Proteasome inhibition restored the reduced expression of fibroblast growth factor receptor 3 in rheumatoid arthritis synovial fibroblasts

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Abstract

Background. Synovial hyperplasia is a hallmark of rheumatoid arthritis (RA). This might be associated with an imbalance of growth-promoting and apoptotic pathways, among them fibroblast growth factors (FGFs) and their receptors (FGFRs) . The aim was to investigate differences in FGFRs and to explore the factors that might explain the differences between RA and osteoarthritis (OA) synovial fibroblasts.

Methods. To assess FGFRs expression, immunohistochemistry, flow cytometry and RT-qPCR were performed. The cells were treated with TNFa, FGF2, a demethylation agent, PKA-mimics or inhibitors, hypoxia-mimics or proteasome inhibitors. Proliferation was measured using the CCK8 assay and 20S proteasome activity by a fluorescent assay.

Results. In RA, FGFR3 protein was decreased in the synovial lining layer ($p<0.005$) and in cultured RA synovial fibroblasts (RASF) ($n=10$, $p < 0.01$). Transcription was unchanged and DNA demethylation decrease its expression. Exposure to TNFa or FGF2 had no effect on FGFR3. PKA-modulation and hypoxia-mimics induced transient changes only. Most interesting, in RASF, the proteasome inhibitor MG-132 restored FGFR3 expression ($p<0.001$) to levels measured in normal or OA synovial fibroblasts. MG-132 abolished the enhanced proliferative response of RASF to FGF2 ($p<0.005$). Increased 20S proteasome activity correlated ($r=- 0.63$, $p<0.05$) with decreased expression of FGFR3.

Conclusions. The expression of FGFR3 is reduced in RA partially due to an increased degradation in proteasomes. This leads to an imbalance in the FGF-related signal pathways and may contribute to synovial hyperplasia. Proteasome inhibitors could represent a novel therapeutic strategy in RA, particularly to prevent synovial hyperplasia.

Introduction

Synovial tissue hyperplasia is a hallmark of rheumatoid arthritis (RA). This might be associated with an imbalance of growth-promoting and apoptotic pathways, among them fibroblast growth factors (FGFs) and their receptors (FGFRs). In RA, the synovial lining cells produced fibroblast growth factor 2 (FGF2) [1]. Increased expression of FGF2 occurred at sites of synovial hyperplasia and joint destruction [2] and the importance of FGFF2 was shown in the adjuvant-induced model of arthritis [3].

Fibroblast growth factor receptors include FGFR1-4, which are cell surface membrane receptors that possess tyrosine kinase activity. FGFR1-2 initiate a cascade of intracellular signals that mediate cell division, growth and differentiation. At opposite, FGFR3 in fibroblasts counter-regulate the Ca^{+} -dependent activating signal by FGFR1 [4] (Fig. 1). For instance, in human articular chondrocytes, differences in responses to FGF2 might be due to changes in the balance between the two major articular cartilage FGF receptors, FGFR1 and FGFR3 [5]. The catabolic and anti-anabolic effects of FGF2 are mediated primarily through FGFR1, whereas the beneficial effects occur through FGFR3. The FGFR1/FGFR3 ratio appears to be a dynamic balance. In RA synovial tissues, overexpression of FGFR1 was reported on perivascular CD4 + T cells [6]. In such tissues, FGFR3 appears expressed on macrophages and T-lymphocytes [7];

synovial fibroblasts were not mentioned. Here, in RA, we report a decreased expression of FGFR3 on synoviocytes in situ and on synovial fibroblasts in vitro. Next, we searched for possible explanation of the lower FGFR3 expression.

FGFR3 can be proteolytically cleaved in response to ligand-induced receptor activation [8]. In rodents, promoter hypermethylation and decreased expression of FGFR3 were associated with liver fibrosis [9]. During the acquisition of the chondrocyte phenotype, synovium-derived mesenchymal stem cells maintain a promoter methylation of the CpG-rich FGFR3 promoter [10]. Thus, decreased FGFR3 expression could be due to gene silencing by DNA methylation. Alternatively, FGFR3 can be downregulated in chondrocytes by cAMP [11] and in non-muscle invasive bladder cancer cells by hypoxia [12]. Finally, intracellularly, FGFR3 is strongly associated with the HSP90 chaperone complex, which confers it stability and correct function [12].

Thus, we tested the hypothesis that reduced FGFR3 in RA synovial fibroblasts can be due a pro-inflammatory or a growth-promoting milieu, hypermethylation of the promoter, decreased transcription, activation of protein kinase A, hypoxia or increased degradation in proteasomes. Indeed, we found that inhibition of proteasomes restored FGFR3 expression and reduced the elevated proliferation rate of RA synovial fibroblasts induced by FGF2. We concluded that degradation of FGFR3 in proteasomes might be a factor contributing to RA synovial hyperplasia.

Material And Methods

Synovial tissues. Synovial tissues of patients with rheumatoid arthritis (RA) and osteoarthritis (OA) were obtained after joint replacement (n = 11 consecutive patients of each diagnosis), and normal synovial tissues after trauma surgery (Balgrist University Clinic, Zurich) (n = 3). Patients fulfilled the ACR classification criteria for RA or OA and were matched for age (71 ± 6 years old) and gender (1 man and 9 women). Synovial tissues from large joints were obtained for this study (shoulder, knee and elbow – hands for RT-qPCR comparison only).

Cell culture. Synovial tissue biopsies were digested with Dispase II (from Bacillus polymyxa, Roche, Switzerland) at 1 mg/ml in PBS for 3 hours at 37°C under rotation 800 rpm. The digested sample was filtered (100 µm Nylon filter) and centrifuged at 350 g for 10 minutes. The pellet was resuspended in DMEM containing 10% fetal calf serum (FCS), 1% antibiotics (penicillin-streptomycin) and L-Glutamin, HEPES buffer and fungicide as described previously [14]. Synovial fibroblasts were cultured over 4 to 6 passages.

All reagents and stimuli below were used at non-apoptotic (according to the Dead Cell Apoptosis Kit with Annexin V FITC and PI, Invitrogen) and non-cytostatic (optical evaluation) concentrations for synovial fibroblasts. Increased Annexin V staining was regarded as an early sign of apoptosis.

Single exposure and chronic stimulation. Cell cultures were 75% confluent. Synovial fibroblasts were stimulated with 1 ng/ml TNFα or 0.1 ng/ml FGF2 (both from R&D Biosystems) either for 24 h or every

third day for 2 weeks in DMEM containing 10% FCS.

DNA demethylation agent. RASF and OASF were treated for 2 weeks with a non-toxic dose of 5-azacytidine (0.1 μM, Sigma-Aldrich), as described previously [15]. DMEM containing 10% FCS and 5-azacytidine was replaced once a week.

Hypoxia-mimics. RASF and OASF were incubated with fresh prepared 50 μM desferrioxamine (DFO) or 50 μM cobalt chloride ($CoCl_2$) (Sigma-Aldrich) in DMEM containing 1% FCS. Cells were analyzed after 1 to 24 hours.

Protein kinase A (PKA) modulation. The PKA Tocritest kit (Tocris Bioscience) was used. Stock solutions of forskolin and KT5720 were prepared in 10 mM DMSO. Two weeks after passaging, RASF and OASF were incubated in DMEM containing 1% FCS plus PKA modulators (100 μM 8-bromo-cAMP, 10 μM Forskolin, 10 μM NKH477, 100 μM KT5720 or 20 μM H89). Cells were analyzed after 1 to24 hours.

Proteasome inhibition. Z-Leu-Leu-Leu-al (50 μM MG-132, Sigma-Aldrich) was prepared in DMEM containing 1% FCS. Cells were analyzed after 1 to 24 hours. Bortezomib (0.1 μM, Calbiochem) for 24 hours was also tested; however, bortezomib increased annexin V staining. Therefore, experiments were performed mostly with MG-132.

Protein synthesis inhibition. Actinomycin D (50 nM, Sigma-Aldrich) was prepared in DMEM containing 1% FCS. Cells were analyzed after 24 and 48 hours.

Flow cytometry. Cells were washed with PBS and detached using Accutase (PAA Lab.). Staining was performed using mouse IgG1 (isotype control), or mouse IgG1 anti-human FGFR1, FGFR2 or FGFR3 (R&D Systems) antibodies conjugated with FITC for 45 minutes at 4 °C in DMEM containing 1% FCS with or without 10% FACS Permeability Solution 2 (BD Bioscience). The fluorescence was measured at 488 nm using a FACSCalibur flow cytometer (Becton-Dickinson). Marker M1 was set at < 5% positive cells in the isotype control. Mean background fluorescence was subtracted from stained mean fluorescence. All cell culture tested were > 90% CD90 + and < 1% CD45+.

Immunohistochemistry. Paraffin sections of synovial tissues were boiled (for antigen retrieval) in 10 mM citrate buffer, pH 6, for 10 minutes. Endogenous peroxidase and endogenous bindings were blocked with PBS containing 3% H_2O_2, 5% horse serum and 1% bovine serum albumin. The sections were incubated either with rabbit IgG1 (negative control) or polyclonal rabbit antibodies to human CD333 /FGFR3 (Acris Antibodies), end-concentration 10 μg/ml in PBS, for 1 hour at 22 °C. The slides were washed with PBS plus Tween20 and incubated with secondary biotinylated donkey anti-rabbit antibodies (Jackson ImmunoResearch) for 10 minutes at 22 °C. Binding was revealed using Vecastain Elite ABC kit for HRP (Vector Laboratories) and 3-amino-9-ethylcarbazole (AEC, Sigma-Aldrich). Positive control was breast carcinoma.

RNA Isolation and semiquantitative Real-Time-PCR. Cells were lysed in QIAzol lysis reagent and total RNA was isolated using a miRNAeasy Mini kit including a DNA digestion step using RNAse-free DNAse (Qiagen). The RNA concentration, integrity and purity were determined with Nanodrop spectrophotometer (VWR, Switzerland). Total RNA was reverse transcripted using random hexamers and MultiScribe reverse transcriptase (Applied Biosystems). Gene expression was quantified by SYBR Green real time-PCR using the ABI Prism 7500 Sequence Detection System (Life Technologies). Samples without enzyme in the reverse transcription reaction (non-RT controls) were used as negative controls. Unspecific signals caused by primer dimers were excluded by non-template controls and by dissociation curve analysis. HPRT1 was used to normalize for the amounts of cDNA within each sample. Primer were synthesized by Microsynth: FGFR3, fw GGC CTT GTT TGA CCG AGT CTA C, rev CCT AAG GAC CAG ACG TCA CTC T; HPRT1, fw CCT GGC GTC GTG ATT AGT GA, rev CGA GCA AGA CGT TCA GTC CT. Relative expression of mRNA was calculated by the comparative threshold cycle method, where dCt = Ct (RNA of interest) – Ct (housekeeping gene). The results were also replicated using a TaqMan assay (ThermoFisher, Hs06632171_s1). This assay was used to estimate the half-life of the FGFR3 transcripts before and after incubation with actinomycin D.

Proliferation assay. Cell Counting Kit-8 (CCK-8, Sigma-Aldrich) was used.

20S Proteasome activity assay. Synovial fibroblasts (0.5×10^4/well in a 96-well plate, n = 3 normal SF, 6 OASF and 6 RASF) were analyzed for 20S proteasome activity using a commercially available kit (Cayman Chemicals).

Statistics. Mann-Whitney U-test, Wilcoxon signed rank test and Pearson's correlation test were used to reveal the level of significance.

Results

Lower expression of FGFR3 protein in RA. Immunohistochemistry revealed lower levels of FGFR3 in RA than in normal or OA synovial tissues (representative examples Figs. 2A-C). In the lining layer, FGFR3 + cells represented 92 ± 2% in OA and 47 ± 21% in RA (Fig. 2D, $p < 0.005$, n = 6 sections). These numbers were obtained by optical counting of the stained nuclei and FGFR3 + cells. Normal synovial membranes showed similar staining as OA sections (95 ± 7%, n = 3, Fig. 2A). Flow cytometry confirmed the reduced expression of FGFR3 on the cell surface in RASF, as well as in the cytoplasm after permeabilization, in comparison to normal (NSF) or OASF (representative examples in permeabilized cells Figs. 3A-C). In RASF, only FGFR3 (Fig. 3F), but not FGFR1 or FGFR2 (Figs. 3D, E), was significantly reduced. FGFR1 and FGFR2 staining in normal synovial fibroblasts are not shown because of the low number of cases but are similar in mean fluorescence to that of OASF. The difference between OASF and RASF was more obvious in permeabilized cells. Receiver operating characteristic curve (ROC) analysis confirmed the highly significant diagnostic predictive value of FGFR3 expression.

Normal transcription of FGFR3 in RASF. Measurement of FGFR3 transcripts showed no difference between RASF and OASF (Fig. 4A). Normal synovial fibroblasts had similar expression (n = 3, not shown). In addition, no significance difference in transcription occurred between joint localizations, i.e hand, shoulder, elbow or knee (n = 3 to 6 each, either RA or OA, ANOVA p = 0.81, supplementary Fig. 1). Treatment with actinomycin D reduced FGFR3 mRNAs in both cell types at the same level (Fig. 4B); half-life of FGFR3 transcripts were about 24 hours.

DNA demethylation and FGFR3 expression. OASF and RASF treated with a low dose of 5-azacytidine for 2 weeks did not increase, but at opposite significantly decreased the expression of FGFR3 (representative examples Fig. 4C). 5-Azacytidine reduced the expression of FGFR3 in all synovial fibroblasts independently of the diagnosis (Fig. 4D).

TNFα or FGF2 did not contribute to low FGFR3 expression. In OASF and RASF, acute stimulation of TNFα for 24 hours or chronically for 2 weeks did not change the expression of FGFR3. Similarly, neither short-term nor chronic exposure to FGF2 lowered the expression of FGFR3. Acute and chronic stimulations with TNFα showed different effects on the expression of FGFR2. Thus, acute exposure resulted in increased FGFR2 expression in permeabilized OASF, while chronic stimulation resulted in decreased expression (mean fluorescence was increased by 56 ± 14% after 24 h and lowered by -24 ± 3% after two weeks, n = 6, $p < 0.05$ for both) (supplementary Fig. 2). However, on the cell surface (without permeabilization), the differences were not significant. TNFα did not affect the expression of FGFR1 or FGFR3.

PKA transiently modulates FGFR3 expression. OASF were exposed to PKA-inhibiting agents (KT5720, H89), while RASF were treated with PKA-mimics (forskolin, NKH477, 8-bromo-cAMP) with the hypothesis that the first will downregulate and the second upregulate FGFR3 expression. Indeed, this was the case, but RASF did not recover to the levels in untreated OASF. PKA-inhibiting agents decreased the FGFR3 expression in OASF. However, the changes were transient only, with maximums within 2 hours, as shown by flow cytometry (supplementary Fig. 3).

Hypoxia mimics transiently reduced FGFR3 expression. RASF and OASF were exposed to DFO or $CoCl_2$. They induced small changes in FGFR3 expression. DFO was more potent than $CoCl_2$ (not shown) and reduced the expression of FGFR3 in OASF within 4–6 hours. The effect was obvious with permeabilized cells only and returned to baseline after 24 hours. Hypoxia mimics had no effect on RASF which already have low expression of FGFR3 (supplementary Fig. 4).

Inhibition of proteasomes restored FGFR3 expression in RASF. In RASF, significant increases of FGFR3 on the cell surface and in permeabilized cells occurred upon treatment with the proteasome inhibitor MG-132 (Figs. 5A). This was observed in RASF with low FGFR3, but not in OASF with normal expression (Fig. 5B). The FGFR1/FGFR3 ratio decreased to levels observed in OASF (Fig. 5C; same observation for the FGFR2/FGFR3 ratio, not shown). The effect in RASF was sustained for at least 24–48 hours and the cells showed no increase of annexin V staining or decrease in cell numbers thereafter. bortezomib also increased the expression of FGFR3, but even at the lowest effective dose (LOED) induced annexin V

staining (and all cells were death after 3 days, not shown). Therefore, MG-132 was used in further experiments.

Inhibition of proteasomes reduced FGF2-induced proliferation of RASF. An increased proliferative response to FGF2 occurred In RASF with reduced expression of FGFR3. This enhanced response was abolished in RASF pre-treated with MG-132 (Fig. 5D). This phenomenon was observed for RASF, but not for OASF. The decreased proliferation in RASF treated with both MG-132 and FGF2 was not accompanied by increased staining of annexin V.

20S Proteasome activity correlated with FGFR3 expression. In RASF, 20S proteasome activity was modestly but significantly increased (Fig. 6A). Most importantly, by analyzing all samples together (3 normal SF, 6 OASF and 6 RASF), the expression of FGFR3 in permeabilized cells negatively correlated with baseline 20S proteasome activity (Fig. 6B).

Discussion

FGFR3 expression was reduced in RA synovial tissues. This was confirmed in RASF in vitro. Thus, the reduction of FGFR3 is conserved in vitro over at least 4–6 passages. This raised the question whether decreased expression reflects reduced transport to the plasma membrane or more generally decreased synthesis or increased degradation.

The ratio of FGFR3 on the cell membrane versus total in permeabilized cells was not reduced, suggesting a normal transport to the cell surface. In addition, quantifications of FGFR3 transcripts showed no difference between RASF and OASF. FGFR3 transcripts can be targeted by microRNAs, e.g. miR-24 and miR-100 [12, 16] but this is probably not the major factor here, since FGFR3 transcript half-life was not different between OASF and RASF. The most prominent differences in vitro are observed in the permeabilized cells, because 1) the transport mechanism to the cell membrane is not affected and 2) the test becomes more sensitive due to the higher proportion of positive cells.

The ROC analysis confirmed the highly significant diagnostic predictive value of FGFR3 expression in permeabilized SF. The benefits or potential use of this finding appear limited by the invasive nature of biopsy or surgery. An alternative could be to check whether fibroblasts in RA synovial fluids [17] obtained by arthrocentesis also have low FGFR3 expression.

During the wound healing process, FGFR3 protein expression on fibroblasts and myofibroblasts is reduced [18]. Low FGFR3 also been described in urothelial bladder cancer associated with poor outcome [19]. In the context of arthritis, low FGFR3 could also have a pathological relevance. This is suggested by FGFR3 knockout mice; thus, the absence of signaling through FGFR3 in the joints of FGFR3(-/-) mice leads to premature cartilage degeneration and early arthritis [20].

Our first hypothesis was that reduced FGFR3 in RA could be due to chronic stimulation by pro-inflammatory mediators, such as TNFα, or to the action of its ligand, particularly FGF2. However, this was

not the case. Together with the fact that RASF conserved the low FGFR3 phenotype after more than 4 passages in vitro, this indicates that pro-inflammatory or growth-promoting environments alone are not sufficient to explain the reduced FGFR3 expression. Regarding FGFR2, short-time exposure to TNFa increases it expression, as reported by others [21], while chronic exposure increased it. Our results indicated that the expression of FGFR2, but not of FGFR1 or FGFR3, can be modulated by the pro-inflammatory milieu. FGFR2 appears to be differently regulated than FGFR1 or FGFR3, e.g. particularly in the context of RA and inflammatory bowel diseases FGFR2 expression could be affected by micro-RNAs [22].

Since FGFR3 promoter can be hypermethylated under certain circumstances, we tested the effect of 5-azacytdine on its expression. However, treatment with 5-azacytidine resulted in a decreased FGFR3 expression in both OASF and RASF. This suggested an indirect effect of a methylation-sensitive factor. Low FGFR3 in RASF could be linked to the global DNA hypomethylation that has been described previously [15]. This possibility has to be investigated in more detail.

The catalytic subunit of PKA (PKACA) is a gene product that is downregulated upon treatment of OASF with 5-azacytidine [15]. Since decreased PKA activity could be associated with reduced FGFR3 expression [11], we tested the effects of PKA-mimics on RASF and PKA-inhibiting agents on OASF. However, the effects were modest and transient only.

In the hyperplastic RA synovial tissue, hypoxia is an important factor influencing cell activity. Since hypoxia can modify FGFR3 expression [12], with tested two chemical hypoxia-mimics. Particularly DFO can reduce the expression of FGFR3 in permeabilized OASF. Again, however, the changes were transient only. In addition, hypoxia would not explain low FGFR3 in RASF kept over 4 passages in normal conditions, i.e. 20% O_2.

Most importantly, we showed that proteasome inhibitors (MG-132 or bortezomib) restored the expression of FGFR3 in RASF to the levels measured in OASF. This increase of FGFR3 remained at least for 24–48 hours and the FGFR1/FGFR3 ratio was normalized for at least 48 hours. An increased proteasome activity might also affect the expression of other proteins in RASF, such as DNMT1, as previously suggested [15]. The inhibition of proteasomes does not affect the expression of FGFR3 in OASF because it is already maximally expressed.

We hypothesized that lower expression of FGFR3 could have an influence on the response to FGF2. To test this, we performed a proliferation assay with or without pre-treatment with MG-132. As expected, MG-132 abolished the enhanced proliferative response of RASF to FGF2. The situation is different in OASF, in which an equilibrated FGFR1-2/FGFR3 balance is able to limit the effect of FGF2. FGFR3, in contrast to other FGFRs, is a strong client of the chaperone HSP90 and is subject of ubiquitination by E3-ubiquitin ligases [13]. This can render it susceptible to lysozymal and proteasomal degradation.

Congruently, in RASF, increased 20S proteasome activity correlated with decreased expression of FGFR3. The measurement of FGFR3 in permeabilized cells allowed to estimate the total expression of the protein,

including the cytoplasmic and cell membrane compartments. This total expression correlates better with the 20S proteasome activity than the cell surface expression, probably because the latter is regulated by additional independent mechanisms influencing the transport on the cell surface.

Interestingly, the expression levels of FGFR1 and FGFR3 declined in synovial fibroblasts after blocking the sonic hedgehog-Gli signaling pathway with a Gli specific inhibitor (Gli-antagonist 61, GANT61) [23]. GANT61 inhibits RASF cell proliferation and increases cell apoptosis but did not the affect the FGFR1/FGFR3 ratio. Unfortunately, the effect of FGF2 in this system was not investigated.

An argument against our hypothesis could be that Kaempferol which inhibit FGF2-FGFR3–ribosomal S6 kinase 2 (RSK2) signalling suppresses the in vitro proliferation and migration of RASF [7]. However, in the hyperplastic synovial tissue, CD68 + macrophages, but not fibroblasts, showed phosphorylated RSK2. This might be due to the downregulation of FGFR3 on fibroblasts. Again, the effect of FGF2 in this system have to be analysed. It is also important to note that Kaempferol has multiple pharmacological properties that at end results in less inflammation, less oxidative stress and more apoptosis [24, 25]. Recently, it has been reported that kaempferol dramatically suppressed TNFα-induced MAPK activation [26], which is certainly a FGFR3-independent process.

Conclusion

FGFR3 protein expression is reduced in RASF in part due to an increased degradation in proteasomes. This might lead to an imbalance in the FGF-related signal pathways, favoring more proliferation and resulting in synovial hyperplasia. The rational to use proteasome inhibitors as anti-inflammatory agents in the treatment of autoimmune diseases, including RA, has been reviewed [27]. We showed here that they could be potentially used to prevent synovial hyperplasia.

Abbreviations

ACR, American College of Rheumatology; DFO, desferrioxamine; DMEM, Dulbecco's Modified Eagle's Medium; FCS, fetal calf serum; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FITC, fluorescein isothiocyanate; GANT61, Gli-antagonist 61; HPRT1, hypoxanthin-phosphoribosyl-transferase 1 gene; HSP90. heat shock protein 90; MAPK, mitogen-activated protein kinase; OA, osteoarthritis; OASF, osteoarthritis synovial fibroblast; PBS, phosphate buffered saline; PKA, protein kinase A; RA, rheumatoid arthritis; RASF, rheumatoid arthritis synovial fibroblast; ROC, receiver operating characteristic curve; RSK2, ribosomal S6 kinase; RT-sqPCR, real time semiquantitative polymerase chain reaction; TNFa, tumor necrosis factor alpha.

References

1. Nakashima M, Eguchi K, Aoyagi T, Yamashita I, Ida H, Sakai M, Shimada H, Kawabe Y, Nagataki S, Koji T, et al. Expression of basic fibroblast growth factor in synovial tissues from patients with

rheumatoid arthritis: detection by immunohistological staining and in situ hybridisation. *Ann Rheum Dis.* 1994;53:45-50.

2. Qu Z1, Huang XN, Ahmadi P, Andresevic J, Planck SR, Hart CE, Rosenbaum JT. Expression of basic fibroblast growth factor in synovial tissue from patients with rheumatoid arthritis and degenerative joint disease. *Lab Invest.* 1995;73:339-46.
3. Yamashita A, Yonemitsu Y, Okano S, Nakagawa K, Nakashima Y, Irisa T, Iwamoto Y, Nagai Y, Hasegawa M, Sueishi K. Fibroblast growth factor-2 determines severity of joint disease in adjuvant-induced arthritis in rats. J Immunol. 2002 Jan 1;168(1):450-7.
4. Nguyen, HB, Estacion M, Gargus JJ. Mutations causing achondroplasia and thanatophoric dysplasia alter bFGF-induced calcium signals in human diploid fibroblasts. *Hum Mol Genet* 1997;6:81-688.
5. Yan D, Chen D, Cool SM, van Wijnen AJ, Mikecz K, Murphy G, Im HJ. Fibroblast growth factor receptor 1 is principally responsible for fibroblast growth factor 2-induced catabolic activities in human articular chondrocytes. Arthritis Res Ther. 2011 Aug 11;13(4):R130.
6. Byrd V, Zhao XM, McKeehan WL, Miller GG, Thomas JW. Expression and functional expansion of fibroblast growth factor receptor T cells in rheumatoid synovium and peripheral blood of patients with rheumatoid arthritis. *Arthritis Rheum.* 1996;39:914-22.
7. Lee CJ, Moon SJ, Jeong JH, Lee S, Lee MH, Yoo SM, Lee HS, Kang HC, Lee JY, Lee WS, Lee HJ, Kim EK, Jhun JY, Cho ML, Min JK, Cho YY. Kaempferol targeting on the fibroblast growth factor receptor 3-ribosomal S6 kinase 2 signaling axis prevents the development of rheumatoid arthritis. Cell Death Dis. 2018 Mar 14;9(3):401.
8. Degnin CR, Laederich MB, Horton WA. Ligand activation leads to regulated intramembrane proteolysis of fibroblast growth factor receptor 3. *Mol Biol Cell.* 2011;22:3861-73.
9. Wu P, Huang R, Xiong YL, Wu C. Protective effects of curcumin against liver fibrosis through modulating DNA methylation. *Chin J Nat Med.* 2016;14:255-264.
10. Ezura Y, Sekiya I, Koga H, Muneta T, Noda M. Methylation status of CpG islands in the promoter regions of signature genes during chondrogenesis of human synovium-derived mesenchymal stem cells. Arthritis Rheum. 2009 May;60(5):1416-26.
11. McEwen DG, Green RP, Naski MC, Towler DA, Ornitz DM. Fibroblast growth factor receptor 3 gene transcription is suppressed by cyclic adenosine 3',5'-monophosphate. Identification of a chondrocytic regulatory element. *J Biol Chem.* 1999;274:30934-42.
12. Blick C, Ramachandran A, Wigfield S, McCormick R, Jubb A, Buffa FM, Turley H, Knowles MA, Cranston D, Catto J, Harris AL. Hypoxia regulates FGFR3 expression via HIF-1α and miR-100 and contributes to cell survival in non-muscle invasive bladder cancer. *Br J Cancer* 2013;109:50-9.
13. Laederich MB, Degnin CR, Lunstrum GP, Holden P, Horton WA. Fibroblast growth factor receptor 3 (FGFR3) is a strong heat shock protein 90 (Hsp90) client: implications for therapeutic manipulation. *J Biol Chem.* 2011;286:19597-604.
14. Ohshima S, Kuchen S, Seemayer CA, Kyburz D, Hirt A, Klinzing S, Michel BA, Gay RE, Liu FT, Gay S, Neidhart M. Galectin 3 and its binding protein in rheumatoid arthritis. *Arthritis Rheum.* 2003;48:2788-

95.

15. Karouzakis E, Gay RE, Michel BA, Gay S, Neidhart M. DNA hypomethylation in rheumatoid arthritis synovial fibroblasts. *Arthritis Rheum.* 2009;60:3613-22.
16. Katoh M. Cardio-miRNAs and onco-miRNAs: circulating miRNA-based diagnostics for non-cancerous and cancerous diseases. *Front Cell Dev Biol.* 2014;2:61.
17. Neidhart M, Seemayer CA, Hummel KM, Michel BA, Gay RE, Gay S. Functional characterization of adherent synovial fluid cells in rheumatoid arthritis: destructive potential in vitro and in vivo. Arthritis Rheum. 2003 Jul;48(7):1873-80.
18. Kanda T, Funato N, Baba Y, Kuroda T. Evidence for fibroblast growth factor receptors in myofibroblasts during palatal mucoperiosteal repair. Arch Oral Biol. 2003 Mar;48(3):213-21
19. Amaral AF, Méndez-Pertuz M, Muñoz A, Silverman DT, Allory Y, Kogevinas M, Lloreta J, Rothman N, Carrato A, Rivas del Fresno M, Real FX, Malats N; Spanish Bladder Cancer/EPICURO Study investigators. Plasma 25-hydroxyvitamin D(3) and bladder cancer risk according to tumor stage and FGFR3 status: a mechanism-based epidemiological study. *J Natl Cancer Inst.* 2012;104:1897-904.
20. Valverde-Franco G1, Binette JS, Li W, Wang H, Chai S, Laflamme F, Tran-Khanh N, Quenneville E, Meijers T, Poole AR, Mort JS, Buschmann MD, Henderson JE. Defects in articular cartilage metabolism and early arthritis in fibroblast growth factor receptor 3 deficient mice. *Hum Mol Genet.* 2006;15:1783-92.
21. D'Amici S, Ceccarelli S, Vescarelli E, Romano F, Frati L, Marchese C, Angeloni A. TNFα modulates Fibroblast Growth Factor Receptor 2 gene expression through the pRB/E2F1 pathway: identification of a non-canonical E2F binding motif. *PLoS One* 2013;8:e61491
22. Krissansen GW, Yang Y, McQueen FM, Leung E, Peek D, Chan YC, Print C, Dalbeth N, Williams M, Fraser AG. Overexpression of miR-595 and miR-1246 in the sera of patients with active forms of inflammatory bowel disease. Inflamm Bowel Dis. 2015 Mar;21(3):520-30.
23. Qin S, Sun D, Li H, Li X, Pan W, Yan C, Tang R, Liu X. The Effect of SHH-Gli Signaling Pathway on the Synovial Fibroblast Proliferation in Rheumatoid Arthritis. Inflammation. 2016 Apr;39(2):503-12.
24. Devi KP, Malar DS, Nabavi SF, Sureda A, Xiao J, Nabavi SM, Daglia M. Kaempferol and inflammation: From chemistry to medicine. Pharmacol Res. 2015 Sep;99:1-10.
25. Imran M, Rauf A, Shah ZA, Saeed F, Imran A, Arshad MU, Ahmad B, Bawazeer S, Atif M, Peters DG, Mubarak MS. Chemo-preventive and therapeutic effect of the dietary flavonoid kaempferol: A comprehensive review. Phytother Res. 2019 Feb;33(2):263-275.
26. Pan D, Li N, Liu Y, Xu Q, Liu Q, You Y, Wei Z, Jiang Y, Liu M, Guo T, Cai X, Liu X, Wang Q, Liu M, Lei X, Zhang M, Zhao X, Lin C. Kaempferol inhibits the migration and invasion of rheumatoid arthritis fibroblast-like synoviocytes by blocking activation of the MAPK pathway. Int Immunopharmacol. 2018 Feb;55:174-182.
27. Verbrugge SE, Scheper RJ, Lems WF, de Gruijl TD, Jansen G. Proteasome inhibitors as experimental therapeutics of autoimmune diseases. Arthritis Res Ther. 2015 Jan 28;17(1):17.

Declarations

All patients signed a consent form approved by the local institutional review board. The procedure was approved by the ethics committee of the University Hospital Zurich and canton of Zurich (Basec 00215-2019 and Basec 2017-00349).

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article. The more detailed datasets are also available from the corresponding author on reasonable request.

Autors' contributions

MN, acquisition of data, writing the manuscript and supervision; EK, acquisition of data and review of the manuscript; SF, providing samples and review of the manuscript; OD, supervision and review of the manuscript; AJ, acquisition of data, supervision and review of the manuscript.

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Funding

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Figures

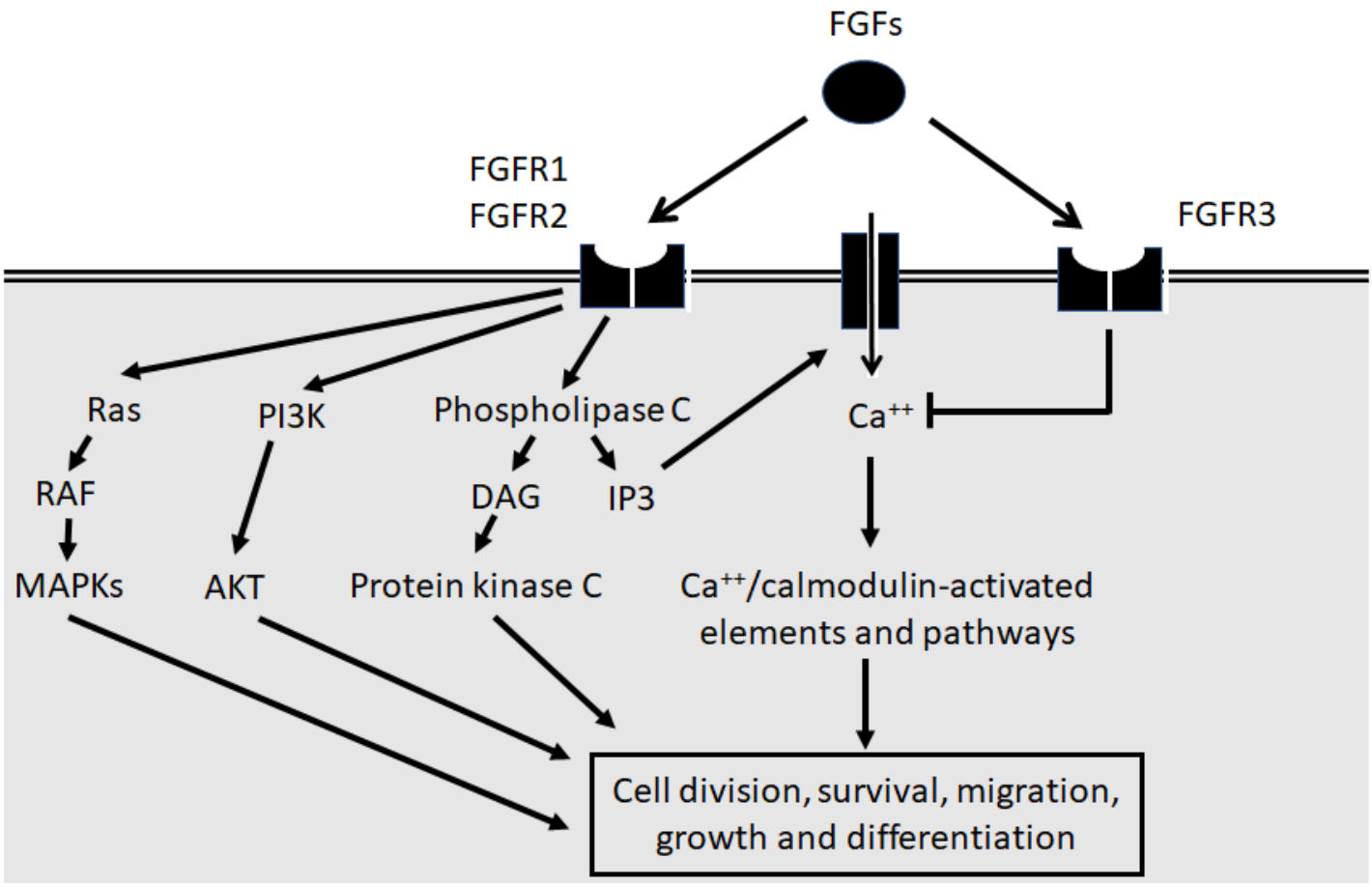

Figure 1

Functions of fibroblast growth receptors. FGFR1-2 initiate a cascade of intracellular signals including MAP kinases, AKT and protein kinase C that mediate cell division, survival, migration, growth and differentiation. At opposite, FGFR3 in fibroblasts counter-regulate the Ca+ -dependent activating signal by other FGFRs to limit the stimulatory signals.

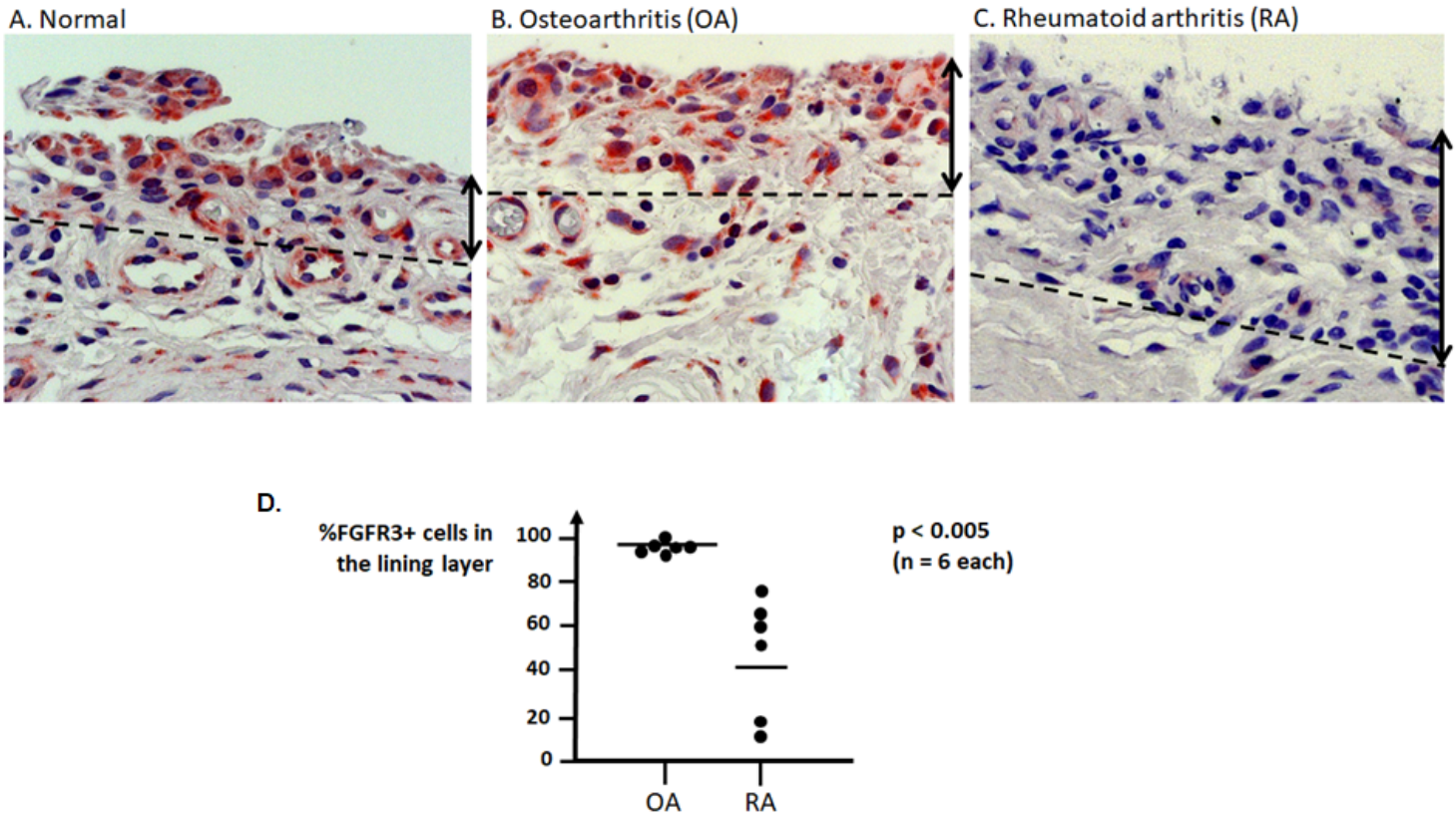

Figure 2

Expression of FGFR3 in synovial tissues. A) Normal synovial tissue, B) osteoarthritis (OA), C) rheumatoid arthritis (RA) (dotted lines and arrows represent the evaluated lining layer) ; D) the expression of FGFR3 is significantly reduced in the synovial hyperplastic lining layer of patients with RA, as revealed by immunohistochemistry (Mann-Whitney U-test, n=6 each diagnosis).

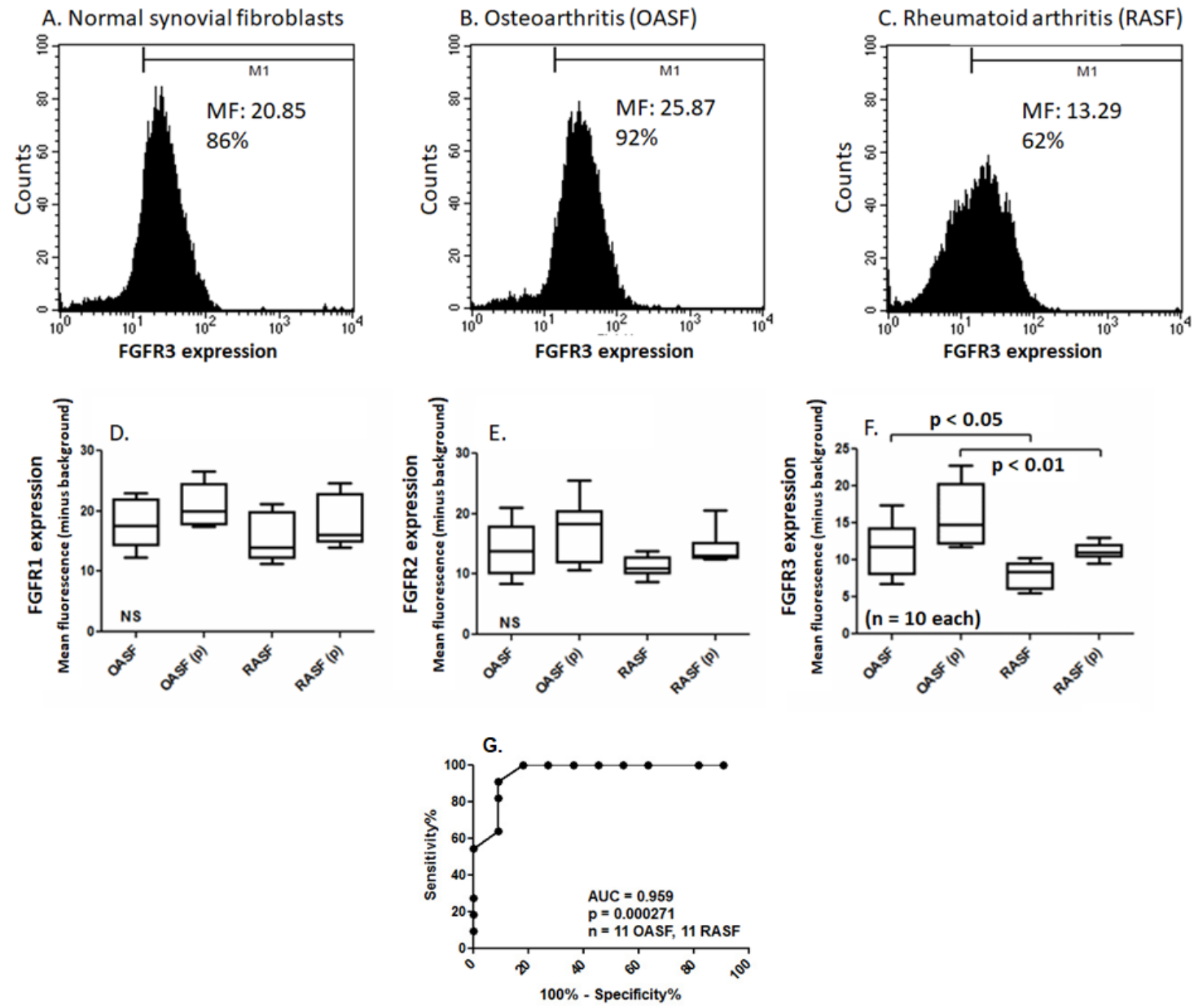

Figure 3

FGFR3 in synovial fibroblasts (SF, flow cytometry). A) Normal SF, B) OASF, C) RASF (representative examples in permeabilized cells, MF: mean fluorescence minus background, and % positive cells; the limit of < 1% positive cells by isotype controls is illustrated by the M1 mark). D-F) Comparison between expressions of FGFR1, 2 and 3 on the cell surface or in permeabilized cells (p); FGFR3, but not FGFR1 or FGFR2, is significantly reduced in RASF (box-whiskers plots, Mann-Whitney U-test, n=10 each). G) Receiver operating characteristic curve (ROC) showing the predictive value of FGFR3 expression in permeabilized SF.

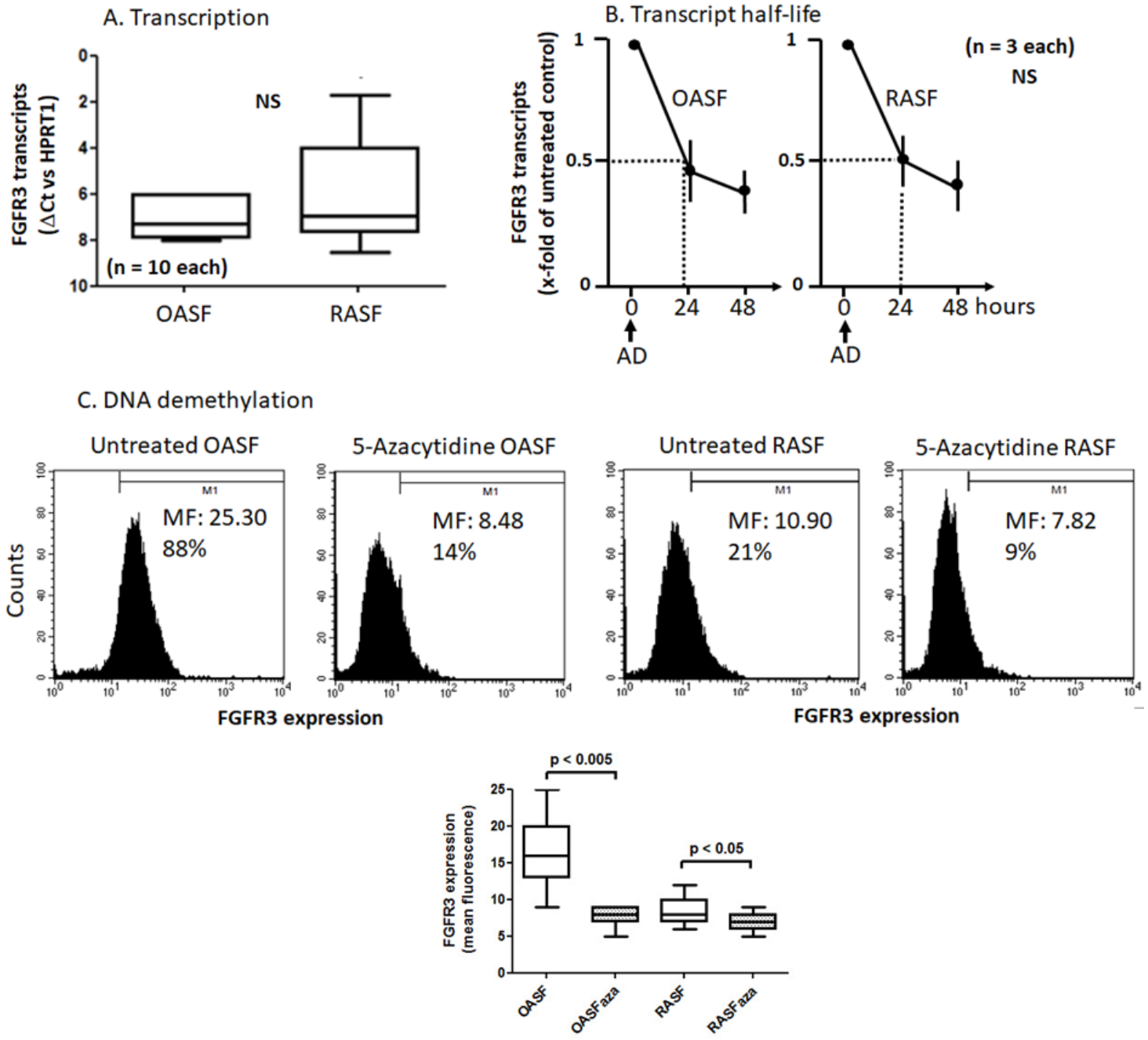

Figure 4

FGFR3 transcription and expression upon DNA demethylation. A) No difference at the mRNA level between OASF and RASF (n=10 each, as determined by SYBR green RT-qPCR), and B) no difference in the transcript half-life upon actinomycin D (AD) (n=3 each, as determined by TaqMan RT-qPCR). These experiments used SYBR Green primers; similar results were obtained with a TaqMan assay. C) Effect of DNA demethylation (2 weeks of treatment with 5-azacytidine), decreased expression of FGFR3 (representative examples in permeabilized cells). DNA demethylation is independent from diagnosis (Box-Whiskers-Plot, Wilcoxon signed rank test, n=10 each).

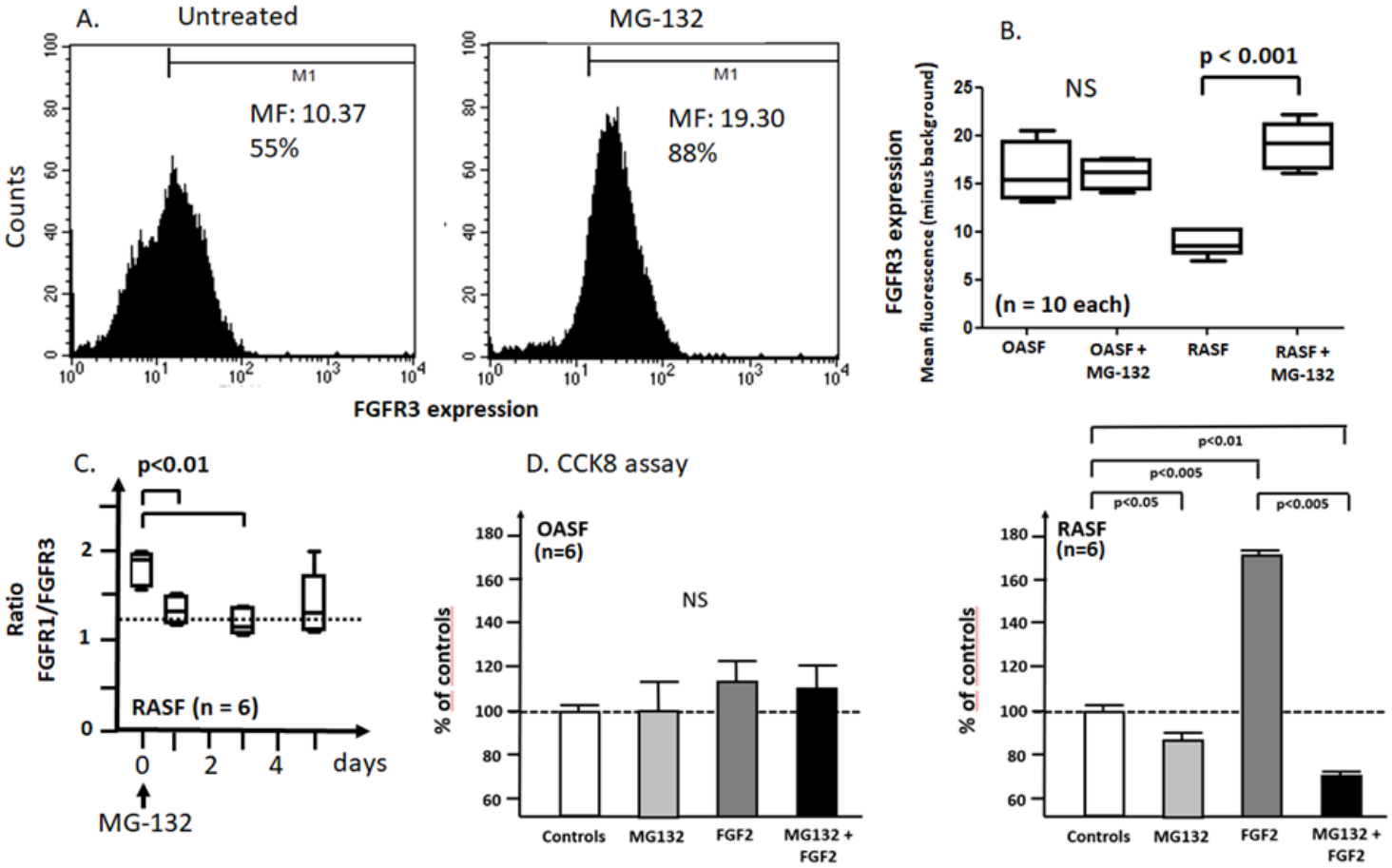

Figure 5

Expression of FGFR3 and response to FGF2 upon proteasome inhibition. A) Effect of MG-132 on FGFR3 expression in permeabilized RASF. B) Restoration of FGFR3 expression in RASF (left) to levels in OASF (right) (n=10 each). C) Decreased FGFR1/FGFR3 ratio in RASF to levels in OASF (dotted line median expression in OASF, n=10 each). D) Proliferation in response to FGF2, OASF (left) and RASF (right), without or with MG-132 (dotted lines represents mean of untreated control cells, bars means and standard deviations); the increased response of RASF to FGF2 is abolished by the inhibition of proteasomes (n=6 each)(Mann-Whitney U-tests).

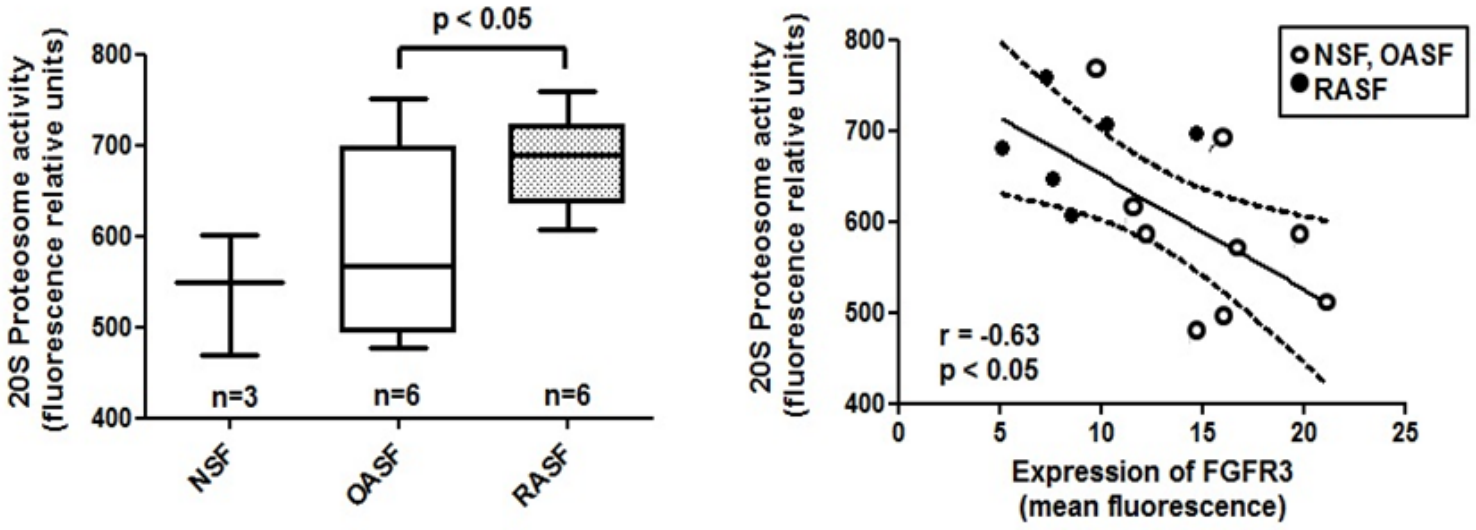

Figure 6

Increased 20S proteasome activity correlated with decreased expression of FGFR3. Synovial fibroblasts were seeded in a 96-well plate in 100 µl culture medium at a density of 0.5x104 cells/well. Tested were 3 cultures of normal synovial fibroblasts (NSF), and 6 each of OASF and RASF. A) After 24 hours, the cells were processed for measurement of 20S proteasome activity. RASF showed significantly more 20S proteasome activity (Box-Whiskers-Plot, Wilcoxon signed rank test). B) Dot-plot of 20S proteasome activity and expression of FGFR3 in permeabilized fibroblasts. 20S proteasome negatively correlated with the expression of FGFR3 (Pearson's correlation coefficient).

Supplementary Files

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- SupplFigure1.tif
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- SupplFigure2.tiff
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