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microRNA-222 Controls Neovascularization by Regulating Signal Transducer and Activator of Transcription 5A Expression

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Objective—Inflammatory stimuli released into atherosclerotic plaque microenvironment regulate vessel formation by modulating gene expression and translation. microRNAs are a class of short noncoding RNAs, acting as posttranscriptional regulators of protein-coding genes involved in various biological processes, including vascular cell biology. Among them, microRNA-221/222 (miR-221/222) seem to negatively modulate vascular remodeling by targeting different target genes. Here, we investigated their potential contribution to inflammation-mediated neovessel formation.

Methods and Results—We used quantitative real-time RT-PCR amplification to analyze expression of 7 microRNAs previously linked to vascular biology, such as miR-17-5p, miR-21, miR-126, miR-210, miR-221, miR-222, and miR-296 and found high levels of expression for all of them in quiescent endothelial cells. However, miR-126, miR-221, miR-222, and miR-296 turned out to be down-modulated in endothelial cells exposed to inflammatory stimuli. Applying a gain-of-function approach, we demonstrated that, among them, only miR-222 was involved in inflammation-mediated vascular remodeling. In addition, we identified signal transducer and activator of transcription 5A (STAT5A) as a bona fide target of miR-222 and observed that miR-222 negatively correlated with STAT5A expression in human endothelial cells from advanced neovascularized atherosclerotic lesions.

Conclusion—We identified STAT5A as a novel miR-222 target, and this finding opens up new perspectives for treatment of vascular diseases. (*Arterioscler Thromb Vasc Biol.* 2010;30:1562-1568.)

Key Words: microRNAs ■ angiogenesis ■ atherosclerosis ■ STAT5 ■ endothelial cells

Inflammation represents a critical and unifying driving force promoting atherosclerotic plaque progression. Macrophages and T lymphocytes modulate lesion growth, by releasing cytokines and growth factors, as suggested by the in vivo angiogenic properties of some of these secreted factors.[1] Early studies demonstrated that T cells infiltrating the atherosclerotic plaque release interleukin (IL)-3[1–3] and basic fibroblast growth factor (bFGF), both promoting in vivo new vessel formation.[3,4] These observations sustain the possibility that, in concert with bFGF or with other soluble factors, IL-3 may contribute to plaque progression. Moreover, as recently demonstrated, like IL-3,[4] bFGF induces vascular endothelial morphogenesis by activating the signal transducer and activator of transcription (STAT)5A signaling pathway.[5] The STAT5 transcription factors migrate into the nucleus and regulate the expression of genes involved in different cell functions, including cell proliferation and cell migration.[6] In particular, despite their high degree of conservation, several lines of evidence indicate that the 2 homologous proteins, STAT5A and STAT5B, exert nonredundant functions in the cardiovascular system.[7–9]

See accompanying article on page 1500

New vessel formation is controlled by a complex network of genes. microRNAs (miRNAs) have recently come into focus as powerful players that control gene expression posttranscriptionally.[10–13] miRNAs are short noncoding RNAs that negatively regulate gene expression, acting on their target mRNAs, in a tissue- and cell type-specific manner.[10] miRNA profiles on endothelial cells (ECs) revealed the presence of specific miRNAs, such as miRNA-17-5p (miR-17-5p), miR-21, miR-126, miR-210, miR-221/222, and miR-296, involved in the regulation of angiogenesis.[10–13] In particular, the miR-221/miR-222 family seems to negatively modulate angiogenesis by targeting the c-Kit receptor.[14] More recently, p27[Kip1] and p57[Kip2] have also been identified as target genes involved in miR-221/miR-222-induced smooth muscle cell (SMC) proliferation.[15]

The present study aimed to investigate the contribution of miR-221/222 in inflammation-mediated neoangiogenesis and

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their potential involvement in atherosclerotic intraplaque neovascularization.

Materials and Methods

Cell Cultures and Cell Proliferation Assay

ECs were isolated from a human umbilical vein and cultured as previously described.[4] To perform the experiments, ECs were synchronized to quiescent state by serum starvation for 12 hours (overnight), then the cells were cultured in the presence of 1% bovine calf serum, untreated or treated for 24 hours to 48 to 72 hours with IL-3 (20 ng/mL) or with bFGF (10 ng/mL). Cell viability was evaluated by trypan blue at the end of each experiment, and proliferative activity was assayed by direct cell count, by 3 individual operators in triplicate, as previously described.[1] Proliferation was evaluated by time-course experiments (24, 48, and 72 hours). Forty-eight hours of IL-3 and bFGF stimulation was used throughout the study. In selected experiments, untreated, bFGF-treated (data not shown), or IL-3-treated ECs were transiently transfected with pre-miR negative control, pre-miR-125a-3p, pre-miR-222, pre-miR-221, or pre-miR-126 precursor oligonucleotides or alternatively with anti-miR negative control, anti-miR-221, or anti-miR-222 inhibitor oligonucleotides (Applied Biosystems), according to manufacturer's recommendation, and analyzed 48 hours later.

RNA Isolation and Quantitative Real-Time PCR (qRT-PCR) for miRNAs Expression

Total RNA was isolated using TRIzol Reagent (Invitrogen) from untreated or treated cells, as indicated, and from human arterial samples- or Matrigel plug-derived ECs, following manufacturer's instructions. The expression of miR-17-5p, miR-21, miR-125a-3p, miR-126, miR-210, miR-221, miR-222, and miR-296 in different experimental conditions was evaluated using the TaqMan miRNA assay kit (Applied Biosystems). Single-stranded cDNA was synthesized from 50 ng of total RNA using looped primers of the TaqMan miRNA assay and TaqMan miRNA reverse transcription kit (Applied Biosystems). Each cDNA generated was amplified by qRT-PCR using sequence-specific primers from TaqMan miRNA assay in the ABI PRISM 7700 Sequence detection system (Applied Biosystems). miRNA expression was normalized to small nuclear RNA, RNU6B.

Reagents and antibodies, EC migration assay, qRT-PCR for STAT5A expression, Northern blot analysis, Western blot analysis, luciferase miRNA target reporter assay, endogenous depletion of STAT5A by small interfering RNAs, in vivo experiments, immunofluorescence analysis, isolation of ECs from Matrigel plugs, human arterial samples and isolation of vascular cells, statistical analysis, and an extended version of cell cultures and cell proliferation assay were described in detail in the supplemental section, which is available online at http://atvb.ahajournals.org.

Results

miRNA Expression in ECs Exposed to Inflammatory Stimuli

T cell-derived cytokine IL-3, like bFGF,[1,2] acts as an inflammatory angiogenic mediator.[3,4] To evaluate the biological functions of miRNAs in inflammatory angiogenesis, a subset of 7 miRNAs, previously known to be involved in angiogenesis,[10–13] was investigated by qRT-PCR. To this end, ECs were exposed or not (quiescent) to IL-3 or bFGF (Figure 1A). Consistent with previous reports, high expression of miR-17-5p, miR-21, miR-126, miR-210, miR-221, miR-222, and miR-296 was found in quiescent ECs, whereas a significant down-regulation of miR-296, miR-126, and miR-221/222 was detected in ECs challenged with both IL-3 and bFGF. No
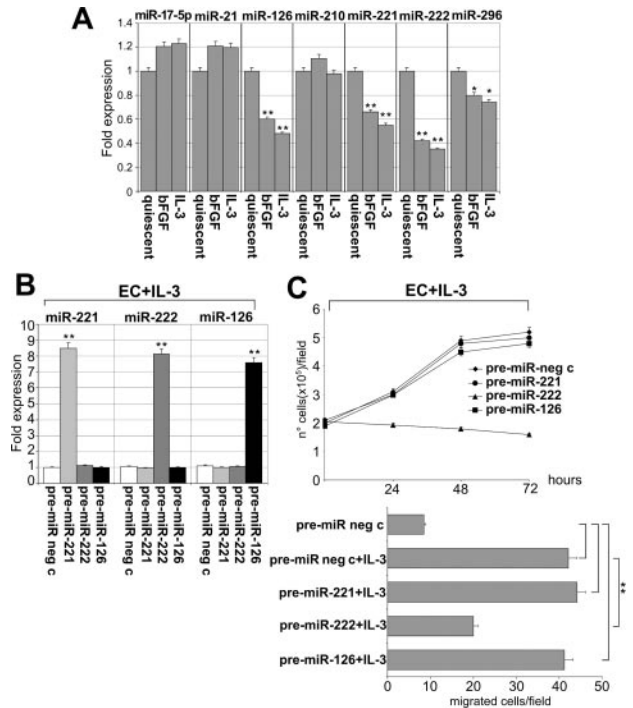

Figure 1. miRNA expression in ECs exposed to inflammatory stimuli. A, Expression of miR-17-5p, miR-21, miR-126, miR-210, miR-221, miR-222, and miR-296 in quiescent or bFGF- or IL-3-treated ECs was analyzed by single assay qRT-PCR. The reported data were normalized to RNU6B. B, qRT-PCR was performed on IL-3-challenged ECs 48 hours after transfection of pre-miR negative control (neg c), pre-miR-126, pre-miR-221, or pre-miR-222 precursors. Fold expression of miRNAs was representative of 3 independent experiments performed in triplicate. C, Cell proliferation (upper) and cell migration (lower) assays were performed as described in Materials and Methods in ECs transfected as above in the presence of IL-3 (*$P<0.05$ and **$P<0.01$, experimental versus control values).

change in miR-17-5p, miR-21, and miR-210 expression was observed either after IL-3 and bFGF stimulation (Figure 1A). Further validation of some of them was obtained by Northern blot analysis (supplemental Figure I), thus suggesting that angiogenic inflammatory stimuli might share a common miRNA signature (Figure 1A). To verify the biological relevance of miR-126, miR-296, and miR-221/222, functional studies were performed in ECs using a gain-of-function approach (Figure 1B). As reported in Figure 1C, ECs overexpressing pre-miR-222, but not pre-miR-221, pre-miR-126, or pre-miR-296 (data not shown), failed to undergo proliferation and to migrate in response to IL-3, compared with negative controls. Similar results were obtained on bFGF stimulation as well (data not shown), suggesting that miR-222 is specifically controlling vascular growth.

STAT5A Is a Novel miR-222 Target in ECs Challenged With IL-3 and bFGF

bFGF and IL-3 trigger vascular EC morphogenesis by activating the STAT5 signaling pathway.[5] According to various prediction software (miRBase-MicroCosm version v5 and miRecords), STAT5A is a predicted miR-222 and miR-221 target (Figure 2A). Thus, biochemical studies were performed
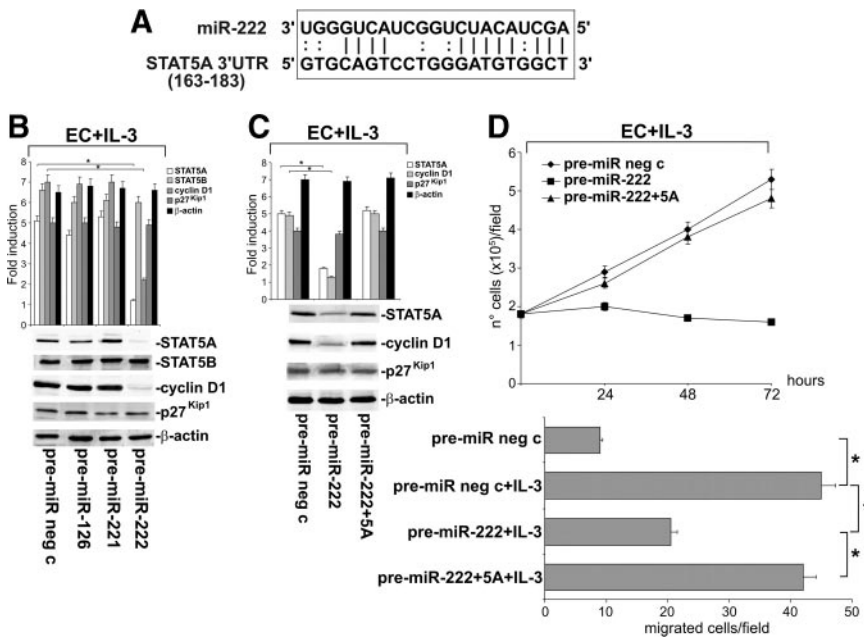
Figure 2. STAT5A is regulated by miR-222. A, Alignment of miR-222 with its potential binding site in the 3′-UTR of STAT5A mRNA. B, IL-3-cultured ECs transfected with pre-miR negative control (neg c), pre-miR-126, pre-miR-221, or pre-miR-222 precursors were lysed and analyzed by Western blot for STAT5A, STAT5B, cyclin D1, p27^{Kip-1}, and β-actin expression. C, ECs cultured with IL-3 were transfected with pre-miR negative control (neg c) or pre-miR-222, only or in combination with a STAT5A expression vector (5A). 48 hours later, cell extracts were prepared and subjected to SDS-PAGE, and the filters were immunoblotted (IB) with the indicated antibodies. D, Cell proliferation (upper) and cell migration (lower) assays were performed in ECs treated as described in C (for B, C, and D, *$P<0.05$, experimental versus control values).

using a gain-of-function approach (Figure 1B). As shown in Figure 2B, despite the fact that STAT5A was also a predicted target of miR-221, miR-222, but not miR-221, overexpression was associated with down-regulation of STAT5A protein levels and, consistently with the biological relevance of this correlation, overexpressing STAT5A, lacking the endogenous 3′-untranslated region (UTR), rescued cyclin D1 expression (Figure 2C) and EC functions, such as proliferation and migration (Figure 2D). No effect of miR-126 was observed on STAT5A in our system (Figure 2B). To validate our data, biochemical and functional studies using a loss-of-function approach were performed (supplemental Figure II). As shown in supplemental Figure IIB through IID, a further increment in STAT5A expression was detected by expressing anti-miR-222 but not anti-miR-221 (supplemental Figure IIA). Consistently, both cell proliferation or cell migration (supplemental Figure IIC) were further improved in the presence of IL-3. However, even in the presence of slightly increased expression of STAT5A, no biological effect was observed following anti-miR-222 transfection in absence of IL-3, possibly because the anti-miR cannot decrease miR-222 levels to rescue IL-3 effect. Similar results were obtained when the experiments were performed in the presence of bFGF (data not shown). Finally, by knocking down STAT5A expression in ECs (supplemental Figure III), by RNA interference, we were able to reproduce miR-222 biological effects. To validate these data, STAT5A and miR-222 expression was evaluated in quiescent ECs following bFGF or IL-3 stimulation. Increased STAT5A protein, but not mRNA levels (Figure 3A, left, and supplemental Figure IVA), and decreased miR-222 (Figure 3A, right) expression were detected. In parallel, increased cyclin D1 protein levels (Figure 3A) and cell proliferation, as well as cell migration (Figure 3B), were observed. No change in c-Kit and p27^{Kip1} were observed (Figure 3A and supplemental Figure IVB). To ascertain the direct effect of miR-222 on STAT5A-3′-UTR, the luciferase reporter vectors containing the wild-type full-length STAT5A- or STAT5B-3′-UTR were transfected in ECs. An increased luciferase activity was detected in STAT5A-3′-UTR expressing ECs exposed to both bFGF or IL-3 (Figure 3C). Decreased gene reporter activity was observed when the same wild-type construct was transfected in HEK293 cells (an easily transfectable miR-222 empty recipient) and when overexpression of miR-222 was forced (supplemental Figure V). However, increased luciferase activity was observed when the same HEK293 cells were transfected with a construct carrying a mutant STAT5A-3′-UTR following miR-222 overexpression (supplemental Figure VB). Nevertheless, pre-miR-221 and pre-miR-222 were able to act on their specific targets. Indeed, when HEK293 cells were transfected with constructs carrying p27^{kip1}-3′-UTR or c-Kit-3′-UTR and overexpressing pre-miR-221 or pre-miR-222, a decreased luciferase activity was detected (supplemental Figure VC). STAT5A is also a predicted miR-125a-3p target (see the prediction software above). As shown in supplemental Figure VIA, miR-125a-3p was down-regulated by inflammatory stimuli. However, we failed to detect changes in STAT5A or cyclin D1 protein expression and in luciferase activity following IL-3 treatment in miR-125a-3p-overexpressing cells (supplemental Figure VIB and VIC), further indicating that STAT5A is mostly regulated by miR-222 during inflammation-mediated neoangiogenesis. Together, these findings led us to focus our in vivo investigations on miR-222.

miR-222 Controls In Vivo Neoangiogenesis and Intraplaque Vessel Formation by Targeting STAT5A

The role of miR-222 in regulating vessel growth was evaluated in vivo by injecting severe combined immunodeficient mice with Matrigel plugs containing bFGF or IL-3 and ECs overexpressing miR-222 or its negative control. As shown in Figure 4A, human CD31-positive ECs, but no functional
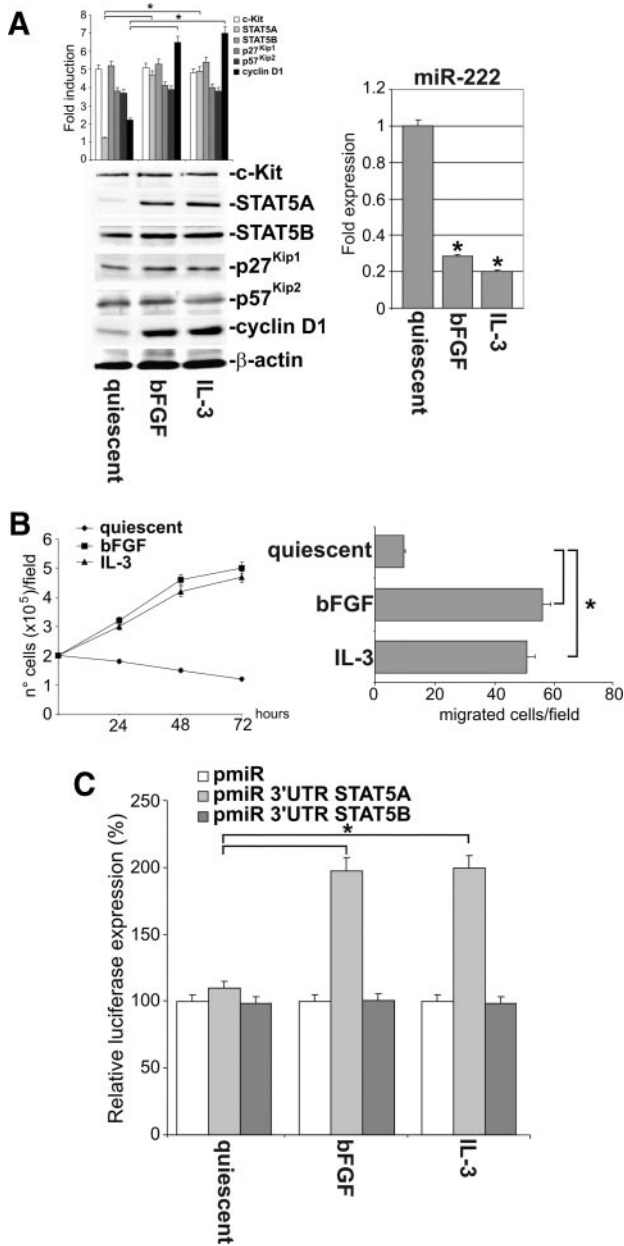
Figure 3. STAT5A is a direct target of miR-222 in ECs treated with IL-3 or bFGF. A, Quiescent or bFGF- or IL-3-stimulated ECs were used to perform qRT-PCR for miR-222 expression (right) and Western blot analysis (left) for c-Kit, STAT5A, STAT5B, p27[Kip-1], p57[Kip2], cyclin D1, and β-actin expression as indicated (left). Fold expression of miRNAs was representative of 3 independent experiments performed in triplicate. B, Proliferation (right) and migration (left) assays of ECs treated as above. C, pmiR vector or pmiR-3′-UTR STAT5A or pmiR-3′-UTR STAT5B luciferase constructs were transfected into quiescent or IL-3- or bFGF-treated ECs. The relative luciferase activity is reported (*$P<0.05$, experimental versus control values).

vessels (no tubular structures containing erythrocytes into the lumen), were detected in Matrigel plugs injected with miR-222-overexpressing cells, compared with negative controls (Figure 4A). Consistently, biochemical analysis on ECs from the same Matrigel plugs revealed almost undetectable levels of STAT5A protein (Figure 4B). Once more, we did not detect any change in c-Kit or p27[Kip1] expression following miR-222 overexpression (Figure 4B). In addition, the finding

that ECs, depleted of endogenous STAT5A by RNA interference, injected in severe combined immunodeficient mice replicated miR-222 overexpressing phenotype further sustains the above data (supplemental Figure VII). Conversely, a rescue of angiogenesis followed STAT5A exogenous expression in miR-222-overexpressing ECs (Figure 4A). Instead, reduced proliferation was observed in plugs injected with miR-222-overexpressing ECs, as measured by Ki67 immunostaining (supplemental Figure VIII).

Inflammatory cells stringently control intraplaque neovascularization and lesion progression.[1,2] IL-3, like bFGF, is released in neointima microenvironment.[3] Thus, the biological relevance of miR-222 in regulating plaque progression was investigated by isolating human ECs from normal and atherosclerotic samples. As already reported,[14,15] miR-222 is highly expressed in CD31-positive ECs recovered from normal vessels (Figure 5A). Conversely, from early to advanced lesions, a progressively decreased expression of miR-222, but not of miR-17-5p, was detected (Figure 5A; for atherosclerotic lesion description, please see supplemental material). Tissue-specificity of miR-222 expression is provided by the following observations: (1) no anti-α-smooth muscle actin staining was revealed in the recovered ECs from advanced lesions, (2) no changes in miR-222 expression could be detected in SMCs exposed to IL-3 or bFGF, and (3) the level of miR-222 was almost undetectable in unstimulated or macrophage colony stimulating factor-stimulated peripheral blood mononuclear cells (Figure 5B). Similar results were obtained by lipopolysaccharide stimulation (data not shown). The possibility that miR-222 expression might negatively control neovascularization is sustained by the proliferation rate of ECs lining vessels inside the advanced lesions (positive immunostaining for Ki67) (Figure 5C) and by the increased number of vessels in low miR-222 expressing plaques (Figure 5D).

ECs recovered from normal vessels and from lesions at different stages of progression were also evaluated for STAT5A expression. Indeed, STAT5A is barely detectable in nonproliferating normal vessel, and its expression increases from early (Figure 5A, lane 1) to advanced lesions (Figure 5A, lanes 2 and 3). A positive STAT5A immunostaining was also detected in vessels from advanced lesions compared with normal tissues (supplemental Figure IX). Moreover, the finding that no changes in c-Kit and p27[Kip1] protein expression could be detected (Figure 5A) again identifies STAT5A as the main regulator of neovascularization, which is mostly regulated posttranscriptionally by miR-222.

Discussion

Atherosclerotic lesion progression requires intraplaque neovascularization,[1,2] and the inflammatory cells contained in the neointima mainly contribute to this process by releasing cytokines and growth factors, such as IL-3 and bFGF.[1–3] Although these mediators are known to control a number of different genes, very little is known about their effects in regulating gene expression and translation during vessel formation. Recently, miRNAs have been recognized as negative regulators of gene expression.[10–12] Key targets of
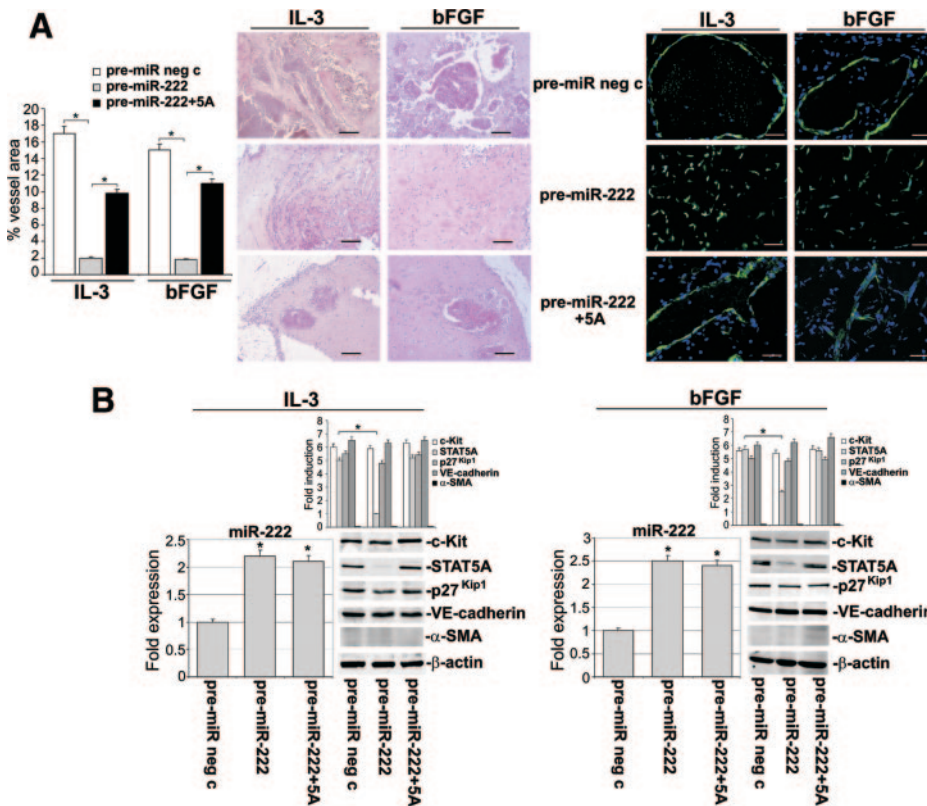
Figure 4. miR-222 controls in vivo neoangiogenesis by targeting STAT5A. A, Matrigel plug sections, containing IL-3 or bFGF and ECs transfected with pre-miR negative control (neg c) or pre-miR-222 oligonucleotide, only or in combination with a STAT5A expression vector (5A), were recovered 4 days after implantation in severe combined immunodeficient mice. Histological (magnification ×10; left) and immunofluorescence (magnification ×20; right) analyses are reported. Immunofluorescence stains were performed with an anti-human CD31 antibody (green) and the 4′,6-diamidino-2-phenylindole DAPI nuclear marker (blue). Quantification of neoformed vessels observed in histological analysis was expressed as percentage±SD of the vessel area to the total Matrigel area. Scale bars indicate 200 μm for histological analysis and 50 μm for immunofluorescence analysis. B, ECs, recovered by CD31-positive magnetic sorting from the Matrigel plugs described above, were analyzed by qRT-PCR and Western blotting for miR-222 and for c-Kit, STAT5A, p27[Kip1], vascular endothelial-cadherin (VE-cadherin), α-smooth muscle actin (SMA), and β-actin expression, respectively. Fold expression of miRNAs was representative of 3 independent experiments performed in triplicate (*$P<0.05$, experimental versus control values).

miRNAs have been identified in cancer cells,[13,16,17] but relatively little is known about miRNAs involved in vascular diseases. So far, only a few specific miRNAs recognized as proangiogenic or antiangiogenic miRNAs have been described.[10–13] Among the latter, miR-221/miR-222 have been reported to negatively regulate vessel formation, by targeting c-Kit.[14] In addition, miR-221/miR-222 have been shown to act as crucial modulators of SMC proliferation by targeting p27[Kip1] and p57[Kip2].[15] However, the impact of hypoxia, inflammation, cardiovascular risk factors, or laminar sheer stress on the expression profile of miRNAs in vascular cell biology is still under investigation.[18–20] Indeed, very recent data demonstrated that in pathological settings, linked to inflammation, growth factors, or cytokines, can differentially regulate miRNA expression in ECs.[21,22] In the present study, we provide evidence that miR-222 is the main regulator of vascular cell biology in an inflammatory microenvironment containing IL-3 or bFGF. Moreover, we identify STAT5A as a novel miR-222 target involved in inflammation-mediated neovessel formation.

The 2 highly homologous proteins, STAT5A and STAT5B, act as signaling components between the plasma membrane and the nucleus and as transcriptional factors by regulating the expression of genes involved in proliferation, survival, and differentiation.[6] STAT5A and STAT5B show a high degree of conservation. However, these 2 homologous proteins exert nonredundant functions during developmental processes and in the adult cardiovascular system.[7] For instance, STAT5B, unlike STAT5A, seems to play a role in

balloon injury-induced neointima formation[8] and to regulate gene expression in diabetes-associated vascular disease.[9] Conversely, STAT5A seems to be involved in postmyocardial infarction remodeling.[23] We found that during vessel growth, STAT5A, but not STAT5B, is posttranscriptionally regulated by miR-222, therefore defining an additional nonredundant function for STAT5 proteins.

Despite the fact that miR-222 and miR-221 contain the same seed and that the expression of both miRNAs was reduced in ECs following IL-3 or bFGF treatment, miR-221 did not appear to be biologically relevant for inflammation-mediated vascular growth (ie, proliferation, migration, STAT5A and cyclin D1 expression, and STAT5A binding 3′-UTR). Different biological activities between miR-221 and miR-222 were previously observed in a model of liver tumorigenesis, in which miR-221, but not miR-222, was able to accelerate tumors in mice.[24] One can make the hypothesis that specific miRNAs activities between miR-221 and miR-222 depend on sequence differences present downstream of the common seed in the 2 miRNAs. In fact, it has been predicted and/or demonstrated that other nucleotides, apart from the seed sequence, can be relevant for miRNA activity as discussed in Filipowicz et al,[25] Bartel,[26] and elsewhere.[27–29] However, functional evaluation of each specific nucleotide downstream of the seed can be performed only by a complete mutagenesis analysis.

miR-222 was shown to be down-modulated in endometrioid and clear cell ovarian carcinomas but not in the serous type of the same tumor.[30] These results indicate that miRNA
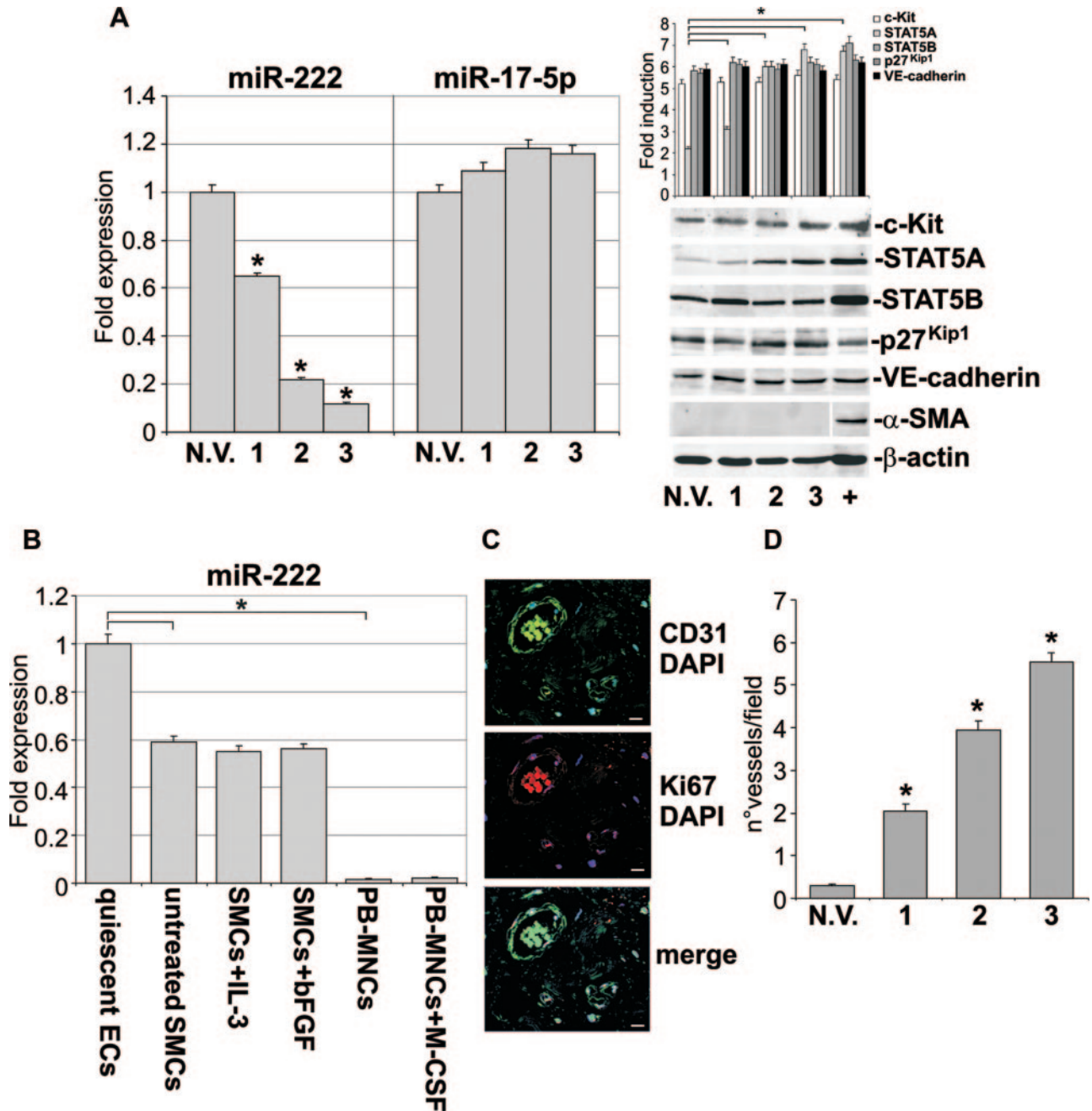
Figure 5. miR-222 correlates with STAT5A expression during lesion progression in humans. A, ECs, recovered from representative atherosclerotic plaque samples (see Materials and Methods), by CD31-positive magnetic sorting, were analyzed for the expression of miR-222 and miR-17-5p (left) or the indicated proteins (right) by qRT-PCR or Western blotting, respectively (N.V.: normal vessel, from 1 to 3 progressively more advanced atherosclerotic lesions, ie, 1: early lesion, 2: fibromuscolar plaques, and 3: advanced lesions). IL-3-treated ECs or SMCs [for α-smooth muscle actin (SMA) expression] were used as positive controls (+). VE-cadherin, vascular endothelial-cadherin. B, miR-222 expression was evaluated by qRT-PCR on peripheral blood mononuclear cells (PB-MNCs), untreated or treated with macrophage colony stimulating factor (M-CSF), or on SMCs, subjected or not to IL-3 or bFGF treatment. Quiescent ECs were used as internal control (*$P<0.05$, experimental versus control values). Fold expression of miRNAs was representative of 3 independent experiments performed in triplicate. C, Representative immunofluorescence staining of advanced lesions. Anti-human CD31 (green) or Ki67 (red) antibodies were used in association with the nuclear marker [4′,6-diamidino-2-phenylindole (DAPI)] (magnification ×40). Scale bars indicate 20 μm. D, Intraplaque vessel number was evaluated by manual counting of CD31 positive vessels by 3 individual operators and reported as number±SD of vessels per field (magnification ×20) (*$P<0.05$, experimental versus control values).

activity can be very dependent on the cellular environment and that miRNA-mediated control can display specificity in terms of functional restriction to a particular cellular context or differentiation pathway.[31,32] Probably, this could be the reason why we were not able to see an effect of miR-222 on

c-Kit in our experimental conditions. Moreover, the fact that we did not observe modulations of miR-222 on p27[Kip1], even if this is a validated miR-222 target, with a proven role in SMC proliferation and cell cycle,[15] sustains the possibility that miR-222 effects and/or its targets are cell type dependent.

Understanding the complex network of miRNAs and their targets may provide new tools to develop therapeutic strategies, not only to enhance neovascularization of ischemic tissues, but also to hamper pathological vessel growth. Indeed, in conditions such as atherosclerosis, intimal angiogenesis occurs as part of the adaptive changes known as vasculature remodeling.[1,2] Our finding demonstrating that miR-222 acts as an antiangiogenic miRNA, by controlling STAT5A expression, provides promising perspectives to interfere with deregulated vascular remodeling and atherosclerotic disease progression.

Sources of Funding

Disclosures

None.

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MS: ATVB/2010/206201 R1

SUPPLEMENTAL MATERIAL

MATERIALS AND METHODS

Reagents and Antibodies. M199 medium (endotoxin-tested), RPMI medium, MEM-D-Val medium bovine serum albumin (BSA), bovine calf serum (BCS), Hank's Buffered Salt Solution (HBSS) and collagenase I were from Sigma-Aldrich (St Louis, MO, USA). Bovine calf serum (BCS) (endotoxin-tested) was from HyClone (Logan, UT, USA). Trypsin was purchased from Difco (Detroit, MI, USA). Nitrocellulose filters, HRP-conjugated anti-rabbit IgG and anti-mouse IgG, molecular weight markers, chemiluminescence reagent (ECL) were from Amersham (Braunschweig, Germany). The presence of endotoxin contamination was tested by the Limulus amebocyte assay (concentration was <0.1 ng/ml). Human Interleukin-3 (IL-3) was a gift from Sandoz Pharma Ltd (Basel, Switzerland). Human basic Fibroblast Growth Factor (bFGF), human Macrophage-Colony Stimulating Factor (M-CSF) and LPS were from Invitrogen.

Anti-β-actin, anti-STAT5A (L-20), anti-STAT5B (G-2), anti-p27^{Kip1}, anti-p57 ^{Kip2}, anti-c-Kit and anti-cyclin D1 antisera were obtained from Santa Cruz Biotechnology, Inc., Heidelberg, Germany. Anti-VE-cadherin, anti-αSMA, anti-human CD31-FITC and secondary FITC- or TRITC-conjugated antibodies were from Sigma-Aldrich. Anti-Ki67 antibody was from Dako (Glostrup, Denmark).

Cell Cultures and Cell Proliferation assay. Endothelial cells (ECs) were isolated from human umbilical vein within 4 h from delivery by Trypsin treatment (0.1%), cultured in M199 medium with the addition of 20% BCS and 5 ng/ml of bFGF and used at early passages (II-III). To perform the experiments ECs were synchronized to quiescent state by serum starvation for 12 h (overnight) then the cells were cultured in the presence of 1% BCS, untreated or treated for 24-48-72 h with IL-3 (20 ng/ml) or with bFGF (10 ng/ml). Cell viability was evaluated by trypan blue at the end of each experiment and proliferative activity was assayed by direct cell count, by three individual operators in triplicate, as previously described [1]. Proliferation was evaluated by time course experiments (24,

48 and 72 h). 48 h of IL-3 and bFGF stimulation was used throughout the study. In selected experiments bFGF-treated (data not shown) or IL-3-treated or untreated ECs were transiently transfected with pre-miR negative control, pre-miR-125a-3p, pre-miR-222, pre-miR-221 or pre-miR-126 precursor oligonucleotides or alternatively with anti-miR negative control, anti-miR-221 or anti-miR-222 inhibitor oligonucleotides (Applied Biosystem, Foxter Cyto, CA), according to manufacturer's recommendation and analyzed 48 h later. Where indicated pre-miR-222 precursor was transiently co-transfected with a STAT5A expression vector [2], using the lipofectin method (Invitrogen), according to the vendor's instructions. Human vascular smooth muscle cells (SMCs) were isolated from the umbilical cord within 4 h from delivery. The vascular SMC layer was identified and characterized as described [3]. SMCs were cultured in MEM-D-Val medium plus 10% BCS and used at early passages (II-IV). Serum-starved SMCs were untreated or treated for 48 h with IL-3 (20 ng/ml) or bFGF (10 ng/ml) to evaluate miR-221/222 expression by qRT-PCR. HEK293 human epithelial cells (American Type Culture Collection) were cultured in RPMI medium supplemented with 10% BCS. In selected experiments HEK293 cells were transiently transfected with pre-miR negative control, pre-miR-222 or pre-miR-221 precursor oligonucleotides (Applied Biosystem, Foxter Cyto, CA) according to manufacturer's recommendation and analyzed 48 h later. Peripheral-blood mononuclear cells (PB-MNCs) were isolated by Ficoll Histopaque 1077 (Sigma-Aldrich, St Louis, MO) from blood donors subjects and used directly or upon 7 days of M-CSF (1000 U/ml) or 24 h of LPS (100ng/ml) stimulation to evaluate miR-221/222 expression by qRT-PCR. The institutional review board of the hospital approved the study.

EC migration assay. Migration of ECs was performed in Boyden's chambers, as previously described [4]. Quiescent ECs that passed across the filter (8-µm pore size) 4 h after addition of bFGF (10 ng/ml) or IL-3 (20 ng/ml) in the lower compartment of the chamber or kept in the vehicle alone (saline containing 0.25% BSA), were counted. Where indicated ECs were transfected for 48 h with pre-miR negative control, pre-miR-126, pre-miR-221, pre-miR-222 or pre-miR-222+STAT5A

expression vector or with anti-miR negative control, anti-miR-221 or anti-miR-222 inhibitor oligonucleotides or alternatively with scramble or STAT5A siRNAs and used 48 h later.

RNA Isolation and Quantitative Real-time PCR (qRT-PCR) for miRNAs or STAT5A expression.
Total RNA was isolated using TRIzol Reagent® (Invitrogen) from untreated or treated cells, as indicated, and from human arterial samples- or Matrigel plug-derived ECs, following manufacturer's instructions. The expression of miR-17-5p, miR-21, miR-125a-3p, miR-126, miR-210, miR-221, miR-222 and miR-296 in different experimental conditions was evaluated using the TaqMan microRNA assay kit (Applied Biosystems, Foxter Cyto, CA). Single-stranded cDNA was synthesized from 50 ng of total RNA using looped primers of the TaqMan microRNA assay and TaqMan microRNA reverse transcription kit (Applied Biosystems). Each cDNA generated was amplified by qRT-PCR using sequence-specific primers from TaqMan microRNA assay in the ABI PRISM 7700 Sequence detection system (Applied Biosystem). miRNA expression was normalized to small nuclear RNA, RNU6B. STAT5A mRNA quantification was also performed by qRT-PCR using specific Taq-Man Gene Expression assay (Applied Biosystem). GAPDH gene was used as standard reference.

Northern Blot Analysis. Total RNA was isolated from ECs, treated as indicated, using TRIzol Reagent® (Invitrogen), following manufacturer's instructions. Northern blot analysis was performed according to standard methods as previously described [5]. Filters were hybridized to ^{32}P random-priming labeled DNA probes corresponding to human STAT5A and β-actin cDNAs, washed for 30 minutes in 0.1XSSC and 1% SDS at 52°C and then exposed to X-ray film for 2-4 days.

Validation of miR-126, miR-221 and miR-222 expression in untreated, or IL-3- or bFGF-treated ECs was obtained by Northern blot. Total RNA samples (25μg each) were subjected to 12.5% TBE/urea gel electrophoresis and transferred to a Hybond N+ membrane (Amersham). Membranes were hybridized (45 °C over-night) with hsa-miR-126, hsa-miR-221 or hsa-miR-222 5'-digossigenin-labeled mercury LNA detection probes (Exiqon, Vedbaek, Denmark). U6 snRNA was

used as endogenous control. Hybridization buffer, wash buffer, blocking buffer and detection buffer were supplied by Roche (Basel, Switzerland). The results were visualized on X-ray film.

Western Blot Analysis. Cells were lysed (50mM Tris HCl ph 8.3, 1% Triton X-100, 10 mM PMSF, 100 U/ml aprotinin, 10 µM/ml leupeptin) and protein concentrations were obtained as previously described [3,4]. 50 µg of proteins were subjected to SDS-PAGE, transferred into nitrocellulose membranes, blotted with the indicated antibodies and revealed by chemiluminescence detection system (ECL).

Luciferase miRNA Target Reporter Assay. The luciferase reporter assay was performed using a construct generated by sub-cloning PCR products amplified from 3'UTR full-length of STAT5A, STAT5B, c-Kit and p27[Kip1] mRNA in the SacI restriction site of the luciferase reporter vector pmiR (Ambion, Applied Biosystem). PCR products were obtained using the following primers:

STAT5A: sense, 5'AAGAGCTCATGTTTGAATCCCACGCT3';

antisense,5'TTGAGCTCACACAAATGTGTGGTCTT3';

STAT5B:sense,5'AAGAGCTCTGACCCCGCGACCTCTCCAT3';

antisense,5'AAGAGCTCCCCCTTCAGAGGAAGGCTTTA3';

c-Kit: sense,5'AAGAGCTCGCAGAATCAGTGTTTGGGTCA3';

antisense,5'TTGAGCTCCTTCAGAACTGTCAACAATTGG3';

p27[Kip1]: sense, 5'AGAGCTCCAGATACATCACTGC;

antisense, 5'TGAGCTCTATACTTGGCTCAG3'.

A site-directed mutagenesis of the 3'UTR STAT5A amplified PCR product was performed to obtain the mutated miR-222 binding site. The sequence was generated using the Quik-Change Site-Direct Mutagenesis kit (Stratagene, La Jolla, CA, USA). The oligonucleotide sense, 5'CAG*A*CCTGG*A*A*A*G*A*GG*A*TGCAGCAGCG3', containing the desired mutation, was designed according to the manufacturer's instructions (the mutated nucleotide is bold and italicized). The insert identities were verified by sequencing. The pmiR, pmiR-3'UTR STAT5A, pmiR-3'UTR STAT5B, pmiR-3'UTR c-Kit and pmiR-3'UTR p27[Kip1] luciferase reporter vectors were transiently

co-transfected in ECs, treated as indicated, or in HEK293 cells at 10:1 molar ratio with the pRL vector, coding for the *Renilla* luciferase, used as internal control of the luciferase assay, by the lipofectin method (Invitrogen), according to the vendor's instructions. Luciferase activities were analyzed 48 h after transfection by Dual-Luciferase Report Assay System (Promega), according to vendor's instructions, using a TD20/20 double injector luminometer (Turner Designs, Forlì, IT). The results are expressed as relative luciferase expression (%), calculated by normalizing the ratio of the firefly/renilla luminescences. Luciferase activities, using the pmiR reporter vectors, above described, or the pmiR-3'UTR STAT5A mutated construct (MUT) were also evaluated in ECs or in HEK293 cells 48 h after transfection of pre-miR negative control, pre-miR-221, pre-miR-222 or pre-miR-125a-3p precursor oligonucleotides or in ECs after transfection of anti-miR negative control, anti-miR-221 or anti-miR-222 inhibitor oligonucleotides, according to Ambion's instructions.

Endogenous depletion of STAT5A by Small Interfering RNAs (siRNAs). To obtain inactivation of endogenous STAT5A, IL-3-cultured ECs were transiently transfected with non specific (scramble) or STAT5A siRNAs purchased by Qiagen (Valencia, CA, USA). Transfection was performed according to the vendor's instructions. 48 h later whole cell extracts were prepared and processed for Western blot. STAT5A depleted ECs were also used to perform cell proliferation or migration assays. Cell viability was evaluated at the end of the experiments.

In Vivo Experiments. For angiogenesis assay SCID mice (five mice for each experimental group) were injected s.c. with growth factor–reduced Matrigel containing IL-3 (50 ng/ml) or bFGF (50 ng/ml) and 2 x 10^6 ECs, previously transfected with pre-miR negative control or pre-miR-222 precursors, alone or in combination with a STAT5A expression construct, or alternatively with non specific (scramble) or STAT5A siRNAs and processed as described [4-6]. Briefly, four days after injection Matrigel plugs were recovered and fixed in 10% buffered formalin and embedded in paraffin for histological and immunofluorescence analysis or digested for EC isolation. The vessel area and the total Matrigel area were planimetrically assessed from hematoxilin-eosin stained

sections as previously described [4-6]. Only the structures possessing a patent lumen and containing RBC were considered vessels. Angiogenesis was expressed as the percentage±SD of the vessel area to the total Matrigel area (% vessel area, 10X magnification). Quantification of neo-formed vessels was also evaluated by CD31 staining of vascular ECs. Any stained EC or EC cluster, clearly separated from connettive tissue elements, was considered as a single microvessel and counted, according to Weidner et al. [7]. Animal procedures conformed to the Guide for Care and Use of Laboratory Resources (National Institutes of Health publication no. 93-23, revised 1985).

Immunofluorescence Analysis. Sections from paraffin-embedded blocks of Matrigel plugs, containing inflammatory stimuli and ECs, transfected with the indicated constructs, were collected onto poly-Lysine-coated slides and processed as previously described [6]. For immunofluorescence assay, the samples were processed using anti-human CD31-FITC antibody and DAPI (Sigma-Aldrich), as nuclear marker, or double stained with anti-human CD31-FITC, DAPI and anti-Ki67 antibody, as proliferative marker. Human CD31 positive vessels were determined by counting 10 randomly selected fields in three different samples (20X magnification). Sections from paraffin-embedded samples of early and advanced lesions were double stained directly with anti-human CD31-FITC antibody and indirectly with anti-STAT5A and TRITC-conjugated secondary antibody (10X magnification). Samples of advanced lesion were also double stained with anti-human CD31-FITC and anti-Ki67 antibodies. Images were acquired with a Zeiss LSM 5 Pascal confocal laser-scanning microscope (Carl Zeiss, Jena, Germany) equipped with a helium/neon laser (543 mm), an argon laser (450-530 mm), and an EC planar Neofluar 40x/1.3 oil-immersion DIC objective lens. Images were analyzed using Zeiss LSM 5 version 3.2 software.

Isolation of ECs from Matrigel Plugs. ECs were recovered from Matrigel plugs four days after injection into SCID mice. After digestion in HBSS containing 0.1% collagenase I for 30 min at 37°C the cells were washed in medium plus 10% BCS and forced through a graded series of meshes to separate the cell component from Matrigel matrix. ECs were isolated via anti-human CD31 antibody coupled to magnetic beads, by magnetic cell sorting using the MACS system (Miltenyi

Biotech Auburn, CA). Briefly, cells were labeled with the anti-human CD31 antibody for 20 min and then were washed twice and re-suspended in MACS buffer (PBS without Ca^{2+} and Mg^{2+}, supplemented with 1% bovine serum albumin and 5 mmol/L EDTA) at the concentration of 0.5X 10^6 cells/80 µl. After washing, the cells were separated on a magnetic stainless steel wool column (Miltenyi Biotech, Auburn, CA) according to manufacturer's recommendation. The endothelial phenotype of cells was verified by FACS analysis using an anti-vonWillebrand antibody (Sigma-Aldrich: data not shown). The recovered cells were subjected to RNA isolation or lysed for Western Blot analysis.

Human arterial samples and isolation of vascular cells. The approval was obtained from the Institutional Review Board of S. Giovanni Battista Hospital, Turin, Italy. Informed consent was provided according to the Declaration of Helsinki. Human non atherosclerotic and atherosclerotic arteries (renal and carotid arteries) were obtained from informed patients who underwent transplantation or endoarteriectomy. By histological analysis, we classified these into non-diseased (n=5), early lesion (n= 3), fibromuscolar plaques (n=4) and atheromatous plaques (n=5), indicated as advanced lesions, according to the American Heart Association histological criteria. Representative examples of early and advanced lesions were reported in Fig. S7. ECs lining the luminal side and intraplaque neo-formed vessels were double stained with anti-CD31-FITC antibody and anti-STAT5A antibody with TRITC-conjugated secondary antibody. Anti-mouse IgG antibody with anti-TRITC-conjugated secondary antibody were used as negative control. Neointima from fresh samples of advanced specimens or intima from non-atherosclerotic specimens were finely minced with scissors and digested by incubation for 1 hour at 37°C in HBSS containing 0.1% collagenase I. After washing in medium plus 10% BCS cell suspension was forced through a graded series of meshes to separate the cell component from stroma and aggregates. Cells were resuspended and ECs were isolated via anti-human CD31 antibody coupled to magnetic beads, by magnetic cell sorting using the MACS system (Miltenyi Biotech), as above described. Anti-

vonWillebrand antibody was used to verify the endothelial phenotype (data not shown). The recovered cells were used for qRT-PCR and for Western Blot analysis.

Statistical Analysis. All i*n vitro* and *in vivo* results are representative of at least three independent experiments. The *in vitro* experiments were performed in triplicate. Densitometric analysis using a Bio-Rad GS 250 molecular imager was used to calculate the differences in the fold induction of protein expression (*p< 0.05 or **p<0.01, statistically significant between experimental and control values) and each Western blot panel and relative densitometric histogram reported in the figures were representative of the results obtained in triplicate. Significance of differences between experimental and control values was calculated using analysis of variance with Newman-Keuls multicomparison test. Similar statistical analysis was performed in the *in vivo* experiments.

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Supplemental Figure I
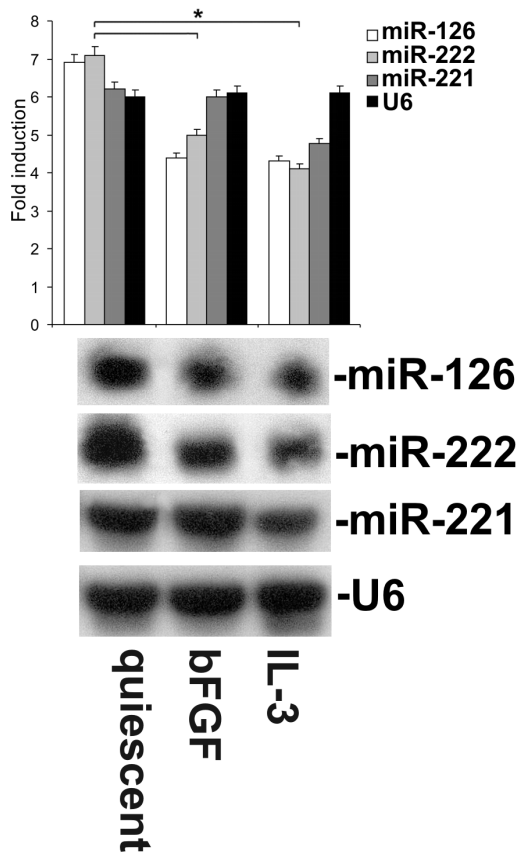
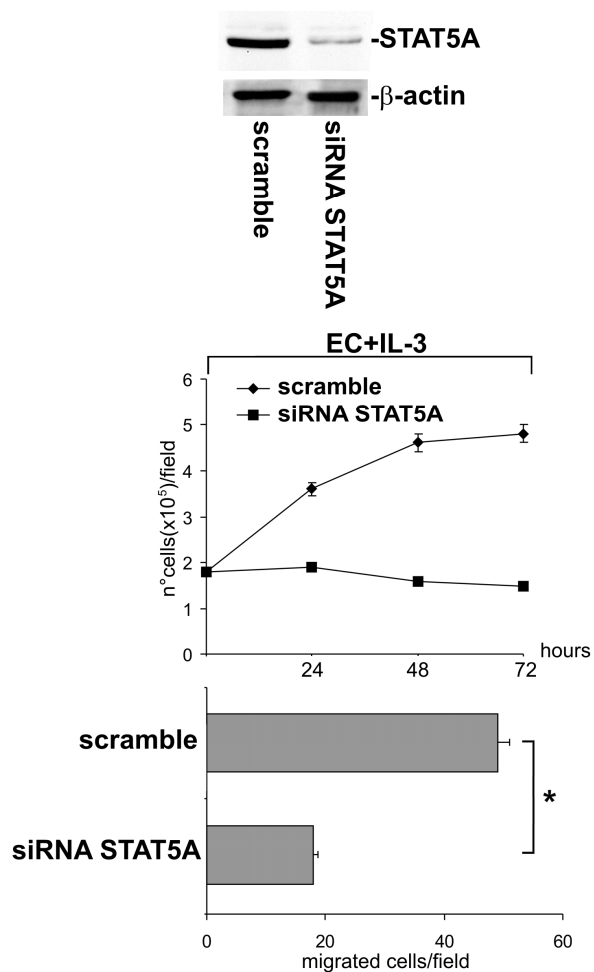**Supplemental Figure I. miRNA expression in ECs exposed to inflammatory stimuli.** Expression of miR-126, miR-221 and miR-222 in quiescent or bFGF- or IL-3-treated ECs was validated by Northern blot analysis. The reported data were normalized to U6. (*$p < 0.05$; experimental vs control values).
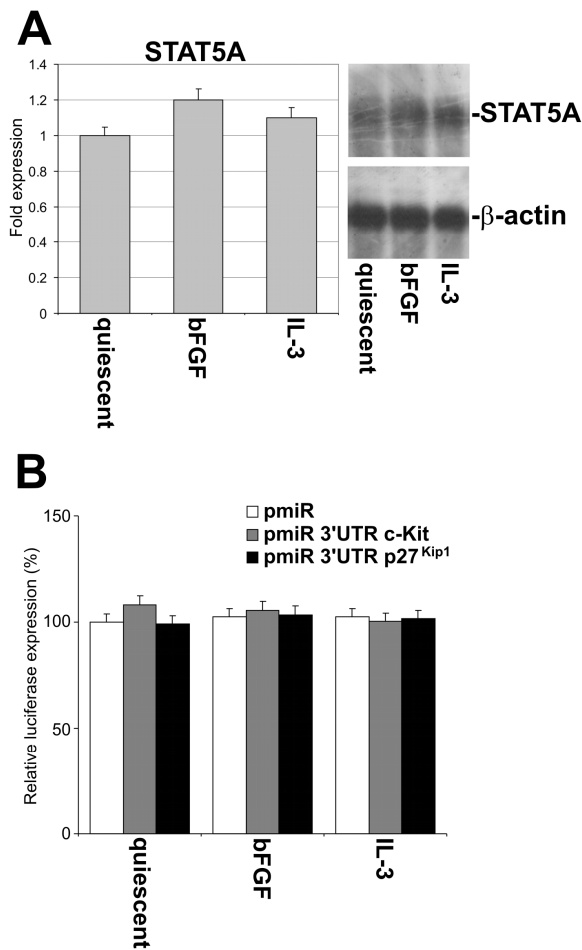
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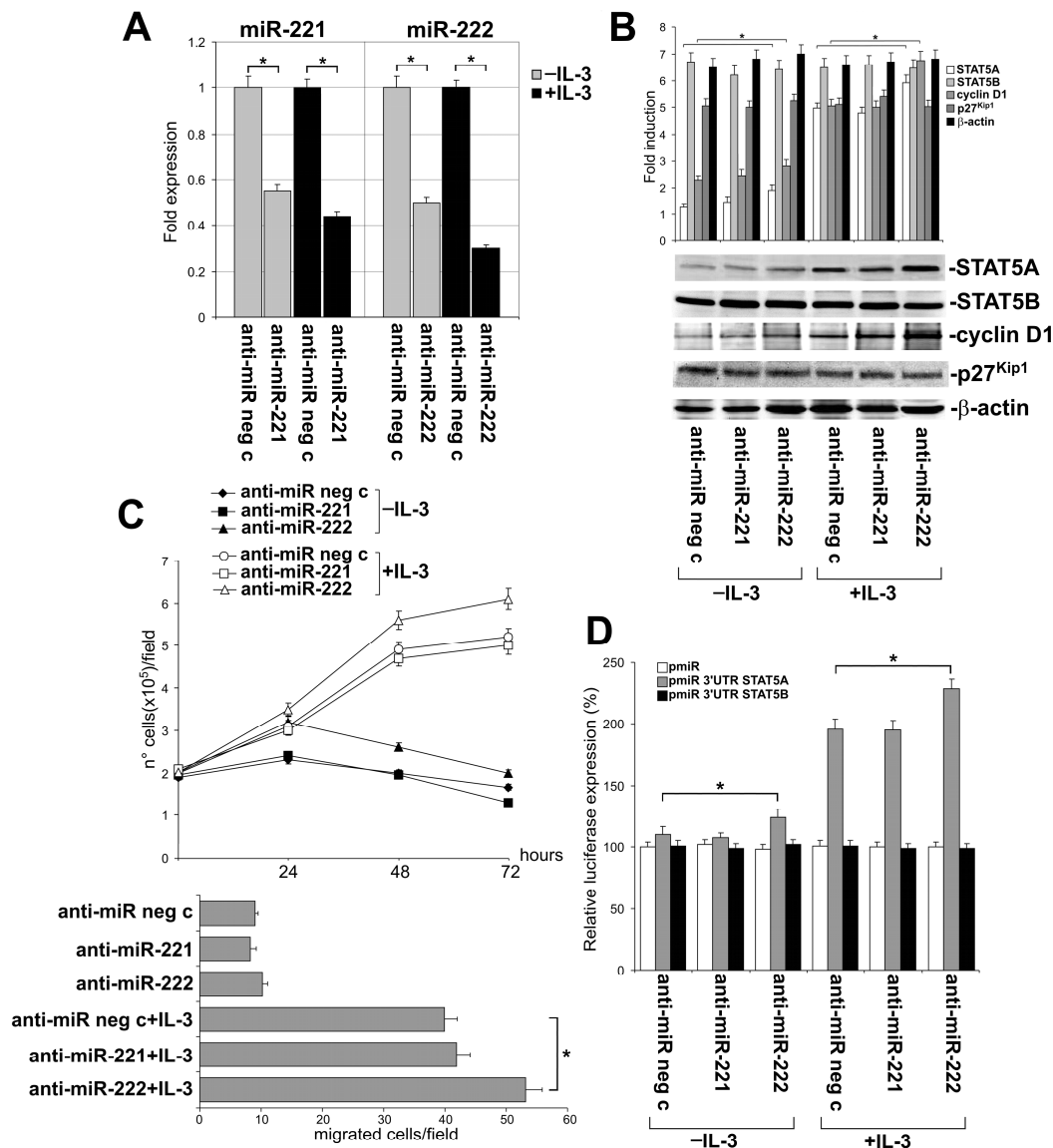
Supplemental Figure II. miR-222 down-regulation further increases EC proliferation and migration only in the presence of IL-3. (A) Expression of miR-221 and miR-222 was analyzed by qRT-PCR on ECs, in the presence or in the absence of IL-3, 48 h after transfection of anti-miR negative control (neg c), anti-miR-221 or anti-miR-222 oligonucleotides. Fold expression of miRNAs was representative of three independent experiments performed in triplicate. **(B)** Untreated or IL-3-cultured ECs transfected with antagonist oligonucleotides, as above, were lysed and analyzed by Western Blot for STAT5A, STAT5B, cyclin D1, p27 Kip-1 and β-actin expression. **(C)** Cell proliferation (upper panel) and cell migration (lower panel) assays were performed in ECs treated as described in B. **(D)** pmiR vector or pmiR-3'UTR STAT5A or pmiR-3'UTR STAT5B luciferase constructs were transfected into ECs treated as above. The relative luciferase activity is reported. (*$p < 0.05$, experimental vs control values).

Supplemental Figure III

Supplemental Figure III. STAT5A depletion phenocopies miR-222 over-expression. (A) ECs were transfected with STAT5A or scrambled sequence (scramble) siRNAs, cultured with IL-3 and lysed. The filters were immunoblotted with anti-STAT5A or anti-β-actin antibodies. **(B)** IL-3-cultured ECs transfected with STAT5A or scramble siRNAs were analyzed for cell proliferation (upper panel) or cell migration (lower panel) assays. (*$p<0.05$, experimental vs control values).
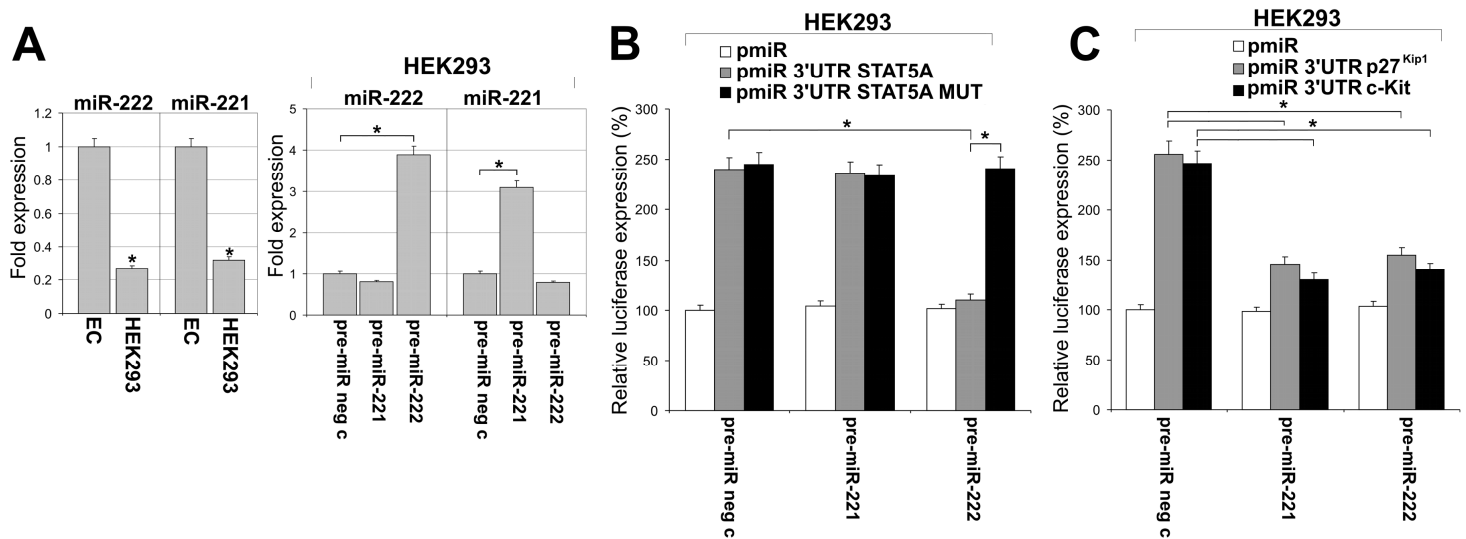
Supplemental Figure IV.

A

B

**Supplemental Figure IV. miR-222 does not affect STAT5A mRNA level and c-Kit or p27** [Kip-1]

luciferase activity (A) qRT-PCR and Northern blot analysis were performed on quiescent or

bFGFor IL-3-stimulated ECs to evaluate STAT5A expression. Data were normalized to GAPDH or

β- actin expression, respectively. Fold expression of STAT5A was representative of three

independent experiments performed in triplicate. **(B)** Luciferase report assay was performed on

ECs, treated as above, 48 h after transfection with pmiR or pmiR-3'UTR c-Kit or pmiR-3'UTR p27

Kip-1 luciferase constructs. Relative luciferase activity (%) was representative of three independent

experiments performed in triplicate.
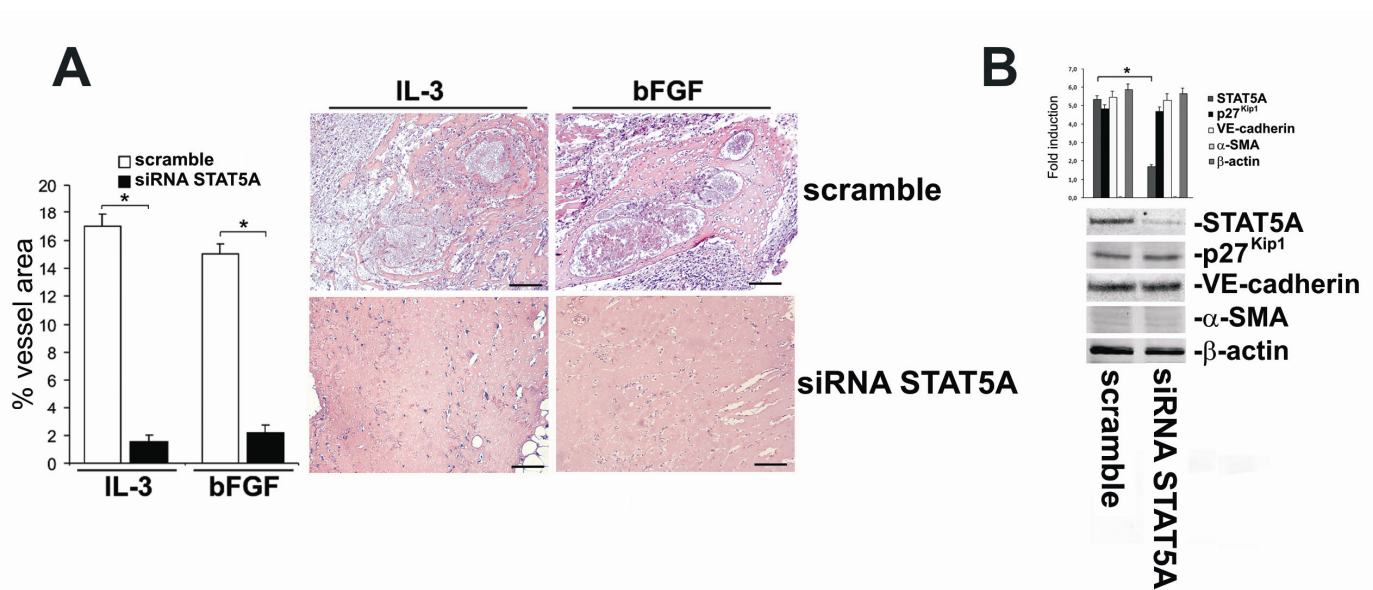
Supplemental Figure V

Supplemental Figure V. STAT5A is a target of miR-222 in HEK293 cells. (A) qRT-PCR was used to measure miR-221 or miR-222 expression in HEK293 cells (left panel). Over-expression of miR-221 or miR-222 in HEK293 cells was obtained 48 h after transfection with miRNAs mimic oligonucleotides as measured by qRT-PCR (right panel). Fold expression of miRNAs was representative of three independent experiments performed in triplicate. **(B)** Luciferase activity was evaluated in HEK293 cells co-transfected with pre-miR negative control (neg c) or pre-miR-221 or pre-miR-222 precursors and pmiR or pmiR-3'UTR STAT5A or pmiR-3'UTR STAT5A mutant binding site (MUT). Relative luciferase activity (%) was representative of three independent experiments performed in triplicate. **(C)** Luciferase activity was evaluated in HEK293 cells cotransfected with pre-miR precursor oligonucleotides as above and pmiR or pmiR-3'UTR p27$_{Kip1}$ or pmiR-3'UTR cKit for 48 h. Relative luciferase activity (%) was representative of three independent experiments performed in triplicate. (*$p<0.05$, experimental vs control values).
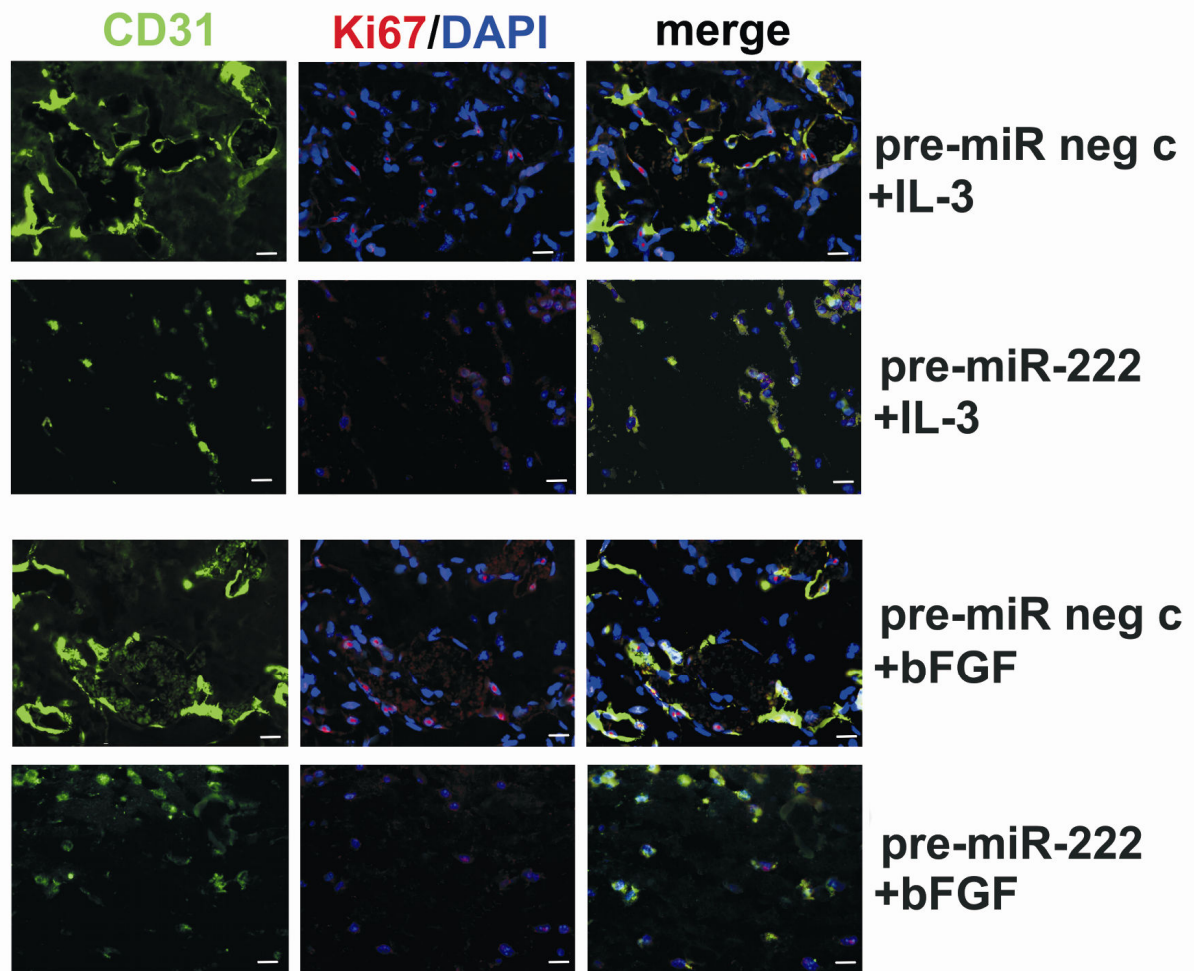
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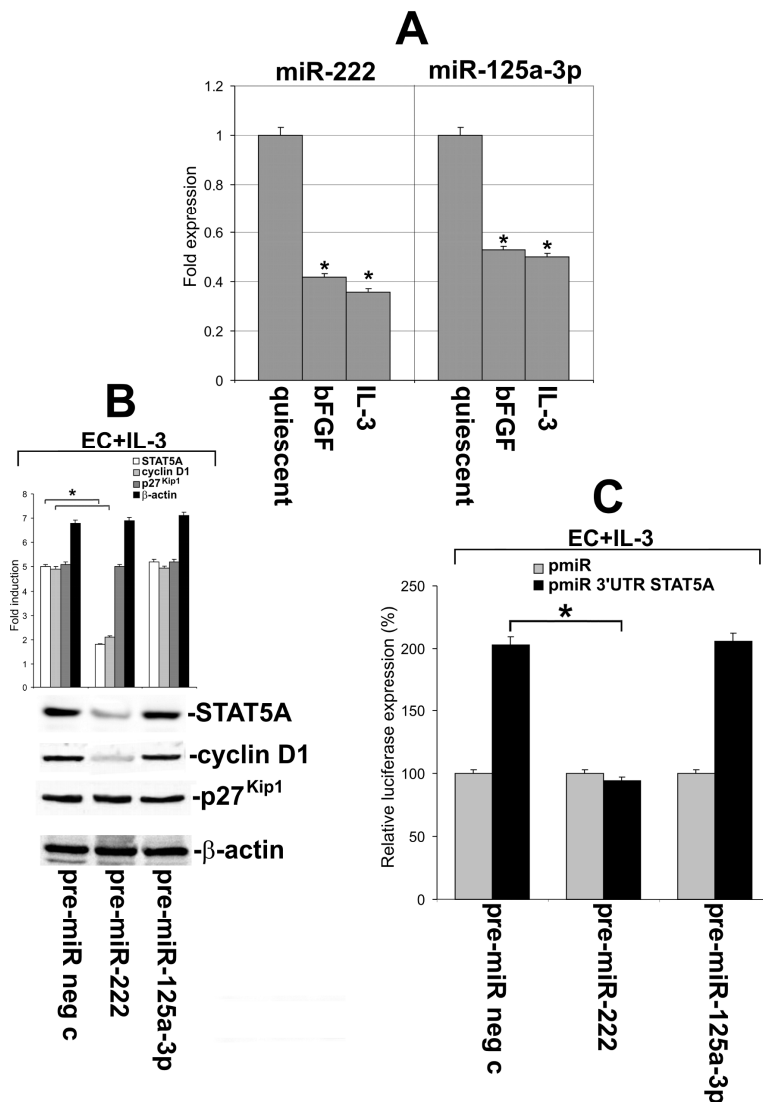
Supplemental Figure VI. miR-125a-3p does not control STAT5A expression in ECs. (A) miR-125a-3p and miR-222 expression was analyzed by qRT-PCR in quiescent or bFGF- or IL-3-treated ECs. Fold expression of miRNAs was representative of three independent experiments performed in triplicate. **(B)** Lysates from pre-miR negative control- (neg c), pre-miR-222- or pre-miR-125a-3p transfected ECs, cultured in the presence of IL-3, were analyzed by Western blots with the indicated antibodies. **(C)** Luciferase activity was evaluated in IL-3-treated ECs 48 h after co-transfection with the indicated pre-miRNA oligonucleotides and pmiR or pmiR-3'UTR STAT5A. Fold expression of miRNAs and relative luciferase activity (%) were representative of three independent experiments performed in triplicate. (*p<0.05, experimental vs control values).
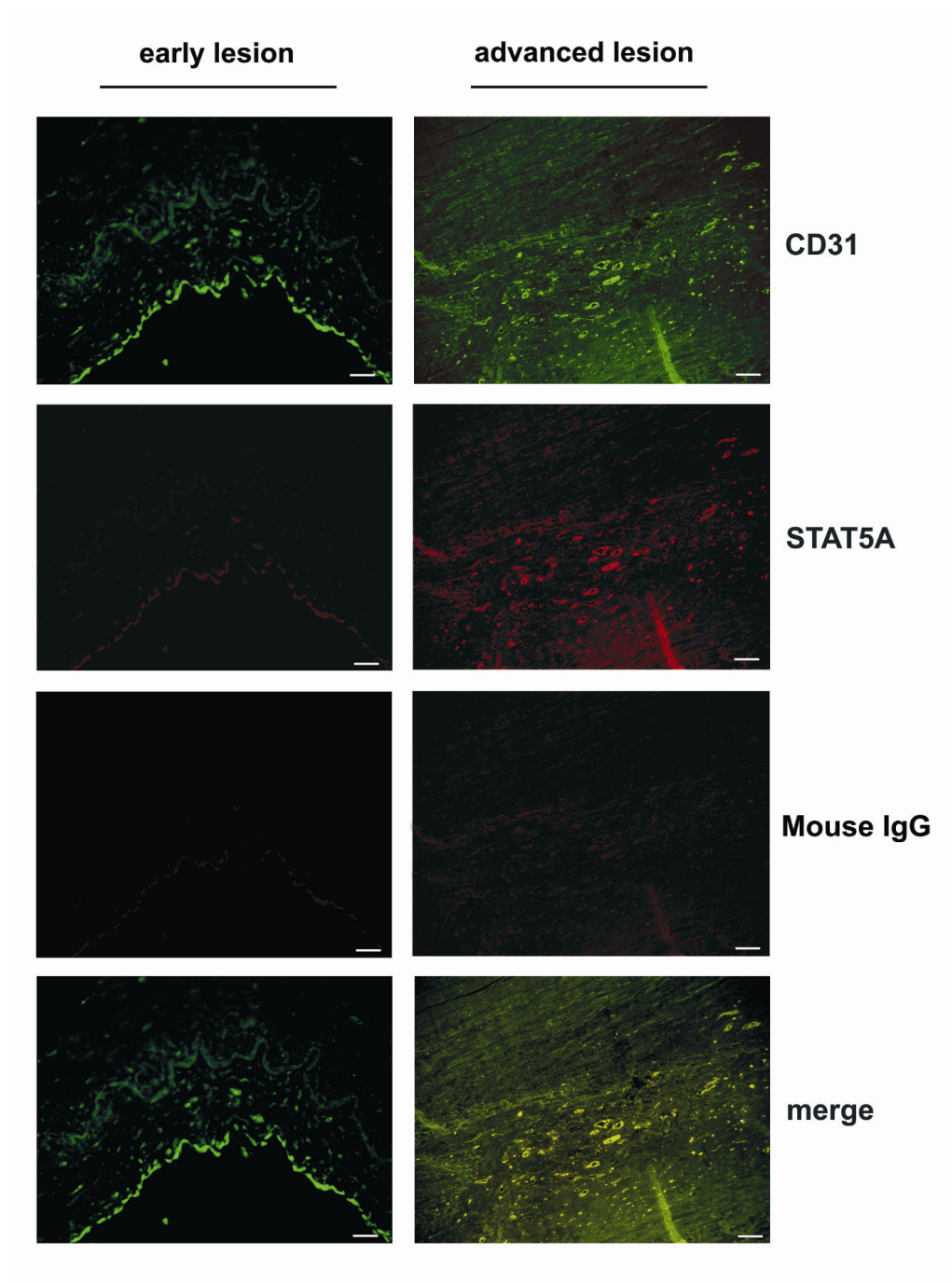
Supplemental Figure VII

Supplemental Figure VII. STAT5A silencing replicated miR-222 over-expressing phenotype *in vivo*. (A) Sections of Matrigel plugs, containing IL-3 or bFGF and ECs transfected with scramble or STAT5A siRNAs were recovered 4 days after implantation in SCID mice and subjected to histological analysis (10X magnification). Quantification of neo-formed vessels was expressed as percentage ±SD of the vessel area to the total Matrigel area. Scale bars indicate 200 μm. **(B)** ECs, recovered by CD31-positive magnetic sorting from the Matrigel plugs described above, were analyzed by Western Blot for STAT5A, p27 Kip1, VE-cadherin, α-SMA and β-actin expression. (*p<0.05, experimental vs control values).

Supplemental Figure VIII.

Supplemental Figure VIII. miR-222 inhibits *in vivo* neo vessel formation by blocking EC proliferation. Sections of Matrigel plugs from SCID mice containing IL-3 or bFGF and ECs transfected with pre-miR negative control (neg c) or pre-miR-222 oligonucleotides were analyzed by immunofluorescence using anti CD31 or Ki67 antibodies (40X magnification). DAPI staining was used as nuclear marker. Scale bars indicate 20 μm.

Supplemental Figure IX.

Supplemental Figure IX. ECs lining intra-plaque neo vessels express STAT5A. Representative samples of early and advanced human lesions double stained with anti CD31 (green) and STAT5A (red) antibodies. Merge (bottom panels); endothelium lining early atherosclerotic lesions expressing CD31 but not STAT5A are green stained; ECs lining intra-plaque vessels in advanced atherosclerotic lesions that co-express CD31 and STAT5A are yellow stained (10X magnification). Anti-mouse IgG antibody was used as negative control. Scale bars indicate 200 μm.