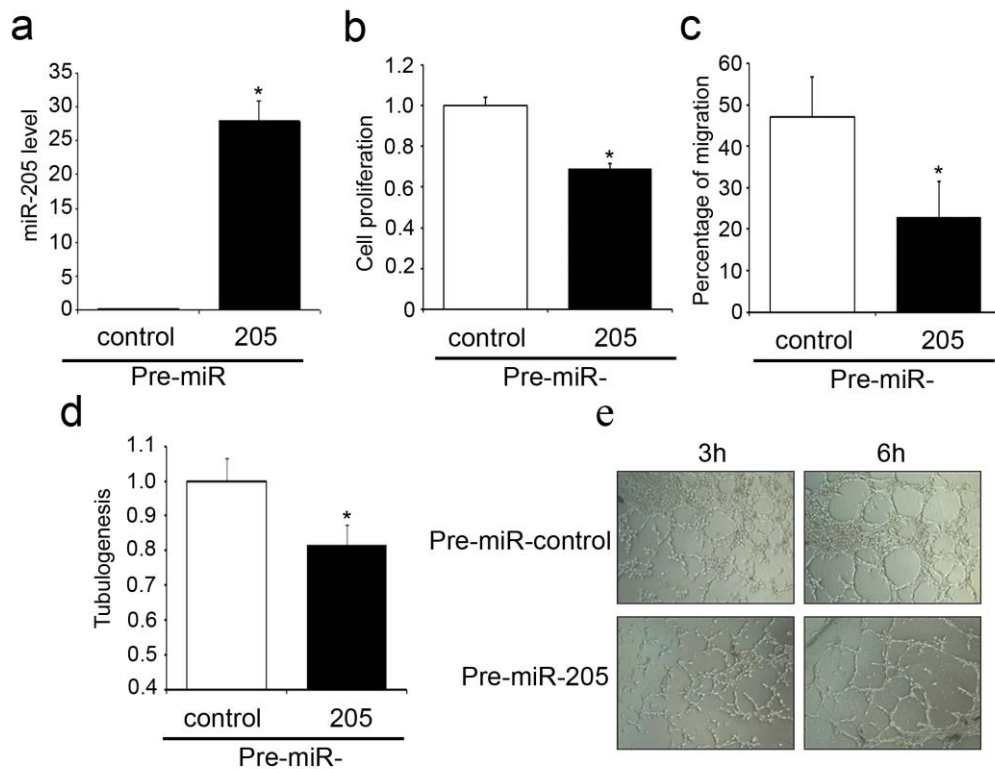
Fig. 2 MiR-205 reduces endothelial cell proliferation, migration and tube formation
a) Expression level of miR-205 in HUVECs transfected with pre-miR-control and pre-miR-205 by qRT-PCR. HUVECs were transfected with pre-miR-control and pre-miR-205 before (**b**) their proliferation status was measured by measuring the amount of incorporated BrdU (**c**) their relative migration in a scratch assay was measured and (**d**) before seeding in matrigel to determine their ability to form tube like structures. Data are expressed as relative values compared to pre-miR-control ± SD. * P<0.05 *vs*. pre-miR-control. n≥3. (**e**) Representative picture of HUVECs tubulogenesis after being transfected with pre-mir-Control and pre-miR-205.

Figure 3
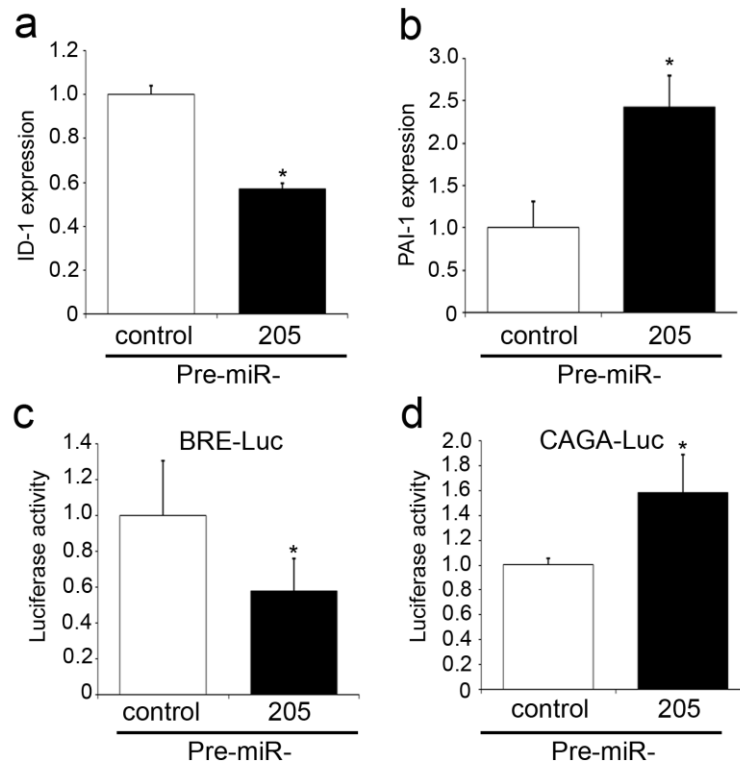
**Fig. 3 MiR-205 favors ALK5 pathway in endothelial cells**

qRT-PCR expression analysis of ID-1 (**a**) and PAI-1 (**b**) in HUVECs transfected with pre-miR-control and pre-miR-205. Luciferase activity in HEK293 cells transfected with (**c**) a BRE-LUC construct (Smad1/5-ALK1 reporter) or (**d**) a CAGA-LUC construct (Smad 2/3-ALK5 reporter) and transfected with pre-miR-control and pre-miR-205. All values are expresses as mean ± SD, n≥3. * P<0.05 *vs*. pre-miR-control.

Figure 4
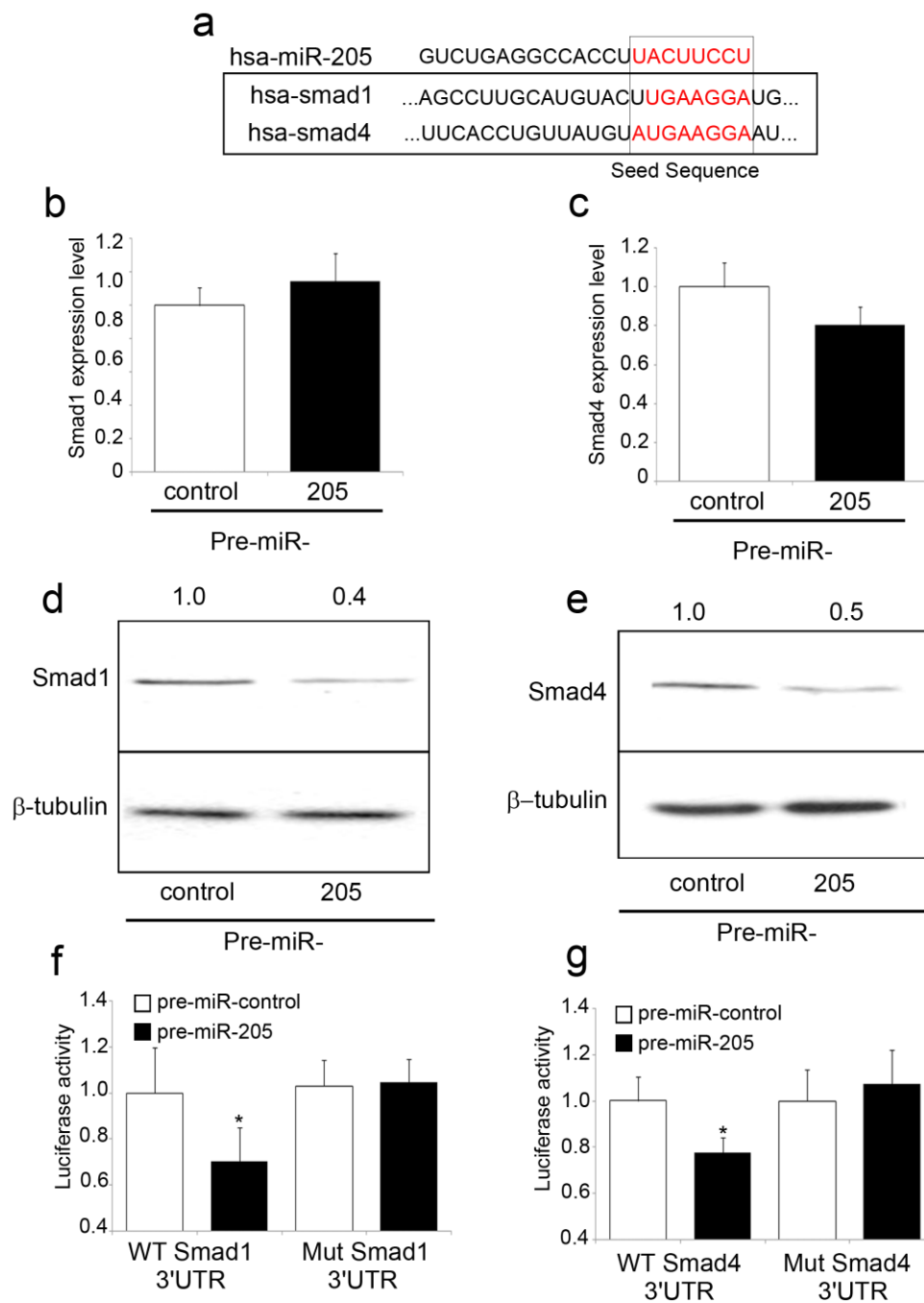
Fig. 4 MiR-205 targets Smad4 and Smad1 in endothelial cells
(**a**) Focus on the pairing sequences of Smad1 and Smad4 3'UTR and miR-205 in human. QRT-PCR expression level of Smad1 (**b**) and Smad4 (**c**) in HUVECs transfected with pre-miR-control and pre-miR-205. $n \geq 3$, no significant difference. Protein level (Western blot) of Smad1 (**d**) and Smad4 (**e**) in total protein extracts from HUVECs transfected with pre-miR-control and pre-miR-205. Luciferase activity from Smad1 3'UTR (**f**) and Smad4 3'UTR (**g**) WT and mutated reporter plasmid and the cotransfected into HEK293T cells with pre-miR-control or pre-miR-146a. Data are means ± SD ($n \geq 3$).* $P<0.05$ *vs.* corresponding pre-miR-control.
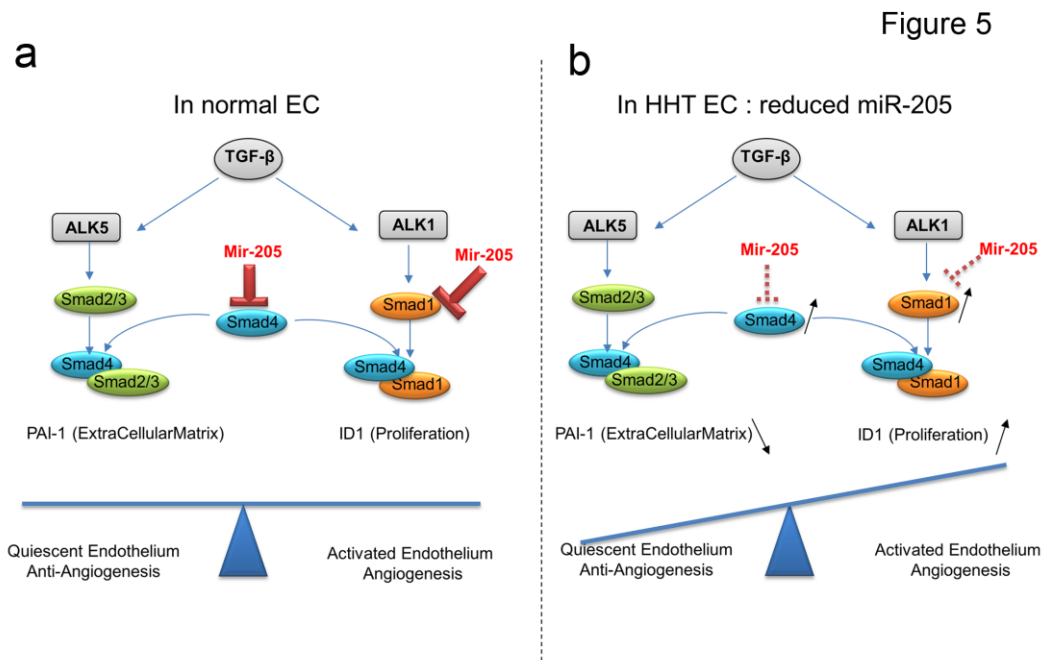
Fig. 5 Mir-205 levels contribute to modulate the equilibrium between proliferation (angiogenesis) and quiescence (deposition of extracellular matrix) in the endothelium by regulating the TGF-β pathway. (**a**) Proposed equilibrium in normal endothelial cells of both TGF-β induced pathways dependent on different type I receptors ALK5 and ALK1. Under normal conditions miR-205 acts modulating the levels of Smad1 and Smad 4 by downregulation. This situation is marked in the figure by the interruption bar for miR205 over the targets Smad1 and Smad4.(**b**) In HHT patients, the lower levels of miR-205 would favour the signalling through Smad1, although this pathway is partially inactive by the ALK1 or Endoglin haploinsufficiency, derived of the disease. The partial recovery of Smad 1 and Smad 4 levels is shown by the arrows.