

# mTOR Regulates the Invasive Properties of Synovial Fibroblasts in Rheumatoid Arthritis

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The invasive properties of fibroblast-like synoviocytes (FLS) correlate with radiographic and histologic damage in rheumatoid arthritis (RA) and pristane-induced arthritis (PIA). We previously determined that highly invasive FLS obtained from PIA-susceptible DA (blood type D, Agouti) rats have increased expression of genes associated with invasive cancers, including Villin-2/ezrin. Villin-2/ezrin mediates invasion via mTOR. In the present study we used the mTOR inhibitor rapamycin to assess the role of the ezrin-mTOR pathway on the invasive properties of FLS. FLS were isolated from synovial tissues from arthritic DA rats, and from RA patients. FLS were treated with rapamycin or dimethyl sulfoxide (DMSO) for 24 h and then studied in a Matrigel-invasion assay. Supernatants were assayed for matrix metalloproteinase (MMP) activity, and cell lysates were used for quantification of mTOR, p70S6K1, 4EBP1 and FAK, as well as their respective phosphorylated subsets. Actin filament and FAK localization were determined by immunofluorescence. Rapamycin decreased FLS invasion in DA and RA tissues by 93% and 82%, respectively. Rapamycin treatment reduced the phosphorylation of mTOR and its substrates, p70S6K1 and 4EBP1, confirming mTOR inhibition. In conclusion, rapamycin prevented actin reorganization in both DA and RA FLS, and inhibited the directional formation of lamellipodia. Phosphorylation of the lamellipodia marker FAK was also reduced by rapamycin. MMPs were not significantly affected by rapamycin. Rapamycin significantly reduced RA and DA rat FLS invasion via the suppression of the mTOR signaling pathway. This discovery suggests that rapamycin could have a role in RA therapy aimed at reducing the articular damage and erosive changes mediated by FLS.

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## INTRODUCTION

Rheumatoid arthritis (RA) is a common chronic autoimmune disease that affects approximately 1% of the population and is commonly associated with disability and deformities (1,2). The basic joint pathology in RA is characterized by pronounced synovial hyperplasia, also called pannus, which produces several proinflammatory cytokines and proteases, and like a malignant tumor, invades and destroys cartilage and bone (2–4).

Synovial fibroblasts, also called fibroblast-like synoviocytes (FLS), have a central role in the formation of pannus,

and in cartilage and bone invasion and destruction (2,5). The invasive properties of RA FLS have been studied *in vitro*, and correlate with radiographic evidence of damage in RA (6), which is a well-established severity and outcome parameter. However, the regulation of the invasive properties of FLS remains incompletely understood.

We have previously reported our discovery that Cia5d is a genetic regulator of disease severity, bone and cartilage destruction, and FLS invasion in rat arthritis (7,8). Microarray analyses of gene expression to compare highly invasive FLS from arthritis-susceptible

DA (blood type D, Agouti) rats with minimally invasive FLS from arthritis-protected DA rats with DA.F344(Cia5d) congenics led to the identification of an invasion-associated signature (9). This invasion-associated signature included several genes implicated in cancer invasion and metastasis, such as *Villin-2* (which encodes the protein ezrin), *Cxcl10* and *Nras* (10–15). *Vil-2/ezrin* is a member of the ezrin-radixin-moesin family of proteins, which are linkers between the actin cytoskeleton and the plasma membrane that also mediate cell signal transduction. *Vil-2/ezrin* was of particular interest because it has been implicated in the regulation of cancer metastasis (15,16) and cancer cell invasion (17,18), but has not been studied in FLS. Ezrin-mediated invasion operates via the mTOR pathway (19,20), and therefore we hypothesized that the *Vil-2/ezrin-mTOR* pathway has a central role in the regulation of the inva-

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sive properties of FLS from rats with arthritis and from patients with RA.

## METHODS

### Rats

DA (DA/BklArbNsi, arthritis-susceptible) rats were housed in a specific pathogen-free environment, with 12-h light-dark cycles and free access to food and water. All experiments involving animals were approved by the Institutional Animal Care and Use Committee of the Feinstein Institute for Medical Research.

DA rats (8–12 wks old) received 150  $\mu$ L of pristane by intradermal injection at the base of the tail (21,22). All rats developed pristane-induced arthritis (PIA) and were euthanized on d 21 after injection. Synovial tissue was dissected from the ankles for FLS isolation.

### RA Patients

Synovial tissues were obtained from RA patients undergoing an elective orthopedic surgery. All patients met the American College of Rheumatology criteria for RA (23) and all completed a signed informed consent form obtained through the Feinstein Institute Tissue Donation Program under an institutional review board-approved protocol.

### Isolation and Culture of FLS

FLS were obtained as previously described (9). Briefly, synovial tissues from DA rats and RA patients were minced and then digested with DNase 0.15 mg/mL, hyaluronidase type I-S 0.15 mg/mL, and collagenase type IA 1 mg/mL (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, CA, USA) for 1 h at 37°C. Cells were washed and resuspended in complete media containing DMEM with 10% fetal bovine serum (Invitrogen), glutamine 30 mg/mL, amphotericin B 250  $\mu$ g/mL (Sigma) and gentamicin 10 mg/mL (Invitrogen). After overnight culture, nonadherent cells were removed and adherent cells cultured. All experiments were performed with FLS after passage four (>95% FLS purity).

### Invasion Assay

*In vitro* invasion of FLS was assayed in a transwell system by using collagen matrix-coated inserts (Matrigel) from BD (Franklin Lakes, NJ, USA) as previously described (8,24,25). Briefly, 70–80% confluent cells were harvested by trypsin-EDTA digestion. Then  $2.0 \times 10^4$  cells were resuspended in 500  $\mu$ L of serum-free DMEM and plated in the upper compartment of the Matrigel-coated inserts. Rapamycin (MP Biomedicals USA, Solon, OH, USA) or the same amount of control solvent (dimethyl sulfoxide [DMSO]) was added to the upper chamber. The lower compartment was filled with complete media and the plates were incubated at 37°C for 24 h. The upper surface of the insert was then wiped with cotton swabs to remove non-invading cells and the Matrigel layer. The opposite side of the insert was stained with Crystal Violet (Sigma) and the total number of cells that invaded through Matrigel counted at 100 $\times$  magnification. Experiments were done in duplicates.

### Zymography

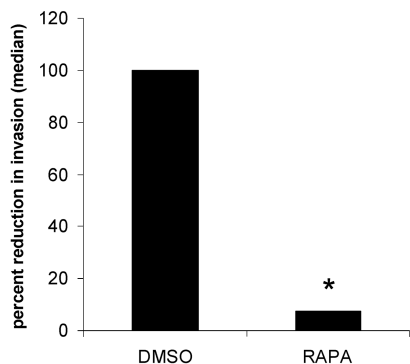
Levels and catalytic activity of MMPs were determined from FLS culture supernatants. Gelatin (MMP-2, MMP-9) and casein (MMP-3) zymography was performed according to previously reported methods (8,26,27). Briefly, equal volumes of supernatants obtained from the upper chambers of the Matrigel-coated invasion system were mixed 1:1 with Tris-glycine-sodium dodecyl sulfate sample buffer loaded into a precasted zymogram gel (Invitrogen). After a 90-min electrophoresis at 125 V, gels were treated with renaturing buffer, followed by overnight incubation in developing buffer (Invitrogen) at 37°C. Gels were stained with SimplyBlue Safe-Stain for 1 h at room temperature and then washed.

### Western Blots

**MMP-1 and MMP-13 detection in cell culture supernatants.** Equal amounts of supernatants from the upper chambers of the Matrigel-coated invasion system were loaded into a NuPAGE 10% Bis-Tris gel (Invitrogen) in the presence of MES buffer

(Invitrogen) in reducing conditions. Proteins were transferred overnight to a polyvinylidene difluoride (PVDF) membrane (Immobilion, Millipore, Bedford, MA, USA), which was subsequently blocked with 5% blotting-grade nonfat dry milk (Bio-Rad, Hercules, CA, USA). Membranes were blotted with either a mouse anti-MMP-1 monoclonal antibody (Calbiochem, EMD, La Jolla, CA, USA) or a mouse anti-MMP-13 monoclonal antibody (Thermo Scientific, Fremont, CA, USA). A horseradish-peroxidase-conjugated goat antimouse IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as secondary antibody.

**Total and phosphoprotein detection in cell lysates.** For detection of intracellular proteins, FLS were plated at 70–80% confluence on 6-well plates and allowed to adhere for a period of 24 h. Cells were then serum starved for 24 h and then treated with the indicated concentrations of rapamycin or DMSO for 5, 30 and 60 min and 24 h in complete media. Total cell lysates were collected with radioimmunoprecipitation assay buffer (RIPA, Thermo Scientific) containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Protein content was determined with a BCA (bicinchoninic acid) protein assay kit (Thermo Scientific), and the same amount of total protein was loaded into a NuPAGE 10% Bis-Tris gel (Invitrogen) in the presence of MES buffer (Invitrogen) in reducing conditions. Proteins were transferred to PVDF membranes and blocked with 5% nonfat milk for 1 h. Membranes were probed with mouse or rabbit antibodies against the phosphorylated form of mTOR, mTOR direct targets for phosphorylation p70S6 kinase (p70S6K) and 4E-BP1, and focal adhesion kinase (FAK) (Cell Signaling, Danvers, MA, USA). Horseradish peroxidase-conjugated goat antimouse IgG or donkey antirabbit IgG (Amersham-GE Healthcare, Piscataway, NJ, USA) were used as secondary antibodies and incubated for 1 h at room temperature in Tris-buffered saline with 0.1% Tween-20. Protein bands were detected with Amersham ECL plus (GE Healthcare) or Western Lightning Chemiluminescence reagent



**Figure 1.** Rapamycin reduces arthritic DA rat FLS invasion. Rapamycin-treated FLS (n = 6, each cell line generated from a different DA rat with pristane-induced arthritis) had a 93% reduction in the median number of invading cells compared with DMSO-treated FLS (\**P* = 0.002, Mann–Whitney test; 25%–75% confidence intervals = 2.42–13.38).

(Perkin Elmer, Boston, MA, USA) and visualized using X-OMAT Kodak films (Eastman Kodak, Rochester, NY, USA). Densitometry was done with Quantity One (Bio-Rad). After the analyses of phosphoproteins, membranes were stripped and reprobed with the respective antibody against the specific total protein (mouse or rabbit antibodies; Cell Signaling) following the same imaging method described above.

**Immunofluorescence**

Confluent FLS (10% to 20%) were cultured on cover slips, starved overnight and treated with either DMSO or rapamycin (0.1 μmol/L for RA FLS and 10 μmol/L for rat FLS) in complete media. FLS were then fixed with 4% formaldehyde (Ted Pella, Redding, CA, USA) for 15 min at room temperature followed by blocking with 5% nonfat milk for 30 min. Cells were incubated with rabbit antibodies against phosphorylated or total FAK (Cell Signaling) for 1 h at room temperature, washed with PBS with 0.1% Triton X-100, and then incubated with TexRed-conjugated antirabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 30 min. Phalloidin–fluorescein isothiocyanate (Sigma-Aldrich, St. Louis, MO, USA) was used to stain actin fila-

ments. Stained cells were washed with PBS and mounted, then observed under fluorescent microscopy (Zeiss Axiovert 200M with Zeiss software Axioversion 4.7) (Carl Zeiss GmbH, Jena, Germany).

**MTT Assay**

FLS were plated in triplicates at 4 × 10<sup>4</sup> cells per well in 96-well plates and allowed to adhere for 24 h. Media were then replaced by media containing either DMSO or different concentrations of rapamycin at 0.1–10 μmol/L. Cell survival was determined with the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Millipore) 24 h later according to the manufacturer’s instructions.

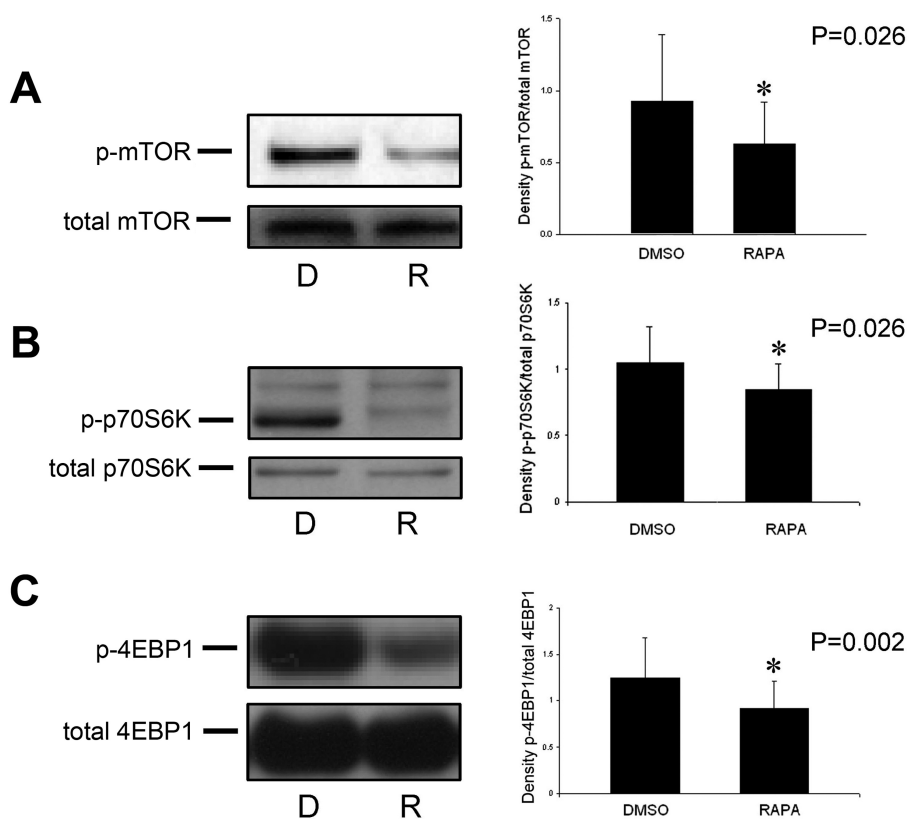
**Statistics**

Means (normally distributed data) were analyzed with the Student *t* test, and medians (nonnormally distributed data) compared with the nonparametric Mann–Whitney test using SigmaStat 3.0 (SPSS, Chicago, IL, USA).

**RESULTS**

**Rapamycin Reduced DA Rat FLS Invasion**

Rapamycin 10 μmol/L significantly reduced the median number of invading FLS by 93% (n = 6; *P* = 0.002, Mann–Whitney test, Figure 1). This concentration of rapamycin was not toxic to FLS (based on results of an MTT assay; data not shown).

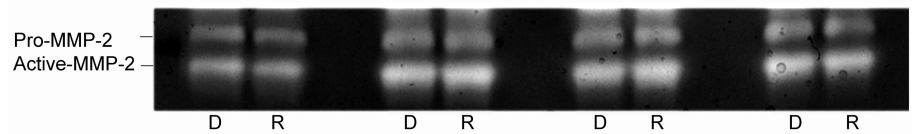


**Figure 2.** Rapamycin treatment inhibits the phosphorylation of mTOR and mTOR target proteins. (A) Rapamycin (10 μmol/L) reduced levels of phospho-mTOR, with a phospho-mTOR/total median reduction of 47% (*P* = 0.026). (B) Levels of phosphorylation of mTOR targets phospho-p70S6K (phospho-p70S6K/total median reduction of 15%, *P* = 0.026) and (C) phospho-4EBP1 (phospho-4EBP1/total median reduction of 29%, *P* = 0.002) were also decreased, confirming the inhibition of the mTOR pathway (data shown are representative of experiments done with six or seven different DA FLS cell lines with 30 min of stimulation; Mann–Whitney test (see Methods)). D = DMSO; R = rapamycin.



### Reduced Invasion in Rapamycin-Treated FLS Was Associated with Reduced Phosphorylation of mTOR Pathway Proteins

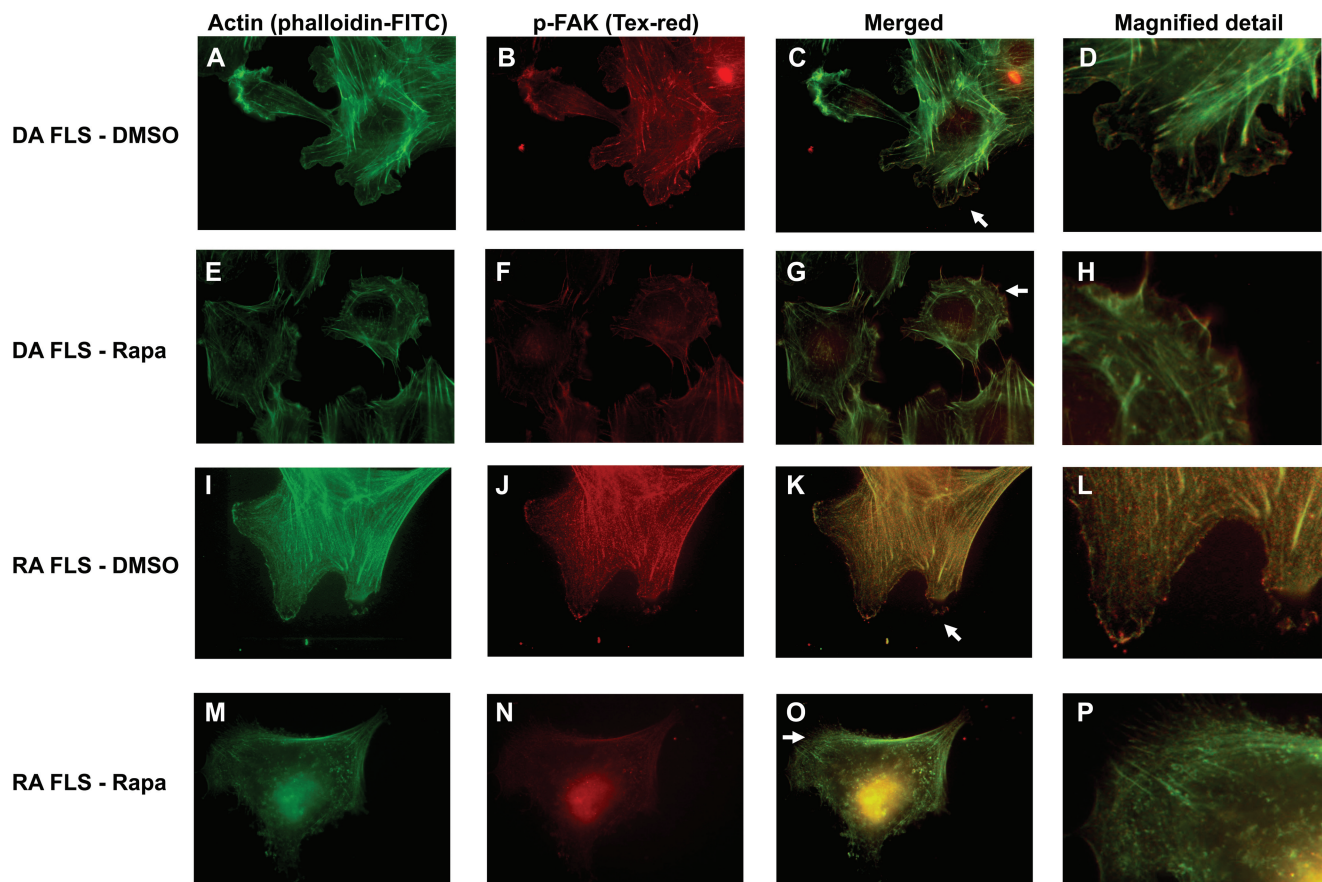
Rapamycin-treated DA FLS had significantly reduced levels of phosphorylated mTOR (Figure 2A, phospho-mTOR/total median reduction of 47%,  $P = 0.026$ ), as well as reduced levels of a phosphorylated form of two mTOR substrates, p70S6K1 (Figure 2B, phospho-p70S6K/total median reduction of 15%,  $P = 0.026$ ) and 4EBP1 (Figure 2C, phospho-4EBP1/



**Figure 3.** Rapamycin treatment did not change the levels of pro or active forms of MMP-2. Gelatin zymogram loaded with FLS culture supernatants from six different DA FLS lines, showing the pro-MMP-2 and active-MMP-2 bands. Results from four cell lines are shown. D = DMSO control; R = rapamycin.

total median reduction of 29%,  $P = 0.002$ ), confirming the inhibition of this pathway in association with reduced in-

vasion. Levels of Akt, Erk, phospho-Akt and phospho-Erk were not affected by rapamycin (data not shown).



**Figure 4.** Rapamycin interferes with actin cytoskeleton and lamellipodia formation and reduces levels of phospho-FAK in FLS from arthritic rats and RA patients. Rapamycin treatment reduces the linear distribution of the actin cytoskeleton (fluorescein isothiocyanate (FITC), panels E, M versus DMSO in panels A and I) and the formation of lamellipodia (E-H and M-P; arrows on C and K point to lamellipodia), compared with control (DMSO-treated, A-D and M-P), creating more compact and less spread-looking cells. Rapamycin reduced levels (F and N) and to a certain extent the focal localization of phospho-FAK (Tex-red, panels F-H and N-P) in FLS from DA rats, and in FLS from RA patients, compared with DMSO (B-D and J-L). Regions marked by arrows on panels C, G, K and O are magnified for detail on the right-hand panels, respectively (D, H, L and P), showing colocalization (yellow) of phospho-FAK (red) with actin (green) in DMSO-treated cells, and lack of colocalization or formation of lamellipodia in rapamycin-treated cells. Pictures taken from cells stimulated for 24 h (four different DA and three different RA FLS cell lines). Images A, B, C, E, F, G, I, J, K, M, N and O were acquired with a 62 $\times$  magnification lens. Images D, H, L and P are 3 $\times$  digital amplifications of details marked with arrows on panels C, G, K and O, respectively.

### Levels of Four Different MMPs Were Not Affected by Rapamycin

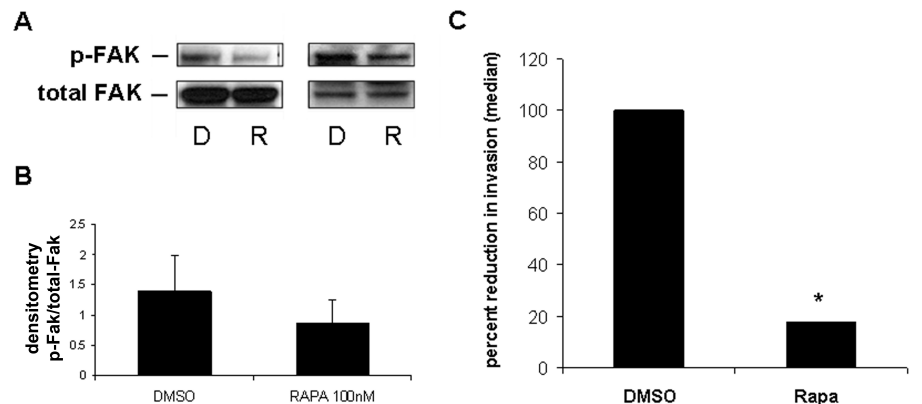
To identify the cellular process accounting for the rapamycin-induced mTOR-mediated inhibition of invasion, we measured levels of pro- and active MMP-1, MMP-2, MMP-3 and MMP-13 in supernatants from DA rat FLS cultures. No significant differences were detected between the supernatants of rapamycin-treated and DMSO-treated FLS (MMP-2 zymogram shown in Figure 3).

### Rapamycin Prevented Actin Cytoskeleton Reorganization and Inhibited the Directional Formation of Lamellipodia

Phalloidin staining revealed that DMSO-treated DA rat FLS had a linear and organized actin distribution (Figure 4A), whereas rapamycin-treated FLS had a less organized and dense distribution of actin (Figure 4E). Furthermore, rapamycin inhibited the directional formation of lamellipodia (Figure 4A, D, E, H), a process required for cell mobility and invasion. Results of immunostaining with anti-FAK and anti-phospho-FAK, which accumulate in and are required for the formation of lamellipodia, confirmed that in rapamycin-treated cells phospho-FAK levels were reduced and had more homogeneous expression in the cytoplasm and nucleus (Figure 4F, G, H), whereas in DMSO-treated cells it had a more intense and polarized (directional) expression (Figure 4B) that more often colocalized with lamellipodia (Figure 4C, D). Western blot analyses using FLS cell lysates confirmed the reduced expression of phospho-FAK in rapamycin-treated DA rat FLS (Figure 5A).

### Rapamycin Inhibited RA FLS Invasion, and Like in DA FLS Modulates Actin Rearrangements, Lamellipodia Formation and Levels of Phospho-FAK

To determine the direct relevance of the observations made in DA rat FLS to human disease, eight RA FLS cell lines were tested in invasion assays. A significantly lower concentration of rapamycin (100 nmol/L) than that required in the rat



**Figure 5.** Rapamycin significantly reduced RA FLS invasion and FAK phosphorylation. (A) Western blot demonstrating reduced levels of phospho-FAK in rapamycin-treated (R) DA FLS, compared with DMSO-treated FLS (D). (B) Densitometry analyses showing phospho-FAK/total FAK in RA cells stimulated for 30 min. (C) Rapamycin (100 nmol/L) also significantly reduced RA FLS median invasion by 82% compared with DMSO alone ( $n = 8$ ;  $*P < 0.001$ , Mann-Whitney test; 25%–75% confidence intervals = 5.42–23.78).

studies (10  $\mu\text{mol/L}$ ) was enough to reduce the levels of phospho-FAK (Figure 5B) in RA FLS, and reduce invasion by 82% ( $P < 0.001$ , Mann-Whitney test; reduction similar to that seen in DA FLS; Figure 5C). Rapamycin also induced morphologic changes in RA FLS that were similar to those detected in DA rat FLS, including the reduced and nonlinear distribution of actin, and the inhibition of the directional formation of lamellipodia (Figure 4, DMSO, panels I–L and rapamycin, panels M–P). In addition, rapamycin reduced the levels and the polarized distribution of phospho-FAK (Figure 4, J–L, N–P). These observations demonstrated that rapamycin induced similar effects in FLS from arthritic DA rats and from patients with RA, making our results directly relevant to human disease and validating the use of the rat-based *in vitro* model of invasion.

### DISCUSSION

FLS have a central role in the formation of the RA pannus, and in pannus invasion and destruction of cartilage and bone (2–5). Yet, the regulation of the invasive properties of FLS remains incompletely understood. The *in vitro* invasive properties of FLS have direct clinical relevance and correlate with radiographic joint damage in RA (6,25), and with his-

tologic damage and cartilage and bone invasion and destruction in rats with PIA (8). Therefore, understanding the regulation of the invasive properties of FLS may lead to the generation of new tools for disease prognostication as well as new targets for therapies aimed at preserving joint architecture and reducing destruction.

Our earlier work demonstrated that the arthritis severity locus *Cia5d* regulates bone and cartilage destruction, and FLS invasion (7,8). DA rats and DA.F344(*Cia5d*) congenics are identical except for the *Cia5d* interval. Nevertheless, that was enough to cause a significant phenotypic difference, so that DA FLS rats are highly invasive, whereas FLS from DA.F344(*Cia5d*) congenic rats are minimally invasive. We used the FLS invasion phenotypic difference as a model system in which background genome noise was reduced, thus facilitating the identification of new and specific FLS invasion regulatory pathways. Microarray analyses of gene expression to compare mRNA from DA and DA.F344(*Cia5d*) congenics led to the identification of a new invasion-associated signature (9).

*Vil-2* (which encodes the protein ezrin) was one of the genes most significantly upregulated in highly invasive DA FLS.

Vil-2/ezrin was of particular interest because it is expressed in several cancers, and regulates cancer cell invasion and metastasis (15–18).

Vil-2/ezrin mediate invasion via the mTOR pathway (19,20). mTOR is an evolutionary conserved serine-threonine kinase that senses various stimuli to control growth, proliferation and protein synthesis (28,29). Like Vil-2/ezrin, mTOR is upregulated in several cancers (30,31) and regulates cancer cell invasion (32,33). mTOR expression also correlates with poor prognosis in cancer (30,34,35).

Therefore, we considered that the Vil2/ezrin-mTOR pathway could have a major role in the regulation of the invasive properties of FLS from arthritic DA rats and from patients with RA. We initially used the mTOR-specific inhibitor rapamycin in rat FLS and then confirmed all findings in RA FLS. Rapamycin significantly reduced the median number of invading FLS from arthritic DA rats and from patients with RA by 93% and 82%, respectively. The phosphorylation of both mTOR and its substrates 4EBP1 and p70S6K was reduced in rapamycin-treated cells, confirming mTOR inhibition.

mTOR inhibition did not affect the FLS production or activity of MMP-1, MMP-2, MMP-3 or MMP-13. However, mTOR inhibition interfered with FLS actin cytoskeleton reorganization, reduced levels of phospho-FAK and prevented the polarized formation of lamellipodia, which are processes central to cell mobility and invasion. Therefore, the inhibition of these cellular processes interfered with the ability of FLS to move and invade in a coordinated and directional manner. Our results also suggest that mTOR regulates the phosphorylation of FAK. This is the first time that mTOR has been implicated in the regulation of these cellular processes in FLS.

Lamellipodia are sheet-like protrusions at the cell front and are composed of mesh and bundles of actin filaments that mediate cell protrusion and migration, and cancer cell invasion (36,37). Lamellipodia formation requires the expression of FAK (38,39), and FAK deletion pre-

vents the polarized formation of lamellipodia (39). FAK, and most importantly phospho-FAK, are upregulated in migrating and invading cells, and tend to transiently colocalize with the lamellipodia protrusions (38,39). These reported findings are in agreement with our observations of rapamycin-induced reduction in levels of total and phospho-FAK, and those correlated with prevention of the polarized formation of lamellipodia and reduced FLS invasion.

The discovery of a role for mTOR in the regulation of FLS invasion suggests a novel target pathway in which available mTOR inhibitors could become new therapies aimed at reducing FLS invasion and preventing and/or reducing joint erosion and damage in RA. Interestingly, rapamycin was shown to reduce pannus formation, and to reduce cartilage erosion and joint damage in adjuvant-induced arthritis in rats (40) through mechanisms that were as yet unknown. In addition, everolimus, another mTOR-specific inhibitor, significantly reduced disease activity (based on ACR20) in a short 12-week, double-blind and placebo-controlled study in RA patients (41). A recent study also demonstrated that FLS from arthritic joints can spread disease to unaffected joints and there invade and erode cartilage (42). To reach another joint the FLS has to move from its location in the synovial tissue to the lumen of a capillary or venule, a process that requires a functional actin cytoskeleton for cell motility and likely invasion through the basement membrane. Therefore, our results provide a mechanism to explain the arthritis-ameliorating and joint-protecting effects of mTOR inhibitors, in which these drugs prevent FLS from reorganizing their actin cytoskeleton and from invading and destroying cartilage and bone, and potentially from spreading disease from one joint to another.

In conclusion, we have demonstrated a new role for mTOR in FLS invasion via the regulation of FAK and the formation of lamellipodia. These observations provide a cellular and molecular explana-

tion for the joint architecture–protecting effects of mTOR inhibitors in rodents, and for the recently reported significant clinical disease amelioration in RA. mTOR antagonists may become important therapeutic agents with joint-protecting properties in RA, and perhaps in other erosive arthropathies as well. Our observations also validate the importance of the study of FLS invasion *in vitro* and microarray analysis of FLS for the discovery of new treatments for RA. Furthermore, we demonstrated that studying phenotypes relevant to RA in rodent models of arthritis can facilitate the discovery of new and relevant processes and new targets for therapy.

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## DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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