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Basic science

HOXA5 is a key regulator of class 3 semaphorins expression in the synovium of rheumatoid arthritis patients

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Abstract

Objective: Class 3 semaphorins are reduced in the synovial tissue of RA patients and these proteins are involved in the pathogenesis of the disease. The aim of this study was to identify the transcription factors involved in the expression of class 3 semaphorins in the synovium of RA patients.

Methods: Protein and mRNA expression in synovial tissue from RA and individuals at risk (IAR) patients, human umbilical vein endothelial cells (HUVEC) and RA fibroblast-like synoviocytes (FLS) was determined by ELISA, immunoblotting and quantitative PCR. TCF-3, EBF-1 and HOXA5 expression was knocked down using siRNA. Cell viability, migration and invasion were determined using MTT, calcein, wound closure and invasion assays, respectively.

Results: mRNA expression of all class 3 semaphorins was significantly lower in the synovium of RA compared with IAR patients. *In silico* analysis suggested TCF-3, EBF-1 and HOXA5 as transcription factors involved in the expression of these semaphorins. TCF-3, EBF-1 and HOXA5 silencing significantly reduced the expression of several class 3 semaphorin members in FLS and HUVEC. Importantly, HOXA5 expression was significantly reduced in the synovium of RA compared with IAR patients and was negatively correlated with clinical disease parameters. Additionally, TNF-α down-regulated the HOXA5 expression in FLS and HUVEC. Finally, HOXA5 silencing enhanced the migratory and invasive capacities of FLS and the viability of HUVEC.

Conclusion: HOXA5 expression is reduced during the progression of RA and could be a novel therapeutic strategy for modulating the hyperplasia of the synovium, through the regulation of class 3 semaphorins expression.

Keywords: RA, individuals at risk, class 3 semaphorins, HOXA5, TCF-3, EBF-1, fibroblast-like synoviocytes, endothelial cells, cell migration, cell invasion

Rheumatology key messages

- HOXA5 is a key transcription factor for the expression of class 3 semaphorins in RA patients.
- HOXA5 expression is reduced in the synovium of RA patients.
- HOXA5 is involved in the aggressive phenotype of RA FLS and in endothelial cell migration.

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Introduction

RA is one of the most frequent rheumatic and musculoskeletal diseases (RMD), characterized by the presence of circulating autoantibodies, inflammation and progressive destruction of joints, which leads to disability and loss of quality of life, and to a significant healthcare burden for patients [1]. The infiltration of immune cells into the joint, the formation of new blood vessels (angiogenesis) and the abnormal growth of synovial stromal fibroblast-like synoviocytes (FLS) induce the hyperplasia of the synovial membrane, which is the hallmark of this disease. Both immune cells and FLS release a plethora of inflammatory mediators that perpetuate the inflammatory process and promote the destruction of cartilage and bone [2, 3].

Class 3 semaphorins are secreted members of the semaphorin family, which is a large family of proteins essential for the neural development [4]. Recent studies have demonstrated that class 3 semaphorins are involved in the pathogenesis of multiple RMD, including RA [5, 6]. The expression of class 3 semaphorins is reduced in the synovial tissue of RA patients compared with healthy controls and other RMD, such as OA and undifferentiated arthritis. Importantly, expression of class 3 semaphorins is negatively correlated with clinical disease parameters such as the swollen joint count of 28 joints (SJC28) and the tender joint count of 28 joints (TJC28) scores, as well as with the CRP and the ESR [7–10]. Moreover, Sema3A, Sema3B and Sema3F are involved in the aggressive phenotype of RA FLS. In fact, Sema3B and Sema3F reduce migratory and invasive ability of RA FLS [10], while contradictory findings have found either that Sema3A induces RA FLS migration and invasion [10] or that it suppresses it [9]. Finally, *in vivo* studies have demonstrated a protective role of Sema3A and Sema3B in arthritis. Adenoviral and plasmid-mediated overexpression of Sema3A reduces the severity of CIA and K/BxN serum-induced arthritis [7, 9]. Also, Sema3B-deficient mice show a higher arthritis severity, while the administration of recombinant mouse Sema3B reduces the clinical severity of serum-induced arthritis [11].

The expression of class 3 semaphorins is regulated by different transcription factors, such as GATA3, SOX4, SOX11, p53, FOXM1 and RORα [12–17]. However, the transcription factors involved in the regulation of class 3 semaphorins in RA synovium are still unknown. Here, we confirmed that the expression of class 3 semaphorins is reduced in the synovial tissue of RA patients and that HOXA5 is a key transcription factor involved in the expression of these semaphorins by FLS and endothelial cells.

Materials and methods

Synovial tissue

Synovial biopsies were obtained at the St Vincent's University Hospital, Dublin, Ireland from individuals at risk (IAR) ($n = 8$) [18], defined as subjects with symptoms of aches and pains, without joint swelling, raised (CRP <5 mg/l) but with positive circulating RF+ and ACPA, and from RA patients ($n = 10$). Synovial biopsies were obtained by needle arthroscopy from the knee joints, as previously described [19]. RA patients fulfilled the ACR/EULAR 2010 ACR/EULAR criteria [20, 21]. Clinical characteristics of patients are detailed in Supplementary Table S1, available at *Rheumatology* online. All patients supplied written informed consent prior to inclusion in the study, and this study was approved by the Institutional Ethics Committees of St Vincent's University Hospital UCD.

RA FLS and human umbilical vein endothelial cells culture and stimulation

FLS were derived from synovial tissue specimens obtained from patients with RA by needle arthroscopy. All patients fulfilled the criteria for the classification of RA and had active disease, including clinical arthritis of the joint from which the synovial biopsies were obtained [20, 21]. FLS were cultured in DMEM (Invitrogen, Nieuwegein, The Netherlands) supplemented with 10% fetal bovine serum (FBS, Biowest, Amsterdam, The Netherlands) and 10 000 I.E penicillin-streptomycin (Thermo Fisher Scientific, Nieuwegein, The Netherlands) and used between passages 5 and 10. Human umbilical vein endothelial cells (HUVEC, Lonza, Basel, Switzerland) were cultured in EBM medium supplemented with EGM bullet kit (both Lonza).

Before stimulation, FLS were cultured overnight in medium containing 1% FBS. FLS and HUVEC were stimulated with IL-1β, (1 ng/ml, R&D Systems, Abingdon, UK) or TNF-α (10 ng/ml, Sigma-Aldrich, Zwijndrecht, The Netherlands) for 4, 24 or 48 h. Alternatively, FLS were pre-incubated with the specific inhibitors for ERK (PD98059, 10 μM, Sigma-Aldrich), p38 (SB203580, 10 μM, Sigma-Aldrich), JNK (SP600125, 10 μM, Sigma-Aldrich), PKB (Wortmanin, 10 μM, Sigma-Aldrich) and nuclear factor (NF)-κB (PDTC, 10 μM, Sigma Aldrich) for 1 h and stimulated with TNF-α (10 ng/ml) for 4 h.

Alternatively, RA FLS were stimulated with the SF of RA patients (10% v/v), previously pre-incubated in the presence or absence of the TNF inhibitor etanercept (10 μg/ml, Amgen, Breda, The Netherlands) for 1 h at 37°C.

siRNA transfection

RA FLS and HUVEC were transfected using DharmaFECT1 (Thermo Scientific). Six hours before transfection, RA FLS were incubated with DMEM containing 10% FBS, which was then replaced with antibiotic-free medium and cells were incubated for 6 h at 37°C. TCF-3, EBF-1, HOXA-5 and control non-targeting siRNAs (100 nM, Thermo Scientific) were mixed with DharmaFECT1 and incubated for 20 min at room temperature prior to transfection for 24 h in OPTI-MEM serum-reduced medium (Dharmafect). Alternatively, HUVEC were transfected for 6 h in OPTI-MEM serum-reduced medium with HOXA5 and control non-targeting siRNAs (100 nM). Experiments were performed 48–72 h after transfection and efficiency of transfections are shown in Supplementary Fig. S1, available at *Rheumatology* online.

RT-PCR and quantitative PCR

RNA from FLS, HUVEC and synovial tissue was isolated using the RNeasy Kit and RNase-Free DNase Set (Qiagen, Hilden, Germany). Total RNA was reverse-transcribed using iScript (Biorad, Hercules, CA, USA). Duplicate PCRs were performed using SYBR green (Applied Biosystem, Foster City, CA, USA) with a StepOnePlus™ Real-Time PCR detection system (Applied Biosystems, Foster City, CA, USA). cDNA was amplified using specific primers [Integrated DNA Technologies, Inc. (IDT), Coralville, IA, USA; Supplementary Table S2, available

at *Rheumatology* online]. Relative levels of gene expression were normalized to expression of 3 housekeeping genes (*B2M*, *RPL13* and *RPL32*) in synovial tissue and to expression of *B2M* in FLS and HUVEC. The relative expression and the relative quantity (RQ) of mRNA were calculated by using the formulas $2^{-\Delta Ct} \times 1000$ and $2^{-\Delta\Delta Ct}$, respectively.

Identification of transcription factor

Identification of transcription factors for co-regulated genes was performed using oPOSSUM-3 web-accessible software system (http://opossum.cisreg.ca) [22].

HOXA5 expression from profiling data

HOXA5 gene expression was retrieved from array profiling data available on the Gene Expression Omnibus (GEO–NCBI; GSE55235, GSE48780 and GSE112658).

Immunoblotting

FLS and HUVEC were lysed in Laemmli's buffer. Equal amount of total protein was subjected to electrophoresis on NuPAGE™ 4-12% Bis-Tris gels (Invitrogen) and proteins were transferred to PVDF membranes (Millipore, Molsheim, France). Membranes were incubated overnight at 4°C in primary antibodies specific for histone3 (H3), TCF-3 (Cell Signalling), EBF-1 (R&D), alpha-tubulin and HOXA5 (Sigma-Aldrich). Membranes were then washed and incubated in TBS/T containing horseradish peroxidase–conjugated secondary antibody. Protein was detected with Lumi-light^{plus} Western Blotting Substrate (Roche Diagnostics, India-napolis, IN, USA) using a ChemiDoc™ MP System (Biorad). Densitometry analysis was performed with ImageJ software. Relative protein expression was normalized to H3 or tubulin.

Sema3B and Sema3F measurement

Sema3B and Sema3F protein levels were measured by ELISA (Biomatik, Huissen, The Netherlands) in cell-free supernatants from RA FLS, according to the manufacturer's instructions.

Immunohistochemistry

Serial sections from paraffin-embedded biopsy samples of IAR ($n = 5$) and RA patients ($n = 5$) were cut with a microtome (5 μm), deparaffinized and rehydrated. After heat-induced epitope retrieval for 20 min at 80°C in EDTA Buffer (1 mM, pH 8.0), endogenous peroxidase and streptavidine/biotin activities were blocked with 3% hydrogen peroxide in PBS and Streptavidin/Biotin Blocking Kit (Vector Laboratories), respectively. Sections were blocked with 5% goat serum and stained overnight at 4°C with anti-HOXA5 Ab (5 µg/ml, Abcam, Cambridge, UK). Equivalent concentration of rabbit serum was used as negative control. Sections were then washed, incubated with goat anti-rabbit IgG (H + L) Biotin (Dako, Amsterdam Netherlands), washed and incubated with VECTASTAIN Elite ABC-HRP KIT (Vector Laboratories, Amsterdam Netherlands). Finally, sections were developed with AEC$^+$ Substrate-Chromogen (Vector Laboratories), counterstained with Mayer's haematoxylin solution (Sigma-Aldrich) and mounted in Entellan mounting medium (Merck).

Viability assay

Transfected FLS were incubated with PrestoBlue™ Cell Viability Reagent (1:10 v/v, Invitrogen) for 10 min, followed by fluorescence measurement (excitation range 560 nm, emission range 590 nm). HUVEC viability was assessed by the calcein assay. Transfected HUVEC were incubated with Calcein-AM (1 μM, Invitrogen) for 2 h, followed by fluorescence measurement (excitation range 490 nm, emission range 520 nm).

MTT assay

Transfected FLS were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 1 mg/ml, Sigma-Aldrich) for 2 h, followed by solubilization of cells in acidified isopropanol solution containing 0.1% Igepal CA-630 (Sigma-Aldrich) and absorbance measurement at 590 nm.

Migration assay

Cell migration was determined using a wound closure motility assay. A linear scratch was made of cultured FLS plated at confluence using a 200-μl micropipette tip and then washed with PBS to remove unattached cells. FLS were cultured in medium containing 1% or 10% FBS. Light microscopy images were taken immediately (time point 0) and 24 h after wounding. The number of migrated cells was averaged from three 10-field-of-view images and normalized to non-stimulated cells.

Invasion assays

The permeable supports of 24-well plates with 8 µM transparent PET membranes (Corning, Madrid, Spain) were coated with Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (3 mg/ml, Corning). To measure cell invasion, 3×10^4 FLS in medium containing 1% FBS were added to the transwells. Medium supplemented with 10% FBS was used as an attractant in the lower chamber. After 24 h, the cells that invaded through the matrix were fixed and stained with crystal violet 0.5%. The number of invading cells was averaged from three 10-field-of-view images and normalized to FLS transfected with scrambled control (Sc) siRNA.

Statistical analyses

Statistical analysis was performed using SPSS v25 software (SPSS, Chicago, DE, USA) and Windows GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA). Potential differences between experimental groups were analysed by parametric two-tailed, paired Student's *t*-test or ANOVA test, or non-parametric, Kruskal–Wallis test or Friedman test, as appropriate. *P*-values <0.05 were considered statistically significant.

Results

Expression of class 3 semaphorins is reduced in the synovial tissue and circulation of established RA patients

In a recent study, we reported a reduced expression of Sema3B in the synovial tissue and serum of established RA patients compared with IAR patients [11]. Here, we first analysed the expression of the other class 3 semaphorins in the same synovial tissue samples and we observed a significant reduction in the mRNA expression of *SEMA3A*, *SEMA3C*, *SEMA3E*, *SEMA3F* and *SEMA3G* in RA patients (Fig. 1).

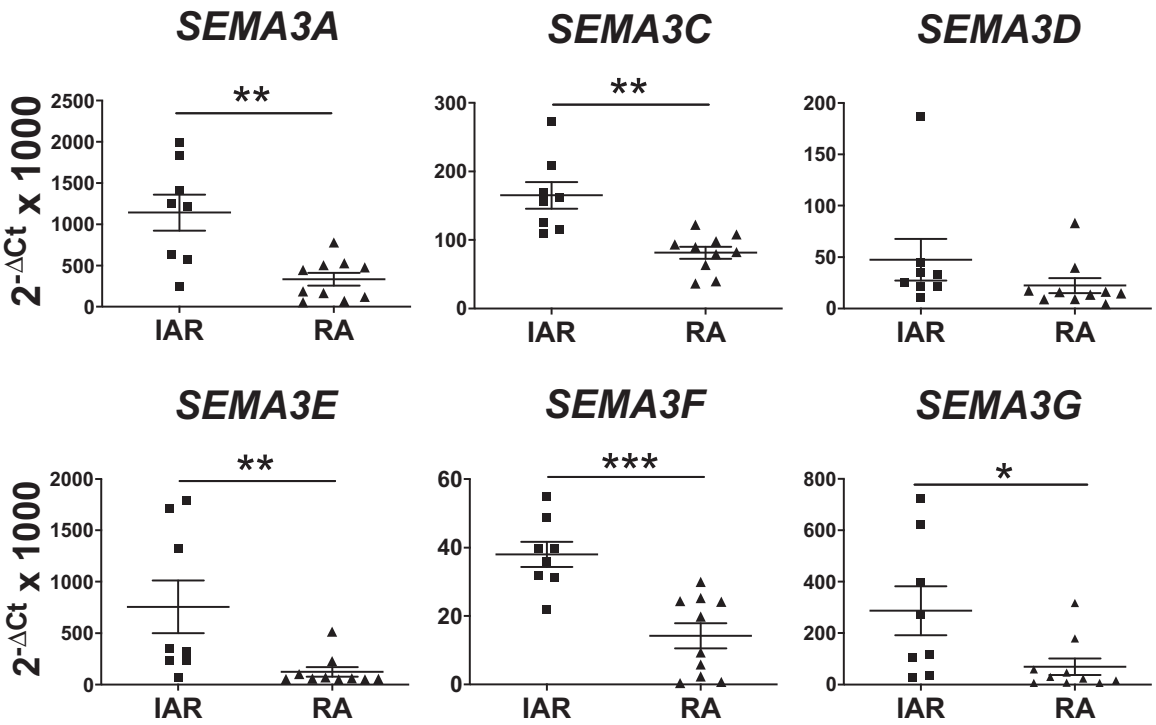

Figure 1. Class 3 semaphorins expression is reduced in the synovial tissue of RA patients. Baseline mRNA expression of class 3 semaphorins in the synovial tissue of IAR ($n = 8$) and RA patients ($n = 10$). Means and SEM are shown. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. IAR: individuals at risk

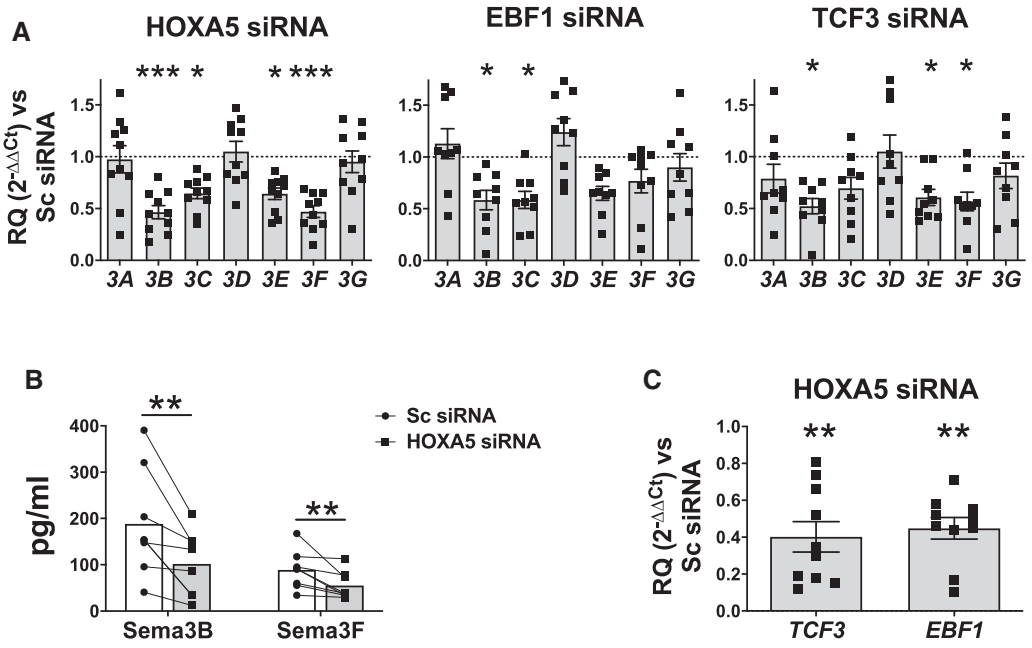

Figure 2. HOXA5 silencing down-regulates the expression of class 3 semaphorins in RA FLS. (**A**) mRNA expression of class 3 semaphorins in RA FLS ($n = 9$–10) transfected with scrambled control (Sc), HOXA5, TCF-3 or EBF-1 siRNA. Data are shown as RQ with respect to Sc siRNA–transfected cells. (**B**) Sema3B and Sema3F secretion by RA FLS ($n = 7$) transfected with Sc or HOXA5 siRNA. (**C**) mRNA expression of *TCF3* and *EBF1* in RA FLS ($n = 10$) transfected with Sc or HOXA5 siRNA. Data are shown as RQ with respect to Sc siRNA–transfected cells. Means and SEM are shown. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. FLS: fibroblast-like synoviocytes; RQ: relative quantity

HOXA5 regulates the expression of class 3 semaphorins by RA FLS

The reduced mRNA expression of most of the class 3 semaphorins members in the synovium of RA patients suggested that a common transcription factor might be involved in the regulation of the expression of these proteins. Analysis of binding sites for the promoter regions of class 3 semaphorins retrieved a list of potential transcription factors (Supplementary Table S3, available at *Rheumatology* online). Two of these transcription factors, FOXI and Nkx5, were not expressed in the synovium of RA or IAR patients, while FOXA1 was not expressed by RA FLS (data not shown), the primary cell type responsible for the

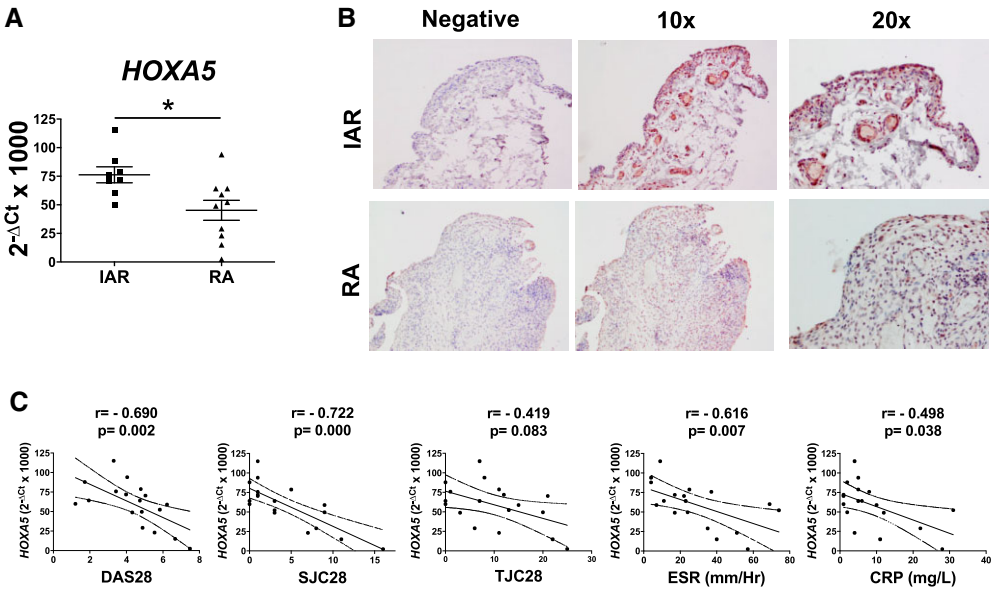

Figure 3. HOXA5 expression is reduced in the synovial tissue of RA patients and negatively correlates with clinical disease parameters. (**A**, **B**) mRNA (A) and protein (B) expression of HOXA5 in the synovial tissue of IAR ($n = 8$) and RA patients ($n = 10$). (**C**) Correlation analysis of HOXA5 mRNA expression with clinical disease parameters in the synovial tissue of IAR and RA patients. mRNA expression is shown as $2^{-\Delta Ct} \times 1000$. Means and SEM are shown. *$P < 0.05$. DAS28: Disease Activity Score 28); SJC28: swollen 28-joint count); TJC28: tender 28-joint count; IAR: individuals at risk

expression of class 3 semaphorins in synovial tissue [10]. Then we focused in HOXA5, TCF-3 and EBF-1 and we analysed the effect of silencing these transcription factors on the expression of class 3 semaphorins by RA FLS. HOXA5 silencing significantly reduced the expression of *SEMA3B*, *SEMA3C, SEMA3E* and *SEMA3F*. TCF-3 silencing significantly down-regulated the expression of *SEMA3B*, *SEMA3E* and *SEMA3F*, while EBF-1 silencing reduced the transcription of *SEMA3B* and *SEMA3C* (Fig. 2A). We attempted to validate these findings at the protein level and observed that HOXA5 silencing reduced the expression of Sema3B and Sema3F by RA FLS (Fig. 2B). Unexpectedly, HOXA5 does not have binding site to the promoter site of *SEMA3B*. However, *in silico* analysis showed that HOXA5 is an important transcription factor involved in the regulation of TCF-3 and EBF-1 (Supplementary Table S4, available at *Rheumatology* online). We confirmed this *in vitro*, as the silencing of HOXA5 significantly down-regulated RA FLS expression of *TCF3* and *EBF1* (Fig. 2C). These data suggest that HOXA5-mediated *SEMA3B* transcription is mediated indirectly through the regulation of the transcription factors TCF-3 and EBF-1.

HOXA5 expression is reduced in the synovial tissue of RA patients

As class 3 semaphorin expression is reduced during the progression of RA, we next analysed the HOXA5 expression in the synovial tissue of IAR and RA patients and we found that mRNA expression was reduced in RA compared with IAR patients (Fig. 3A). Immunohistochemistry analysis of the IAR synovium showed that HOXA5 was mainly expressed in the intimal lining and blood vessels. In the case of RA patients, HOXA5 was also expressed in the sublining. Importantly, the expression of HOXA5 was reduced in the synovial tissue of RA patients, confirming the results found at the mRNA level (Fig. 3B). As expected, there was a positive correlation between the expression of *HOXA5* and the levels of *SEMA3B*, *SEMA3C*, *SEMA3E* and *SEMA3F*, as well as the levels of *EBF1* and *TCF3* (Supplementary Fig. S2, available at *Rheumatology* online). Importantly, *HOXA5* expression showed a strong and significant negative correlation with clinical disease parameters, such as DAS of 28 joints (DAS28) and SJC28 scores, and a trend in the case of TJC28 scores. A significant negative correlation was also observed between the expression of *HOXA5* and ESR and CRP levels (Fig. 3C).

We next compared HOXA5 expression levels in the synovial tissue of healthy controls, OA and RA patients. For this purpose, we made use of publicly available array profiling data from Woetzel and colleagues (GSE55235) [23] and we found that the *HOXA5* expression was significantly reduced in the synovial tissue of RA patients compared with the synovium of healthy controls and OA patients (Supplementary Fig. S3A, available at *Rheumatology* online). Interestingly, in an additional array profile (GSE48780) [24] the expression of *HOXA5* was lower in the inflamed sections of RA synovium compared with non-inflamed sections, although differences were not significant (Supplementary Fig. S3B, available at *Rheumatology* online). Finally, we compared the expression of *HOXA5* in FLS and found a significantly reduced expression in RA FLS compared with OA FLS (Supplementary Fig. S3C available at *Rheumatology* online, GSE112658) [25].

Together, these data confirm the association in expression between HOXA5 and several class 3 semaphorin members and suggest a down-regulation of HOXA5 expression during the inflammatory processes observed in the pathogenesis of RA.

Inflammatory mediators reduce the expression of HOXA5 by RA FLS

We next determined whether TNF-α and IL-1β, both key mediators in the pathogenesis of RA [26, 27], regulate the expression of HOXA5. Stimulation of RA FLS with either IL-1β or TNF-α, significantly down-regulated the expression of *HOXA5* (Fig. 4A). At the protein level, we also found that TNF-α significantly reduces the expression of HOXA5 (Fig. 4B and C). We determined which signalling pathway is involved in the TNF-α-induced HOXA5 down-regulation and we found that PKB inhibition abrogated this

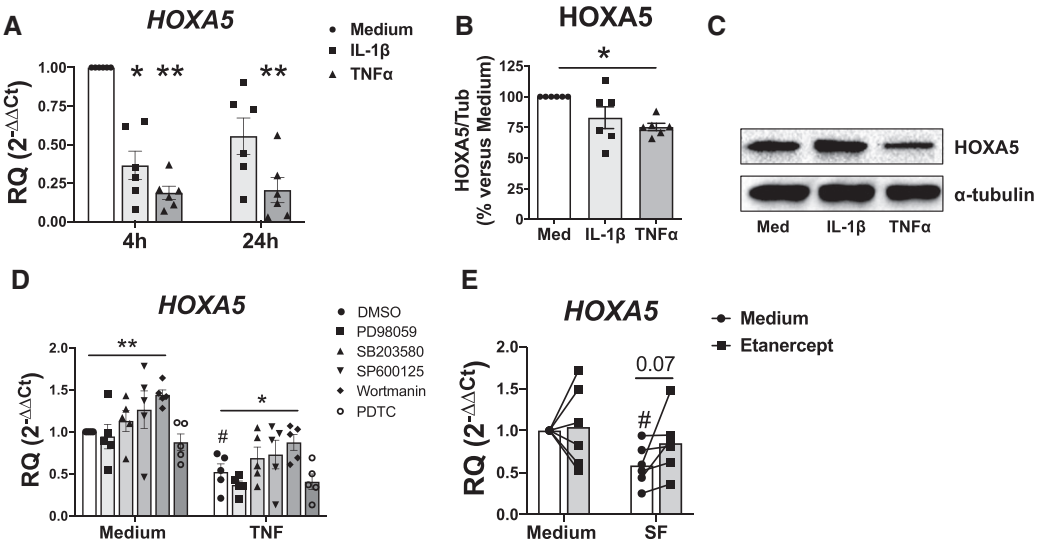

Figure 4. HOXA5 silencing down-regulates the expression of class 3 semaphorins in RA FLS. (**A**) mRNA expression of *HOXA5* in RA FLS ($n=6$) stimulated 4 h and 24 h with IL-1β (1 ng/ml) or TNF (10 ng/ml). Data is shown as RQ with respect to unstimulated cells. (**B**, **C**) Densitometric analysis (B) and representative immunoblot (C) of HOXA5 protein expression in FLS ($n=6$) stimulated 24 h with IL-1β (1 ng/ml) or TNF (10 ng/ml). Data is shown as relative expression with respect to tubulin expression. (**D**) mRNA expression of *HOXA5* in RA FLS ($n=5$) pre-incubated 1 h with inhibitors for ERK (PD98059), p38 (SB203580), JNK (SP600125), PKB (Wortmanin) and NF-κB (PDTC), and stimulated with TNFα (10 ng/ml) for 4 h. (**E**) mRNA expression of *HOXA5* in RA FLS ($n=6$) stimulated 4 h with the SF of RA patients (10% v/v), previously pre-incubated 1 h with etanercept (10 μg/ml). Data are shown as RQ respect to unstimulated cells. Means and SEM are shown. $^{\#}P<0.05$, as compared with medium. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. FLS: fibroblast-like synoviocytes; RQ: relative quantity

down-regulation, while the inhibition of ERK, p38, JNK and NF-κB had no effect (Fig. 4D). These data indicate that PKB activation is involved in the reduced expression of HOXA5. As HOXA5 is reduced in the synovium of RA patients, we also analysed whether SF modulates the expression of HOXA5. RA FLS stimulation with the SF of RA patients significantly down-regulated the expression of *HOXA5*. Interestingly, pre-incubation of SF with a TNF-α inhibitor (etanercept) abrogated this effect on HOXA5 expression (Fig. 4E), demonstrating that inflammatory mediators present in SF reduce the expression of HOXA5 in RA FLS.

HOXA5 regulates the expression of class 3 semaphorins in endothelial cells

As HOXA5 is also expressed in synovial blood vessels (Fig. 3B) we determined whether inflammatory mediators also modulate the expression of endothelial HOXA5 and whether class 3 semaphorin expression is also regulated by HOXA5 in this cell type. Similar to results found in RA FLS, TNF-α stimulation significantly reduced the mRNA and protein expression of HOXA5 in HUVEC (Fig. 5A–C). Importantly, the silencing of HOXA5 reduced the expression of *SEMA3B* and *SEMA3F* by this cell type (Fig. 5D). In contrast to FLS, *SEMA3C* and *SEMA3E* are not expressed in this cell type. These data demonstrate that HOXA5 in an important regulator of class 3 semaphorin expression in both endothelial cells and FLS.

Functional consequences of HOXA5 silencing

Finally, we determined the functional consequences of HOXA5 silencing in RA FLS and endothelial cells. HOXA5 silencing did not modulate the viability or metabolic activity of RA FLS (Fig. 6A and B). As class 3 semaphorins regulate RA FLS cell migration and invasiveness [10], we analysed the effect of HOXA5 silencing on these processes. HOXA5 siRNA-transfected FLS demonstrated a significantly higher migratory capacity and a trend towards an enhanced invasiveness induced by FBS, although differences were not significant (Fig. 6C and D). In HUVEC, HOXA5 silencing had no effect on cell viability, but increased cell migration (Fig. 6E and F). These results suggest that HOXA5 regulates FLS migration and invasion, and endothelial cell migration, through the regulation of class 3 semaphorins expression.

Discussion

In this study, we demonstrated that HOXA5 is a key regulator of class 3 semaphorins expression in RA FLS and HUVEC, and that the reduced HOXA5 expression observed in the synovial tissue of RA patients may contribute to the aggressive phenotype of RA FLS and to the pathogenic angiogenesis found in the synovium of these patients.

First, we found that class 3 semaphorins expression is reduced during the progression of RA, confirming previous results from our and other groups [7–11]. The reduced expression in 5/6 of class 3 semaphorins members suggests a common transcription factor involved in their expression. Previous studies have shown that several transcription factors regulate the expression of different class 3 semaphorins, such as GATA3, SOX11, p53 (Sema3B) [12–14]; FOXM1 [15], SOX4 (Sema3C) [15, 17]; RORα (Sema3E) [16]; or NR4A family members, which are involved in the expression of Sema3C and Sema3E [28, 29]. However, to our knowledge, there are no studies showing a transcription factor responsible for more than two Sema3 family members. Here, we demonstrate that HOXA5 is a key regulator of *SEMA3B*, *SEMA3C*, *SEMA3E* and *SEMA3F* transcription in RA FLS, and *SEMA3B* and *SEMA3F* in HUVEC. HOXA5-mediated *SEMA3C*, *SEMA3E* and *SEMA3F* expression is likely due to a direct interaction, as HOXA5 has binding sites in the promoter regions of these semaphorins. However, HOXA5 lacks binding sites in the *SEMA3B* promoter. Our data indicate

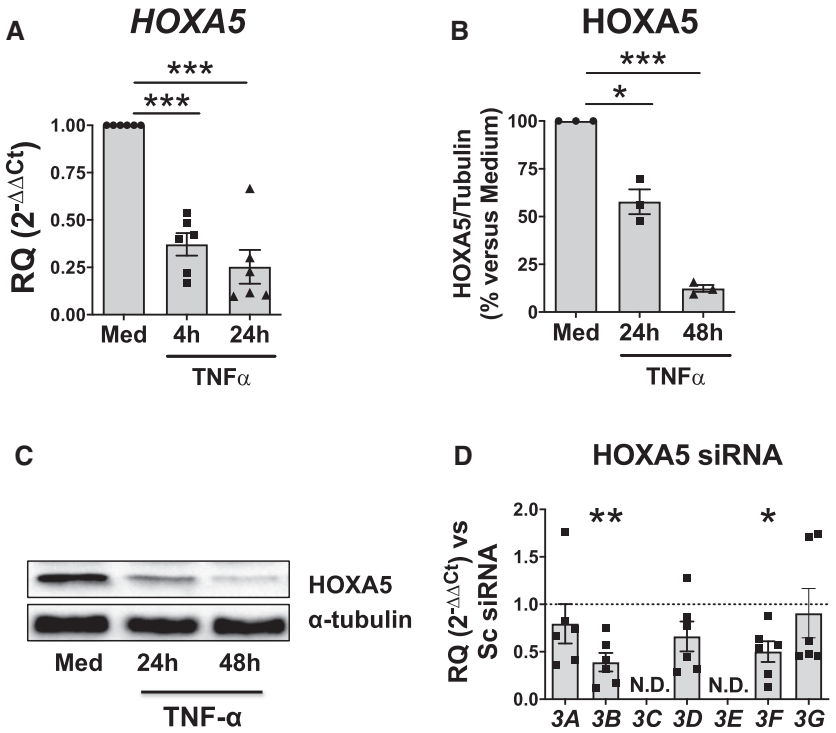

Figure 5. TNF down-regulates the expression of HOXA5 in HUVEC. (**A**) mRNA expression of HOXA5 in HUVEC ($n = 6$) cultured in medium (Med) stimulated 4 h and 24 h with TNF (10 ng/ml). Data are shown as RQ with respect to unstimulated cells. (**B**, **C**) Densitometric analysis (B) and representative immunoblot (C) of HOXA protein expression in HUVEC ($n = 3$) cultured in medium (Med) or stimulated 24 h and 48 h with TNF (10 ng/ml). Data are shown as relative expression with respect to tubulin expression. (**D**) mRNA expression of class 3 semaphorins in HUVEC ($n = 6$) transfected with scrambled control (Sc) or HOXA5 siRNA. Data are shown as RQ with respect to Sc siRNA–transfected cells. Means and SEM are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. RQ: relative quantity; N.D., not detected

that HOXA5 indirectly regulates the expression of *SEMA3B* through the regulation of the transcription factors TCF-3 and EBF-1.

Secondly, we found that HOXA5 expression is reduced in the synovial tissue of RA patients compared with healthy individuals, IAR subjects and OA patients, as well as in the inflamed synovium compared with non-inflamed tissue of RA patients. Moreover, we observed a positive correlation between the expression of HOXA5 and the expression of *SEMA3B*, *SEMA3C*, *SEMA3E* and *SEMA3F*, consistent with the idea that expression of these class 3 semaphorins in the synovial tissue is dependent upon HOXA5.

We found that crucial inflammatory mediators in the pathogenesis of RA, mainly TNF-α [2, 26, 27], down-regulated the expression of HOXA5 in FLS and endothelial cells in a PKB-dependent manner. In addition, the SF of RA patients also reduced the *HOXA5* expression and, more importantly, Etanercept, a TNF-α inhibitor widely used in the clinical practice [20, 30], abrogated this down-regulation. These data suggest that TNF-α levels present in the SF are involved in the reduced expression of HOXA5 found in the synovium of RA patients. Thus, the beneficial effect of TNF-α inhibition therapy may be due, at least in part, to the restoration of HOXA5 and class 3 semaphorins expression levels.

Lastly, we determined the functional consequences of HOXA5 silencing in RA FLS. Several studies have identified HOXA5 as an important tumour suppressor, for which expression is reduced in lung, colon, breast and gastric tumours, among others [31–35]. These cancer studies have demonstrated that HOXA5 inhibits key processes involved in tumour initiation and progression, such as apoptosis and cell survival, proliferation and invasiveness. In the context of RMDs, a recent study has shown that different HOX family members are involved in the specific location and characteristics of RA and OA FLS, but the functional role of HOXA5 in RA was so far unexplored [36]. Here, we demonstrated that HOXA5 did not regulate RA FLS viability or proliferation, but did regulate migration and invasiveness. We have previously shown the role of Sema3B and Sema3F in controlling the aggressive phenotype of RA FLS [10, 11]. The role of Sema3C and Sema3E in this process is still unknown. Data from tumour cells indicate that both Sema3C and Sema3E induce tumour growth and metastasis, through the inhibition of tumour cell apoptosis and the induction of cell migration and invasion [29, 37–41]. Then, Sema3C and Sema3E might be also involved in the aggressive phenotype of RA FLS.

Our data also show that HOXA5 silencing induces endothelial cell migration, pointing out a potential role in angiogenesis. The role of class 3 semaphorins has been extensively studied and these studies have shown that Sema3A, Sema 3B, Sema 3D, Sema 3E and Sema3F are exclusively inhibitors of pathological angiogenesis [42]. In the case of Sema3C, *in vitro* data showed that Sema3C induced endothelial cell proliferation, adhesion and migration [43], but further works found that this semaphorin inhibited pathogenic angiogenesis [44, 45].

HOXA5 is also a regulator of the transcription factors EBF-1 and TCF-3. EBF1 is essential for B cell development and maturation [46], meanwhile TCF3 controls germinal centre B cell and plasma cell development [47]. The potential roles of both transcription factors in RA pathogenesis are so far unexplored, but their role in regulating class 3 semaphorins expression and as tumour suppressors [48–50]

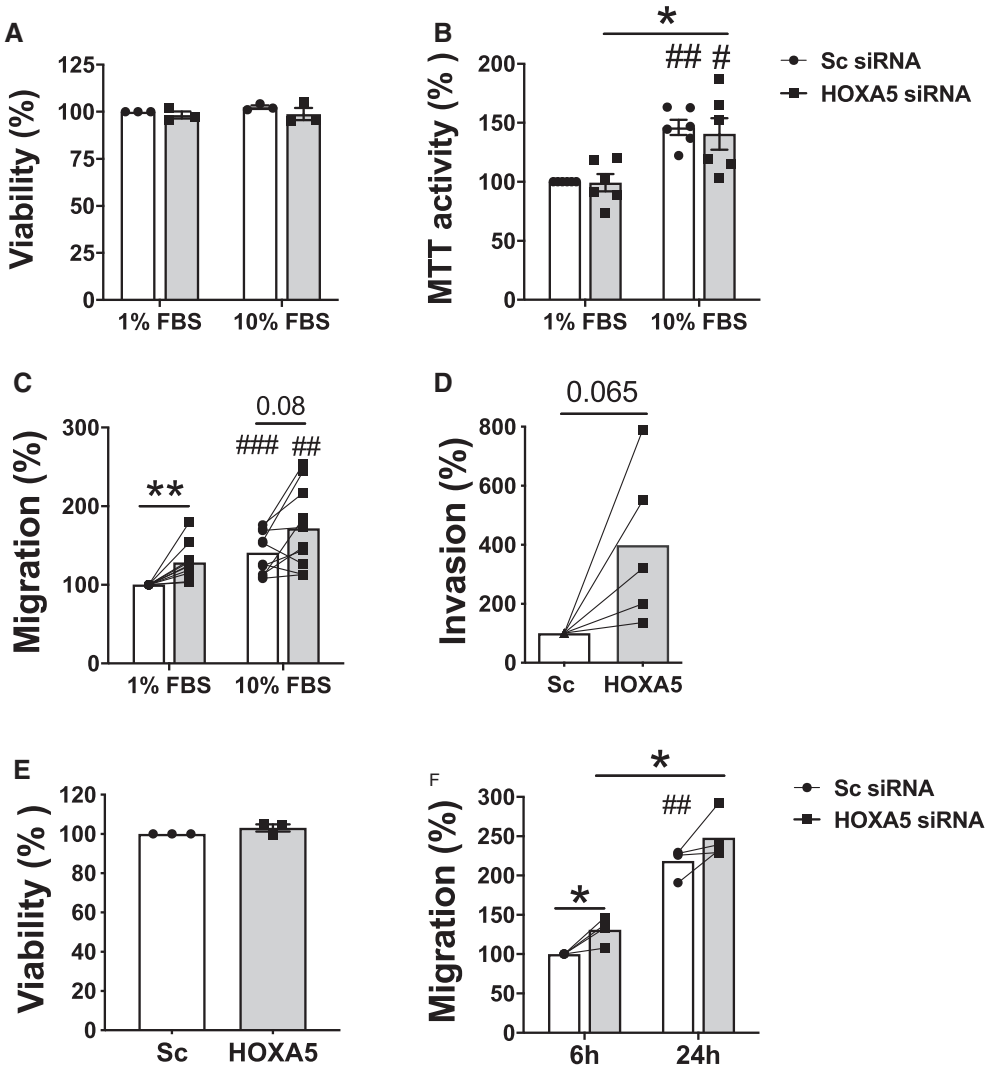

Figure 6. HOXA5 silencing induces RA FLS migration and invasion and EC migration. (**A**–**D**) Viability (A), metabolic activity (B), migration (C) and invasion (D) of RA FLS ($n = 3$–6) transfected with scrambled control (Sc) or HOXA5 siRNA in the presence or absence of 10% fetal bovine serum (FBS) for 24 h. Data are shown as percentage with respect to Sc siRNA-transfected cells. Means and SEM are shown. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, as compared with Sc siRNA 1% FBS. $^{*}P < 0.05$ and $^{**}P < 0.01$. (**E**, **F**) Viability (E) and migration (F) of HUVEC ($n = 3$–4) transfected with scrambled control (Sc) or HOXA5 siRNA for 6 and 24 h. Data are shown as percentage with respect to Sc siRNA–transfected cells. Means and SEM are shown. $^{\#\#}P < 0.01$, as compared with Sc siRNA 6 h. $^{*}P < 0.05$. EC: Endothelial cells; FLS: fibroblast-like synoviocytes

suggest that both TCF-3 and HOXA5 may have a protective role in RA.

Altogether, our work suggests that HOXA5 is a key factor involved in direct and indirect maintenance of synovium homeostasis, due to the expression of class 3 semaphorins, which inhibit FLS aggressiveness phenotype and may also impair pathological angiogenesis. In addition, HOXA5 also regulates the expression of TCF-3 and EBF-1, which in turn alter the expression of the semaphorins mentioned above. Therefore, restoring HOXA5 levels could be a novel approach for the treatment of RA.

Supplementary data

Supplementary data are available at *Rheumatology* online.

Data availability

Data available on request. The data underlying this article will be shared on reasonable request to the corresponding author.

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