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# Altered expression of microRNA-23a in psoriatic arthritis modulates synovial fibroblast pro-inflammatory mechanisms via phosphodiesterase 4B

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ABSTRACT

*Objectives:* To investigate the functional role of miR-23a in synovial fibroblasts (SFC) activation in psoriatic arthritis (PsA).

*Methods:* Differential expression of the miR-23a-27a-24-2 cluster was identified by real-time quantitative PCR in PsA synovial tissue and peripheral blood mononuclear cells (PBMC) compared to osteoarthritis (OA) and correlated with disease activity. For regulation experiments, PsA synovial fibroblasts (SFC) were cultured with Toll-like receptor (TLR) ligands and pro-inflammatory cytokines. PsA SFC were transfected with a miR-23a inhibitor to assess the functional effect on migration, invasion and expression of pro-inflammatory meditators. The direct interaction between miR-23a and predicted target mRNA, phosphodiesterase 4B (PDE4B), was examined by luciferase reporter gene assay, with the expression and regulation confirmed by RT-PCR and western blot. A PDE4 inhibitor was used to analyse the function of PDE4B signalling in both miR-23a and Poly(I:C)-induced PsA SFC activation.

*Results:* Synovial tissue expression of miR-23a was lower in PsA compared to OA and correlated inversely with disease activity and synovitis. TLR activation via Poly (I:C) and LPS, but not Pam3CSK4, significantly decreased miR-23a expression, with no significant effect observed in reponse to stimulation with pro-inflammatory cytokines. Decreased miR-23a expression enhanced PsA SFC migration, invasion and secretion of IL-6, IL-8, MCP-1, RANTES and VEGF. We identified PDE4B as a direct target of miR-23a and demonstrated enhanced mRNA and protein expression of PDE4B in anti-miR-23a transfected PsA SFC. Poly(I:C) and/or miR-23a-induced migration and enhanced cytokine expression was suppressed by the blockade of PDE4 signalling.

*Conclusions:* In PsA, dysregulated miR-23a expression contributes to synovial inflammation through enhanced SFC activation, via PDE4B signalling, and identifies a novel anti-inflammatory mechanism of PDE4 blockade.

## 1. Introduction

Psoriatic arthritis (PsA) is a chronic and destructive arthropathy of the peripheral joints, spine and entheses associated with psoriasis. Synovial inflammation is central in disease progression, joint damage and reduced mobility [1]. There is an established role for synovial fibroblast cells (SFC) in disease initiation and progression in synovial inflammation [2]. In early disease, SFC are a major source of matrix-degrading enzymes and pro-inflammatory mediators which orchestrate the persistent infiltration of immune cells and transform the synovial tissue into a tumour-like 'pannus' that invades adjacent bone and cartilage [3].

While the precise molecular mechanisms regulating the activation of PsA SFC are currently unclear, dysregulated expression of microRNAs (miRNA) have been strongly associated with SFC activation in rheumatoid arthritis (RA) [4,5]. miRNA are small, non-coding RNA molecules involved in post-transcriptional modification to regulate various biological processes such as angiogenesis, immune cell activation, cell motility and cell death [6]. Abnormal expression of miRNA in disease is well documented [7], however little is known about their role in PsA. Evidence that miR-23a, a prominent member of the miR-23a-27a-24-2 cluster, can regulate cellular activation, cell motility and immune cell infiltration has been observed in cancer [8,9]. Recently, Hu et al., identified decreased levels of miR-23a in articular cartilage of RA patients, and demonstrated elevated miR-23a levels repressed IL-17-mediated pathogenic mechanisms, through the targeted regulation of IKKα [10].

In this study, we provide evidence that miR-23a expression is dysregulated in the synovium of PsA patients and modulates PsA SFC activation *ex vivo* including enhanced cell motility and elevated expression of pro-inflammatory mediators. We identify phosphodiesterase 4B (PDE4B) as a direct target of miR-23a through which it modifies PsA SFC phenotype. In addition, we demonstrate that PDE4 blockade, a treatment strategy recently approved for PsA [11], can suppress miR-23a-mediated pathogenic mechanisms. These data suggest the miR-23a-PDE4B axis plays a key role in regulating SFC invasive mechanisms in

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PsA synovium.

## 2. Methods

### 2.1. Patient recruitment

Patients fulfilling the CASPAR classification criteria for PsA [12] with active disease were recruited from the Rheumatology Department, St. Vincent's University Hospital. Synovial tissue biopsies were obtained at arthroscopy under local anaesthetic using a Wolf 2.7 mm telescope as previously described [13]. For comparison, synovial biopsies were also obtained from patients with osteoarthritis (OA). Biopsies were either utilised for isolation of primary PsA SFC, snap-frozen or placed in RNAlater. Macroscopic synovitis and vascularity were quantified using a visual analogue scale (VAS) 1–100 mm. Clinical assessment included tender and swollen joint count (TJC/SJC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and global health visual analogue scale (VAS) to assess disease activity. Peripheral blood mononuclear cells (PBMC) were also obtained from PsA and OA patients.

### 2.2. Culture, treatment and transfection of primary PsA SFC

Primary SFC were isolated from PsA synovial biopsies by digestion with 1 mg/ml collagenase type 1 (Worthington Biochemical, Freehold, NJ, USA) in RPMI 1640 (Gibco-BRL, Paisley, UK) for 4 h at 37 °C in humidified air with 5% $CO_2$. Dissociated cells were maintained in RPMI 1640 supplemented with 10% FCS (Gibco-BRL), 10 ml of 1 mmol/l HEPES (Gibco-BRL), penicillin (100 units/ml; Bioscience), streptomycin (100 units/ml; Bioscience) and fungizone (0.25 μg/ml; Bioscience). For all experiments, cells were generated from a number of different patients and utilised between passages 3–8. For the stimulation experiments, cells were stimulated with TLR-2 agonist Pam3CSK4 (1 μg/ml), TNFα (10 ng/ml), IL-1β (1 ng/ml), IL-17A (20 ng/ml), IL-6 (20 ng/ml), TLR-3 agonist Poly(I:C) (10 μg/ml) and TLR-4 agonist LPS (1 μg/ml) for 24hr. For blockade experiments, cells were cultured in the presence of PDE4 inhibitor rolipram (10 μM) or DMSO (10 μM) vehicle control for 15min prior to stimulation with Poly(I:C) (10 μg/ml). To examine the effect of decreased miR-23a expression on PsA SFC activation, PsA SFC were cultured in antibiotic free RPMI 1640 medium and transfected with 100 nM anti-miR-23a or corresponding negative control using Lipofectamin 2000 Reagent (Invitrogen) in accordance with the manufacturer's instructions. Following 48hr or 72hr transfection, cells were directly lysed for RNA and protein quantification or washed and cultured in fresh medium for a further 24hr, then supernatants harvested, and secretion of pro-inflammatory mediators assessed. Transfected PsA SFC were also cultured in the presence or absence of rolipram (10 μM) or DMSO control (10 μM) where indicated.

### 2.3. 3′UTR luciferase reporter gene assay

Using TargetScan 7.0 (www.targetscan.org), the potential binding sites of miR-23a in the 3′ UTR of the PDE4B mRNA were predicted. To create a 3′UTR luciferase reporter construct, a 500bp fragment of the 3′UTR of PDE4B containing the putative miR-23a binding site, was cloned downstream of the PGK-driven luciferase gene in the pmiRGLO Vector (Promega). For miR-23a/PDE4B target validation, human embryonic kidney 293 (Hek293) cells were transfected with 575 ng of the 3′UTR-pmiRGLO_PDE4B vector together with 30 nM Pre-miR-23a or Pre-miR negative control. After 24hr, cells were lysed, and the relative luciferase activity was measured by a Dual Luciferase Reporter Assay system (Promega). Firefly luciferase activity was normalised to the constitutively expressed Renilla luciferase.

### 2.4. RNA isolation and real time quantitative PCR (RT-qPCR)

Total RNA, including miRNA, was isolated using miRNeasy Kit (Qiagen, Germany) according to the manufacturer's specifications. To determine expression of mature miRNAs, stem-loop primers were designed for miR-23a, miR-27a, miR-24-2, RNU44 and RNU48 as previously described [14]. The corresponding miRNA stem-loops were reverse-transcribed using the Taqman microRNA Reverse Transcription Kit (Ambion) and quantified by a SYBR Green Roche or Applied Biosystems qPCR system. For RT-qPCR analysis of pro-inflammatory mediators and putative signalling molecules, total RNA was reverse-transcribed and quantified using specific primers designed for MMP1, MMP3, RANTES, MCP-1, IL-6, IL-8 and PDE4 (A-D) (Supplementary Table 1).

### 2.5. Chemokine/cytokine expression

To assess the effect of miR-23a expression on PsA SFC cytokine production, following 48hr transfection, cells were subsequently cultured in fresh medium for a further 24hr. For Poly(I:C) stimulated PsA SFCs, cells were pre-treated with rolipram (10 μM) or DMSO (10 μM) for 30min prior to 24hr stimulation with Poly(I:C) (10 μg/ml). Supernatants were harvested and IL-6, IL-8, MCP-1, RANTES and VEGF were measured by specific ELISA according to the manufacturer's conditions (R&D Systems).

### 2.6. Wound repair assay

PsA SFC were seeded onto 48 well plates and grown to confluence. After 48hr anti-miR-23a transfection, a single scratch wound was induced through the middle of each well and migration across the wound margins was assessed and photographed (10x Magnification) at 24–36hr using a phase-contrast microscope (Nikon TMS microscope linked to a Canon S70 camera). For miR-23a or Poly(I:C) and PDE4 blockade experiments, cells were subsequently stimulated for 24–36hr with rolipram (10 μM) or DMSO (10 μM) control. Semi-quantitative analysis of cell repopulation of the wound margins was performed by counting the number of cells migrating into the wound space. This process was repeated for all technical replicates.

### 2.7. Transwell invasion assay

Biocoat Matrigel™ Invasion Chambers (Becton Dickinson, UK) were used to assess PsA SFC invasion in response to miR-23a expression. Cells transfected with anti-miR-23a or anti-Control for 48hr were subsequently seeded at a density of 30,000 cells per well in the migration chamber on 8 μm membranes pre-coated with matrigel. Initial experiments assessed the effect of anti-miR-23a on PsA SFC invasion by placing cells in complete RPMI media for 48hr. Subsequent experiments were also performed in the presence or absence of Rolipram (10 μM) or DMSO control (10 μM). Non-migrating PsA SFC were removed from the upper surface by gentle scrubbing. Migrating cells attached to the lower membrane were fixed with 1% glutaraldehyde and stained with 0.1% crystal violet. Cells from five random high-power fields for each well were counted to assess the average number of migrating cells.

### 2.8. Immunofluorescence staining for F-actin

Anti-miR-23a transfected PsA SFC or SFC cultured in the presence of Poly(I:C) (10 μg/ml) and in the presence or absence of rolipram (10 μM) were sparsely seeded into 8-well chamber slides. Slides were washed with PBS and fixed with 1% paraformaldehyde in PBS for 20min. To visualise F-actin, slides were stained with Oregon Green® phalloidin (Molecular Probes, Leiden, The Netherlands) and nuclei were counterstained with DAPI (Sigma, MO, USA). Slides were washed in PBS and mounted in ProLong Antifade (Applied Biosystems). Stained cells were visualised with a Leitz DM40 microscope and images were captured using the AxioCam system and AxioVision 3.0.6. Software (Carl Zeiss, Inc., NY, USA).

### 2.9. Western blot analysis

Total proteins from PsA synovial tissue and SFC were separated on a 10% SDS polyacrylamide gel and the proteins transferred to a PVDF membrane (ThermoFisher Scientific). Non-specific binding sites were blocked with 5% BSA for 1 hr at room temperature. Membranes were subsequently incubated overnight at 4 °C with rabbit anti-PDE4B (FabGennix, TX, USA) and mouse anti-β-Actin (Sigma-Aldrich) antibodies conjugated with horse-radish peroxidase. Band densities were quantified using the ChemiDoc MP Imaging System (Bio-Rad, CA, USA).

### 2.10. Statistical analysis

Statistical analyses were performed using Prism 5 software. Wilcoxon Signed Rank test or Mann Whitney was used for analysis of non-parametric data. Student t-test was used for parametric data. P-values of less than 0.05 (*$p < 0.05$) were determined as statistically significant.

## 3. Results

### 3.1. Dysregulated miRNA expression in PsA synovial tissue and PBMC

To investigate the expression of miR-23a-27a-24-2 cluster in cells from PsA patients, the expression of mature miR-23a cluster strands, miR-23a, miR-27a and miR-24-2, were quantified in PsA PBMC and synovial tissue compared to OA disease comparator by RT-qPCR. At the systemic level, we found that only the relative expression of miR-23a was significantly increased in PsA compared to OA ($p < 0.05$; Fig. 1A). In contrast, we demonstrated that in synovial tissue biopsies, the relative expression of miR-23a ($p < 0.05$), miR-27a ($p < 0.05$) and miR-24-2 ($p < 0.01$) were significantly decreased in PsA ST compared to OA controls (Fig. 1B).

### 3.2. Synovial miR-23a negatively correlates with clinical disease

To assess whether synovial expression of miR-23, miR-27a and miR-24-2 reflects disease activity, we examined the relationship between synovial tissue miRNA expression and clinical parameters. Synovial tissue expression of miR-23a inversely correlated with disease activity ($r = -0.5294$; $p < 0.05$; Fig. 1C). In contrast, no significant correlations were observed between synovial expression of miR-27a and miR-24-2 and any of the clinical parameters (data not shown. An inverse relationship was also observed between synovial miR-23a expression levels and macroscopic synovitis, but this did not reach statistical significance ($r = 0.48$; NS; Fig. 1C). Representative photomicrographs demonstrate increased macroscopic synovial inflammation (indicated by the black arrows) in PsA patients with low expression of miR-23a compared to patients with moderate synovitis and high relative expression of miR-23a (Fig. 1D).

### 3.3. Down-regulation of miR-23a and miR-27a upon TLR-3 and TLR-4 activation in PsA SFC

To study the regulation of miR-23a cluster in PsA SFC, the mature strands of miR-23a, miR-27a and miR-24-2 were quantified in PsA SFC following stimulation with key pro-inflammatory mediators of PsA pathogenesis. LPS ($p < 0.05$) and Poly(I:C) ($p < 0.005$) significantly repressed expression of miR-23a and miR-27a in PsA SFC (Fig. 2A and B). IL-17A treatment showed no consistent effect on miR-23a and miR-27a expression across the different patient donors and while the combined data showed a slightly increased miR-23a expression, this was due to one outlier and thus is not statistical significance. MiR-24-2 expression levels remained unchanged under all stimulatory conditions (Fig. 2D).

### 3.4. Deficiency of miR-23a promotes cytoskeletal rearrangement, migration, invasion and pro-inflammatory cytokine expression in PsA SFC

To study the contribution of decreased expression of miR-23a to PsA SFC migratory mechanisms, PsA SFC were transfected with anti-miR-23a or control anti-miR. A dramatic decrease in miR-23a expression, as detected by qRT-PCR, in the anti-miR-23a transfected PsA SFC was demonstrated compared to control cells (Supplementary Fig. 1A). To study the role of miR-23a on PsA SFC cell invasion and motility, we first assessed the cytoskeletal dynamics of PsA SFC following transfection

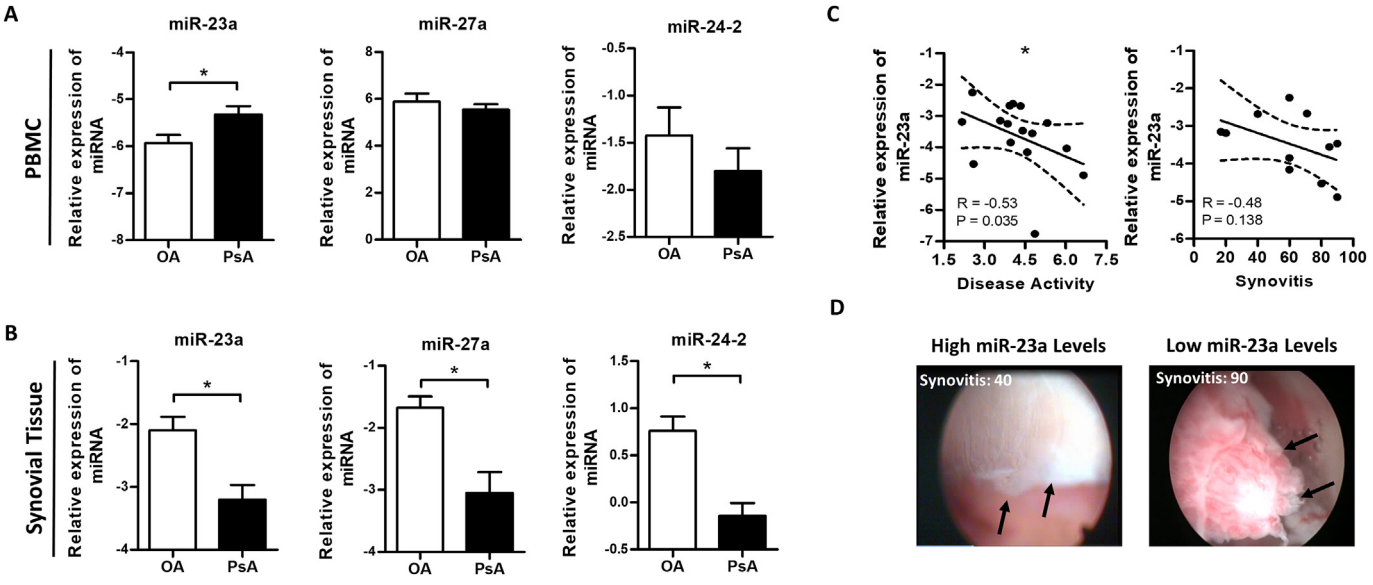


**Fig. 1. Deregulated expression of miR-23a family in patients with PsA and correlation of miRNA expression with disease activity.** Expression profile of the miR-23a cluster, miR-23a, miR-27a and miR-24-2 in (A) Peripheral blood cells (PBMC; n = 5–7 OA; n = 6–12 PsA) and (B) synovial tissue biopsies (n = 4–5 OA; n = 8–11 PsA), as determined by real-time PCR. (C) Synovial expression of miR-23a were plotted against disease activity and synovitis scores and the Spearman correlation (R) and P values (P) were computed. Data are plotted with regression coefficients (solid line) and 95% confidence intervals (broken lines). (D) Representative arthroscopy synovitis photographs in a patient with low miR-23a levels as compared to a patient with high synovial expression of miR-23a. Data are expressed as Mean ± SEM. *$p < 0.05$ significantly different to control.

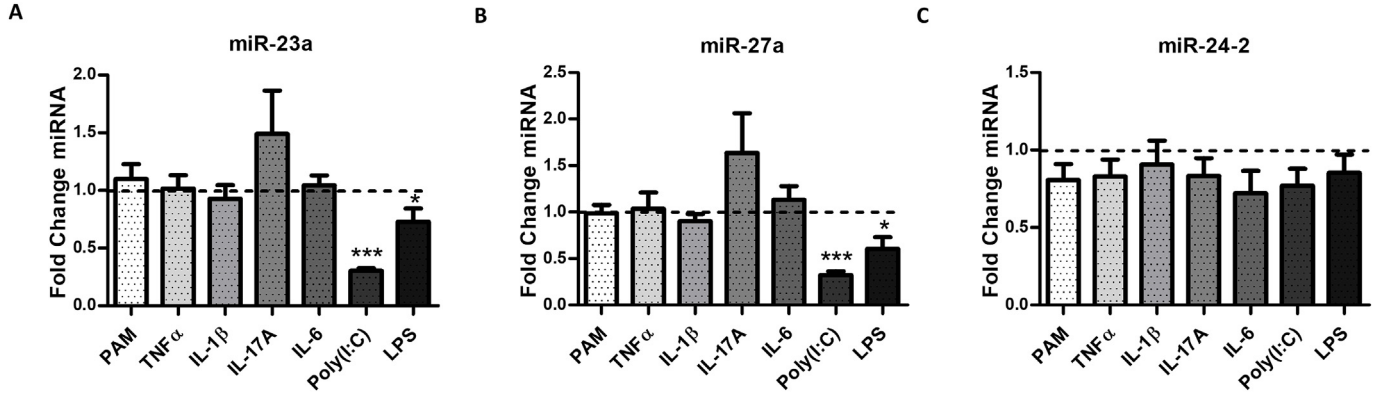


**Fig. 2. Decreased expression of miR-23a and miR-27a in PsA SFC following TLR-3 and TLR-4 activation.** PsA SFC (n = 8) were left unstimulated (Control) or stimulated for 24hr with Pam3CSK4, TNFα, IL-1β, IL-17A, IL-6, Poly(I:C) and LPS. Expression of (A) miR-23a, (B) miR-27a and (C) miR-24-2 were quantified by RT-PCR. Control expression is indicated by the dashed line. Data are represented as fold difference compared to basal. RNU48 was utilised as an endogenous control. Data are expressed as Mean ± SEM. $*p < 0.05$, $***p < 0.005$ significantly different to control.

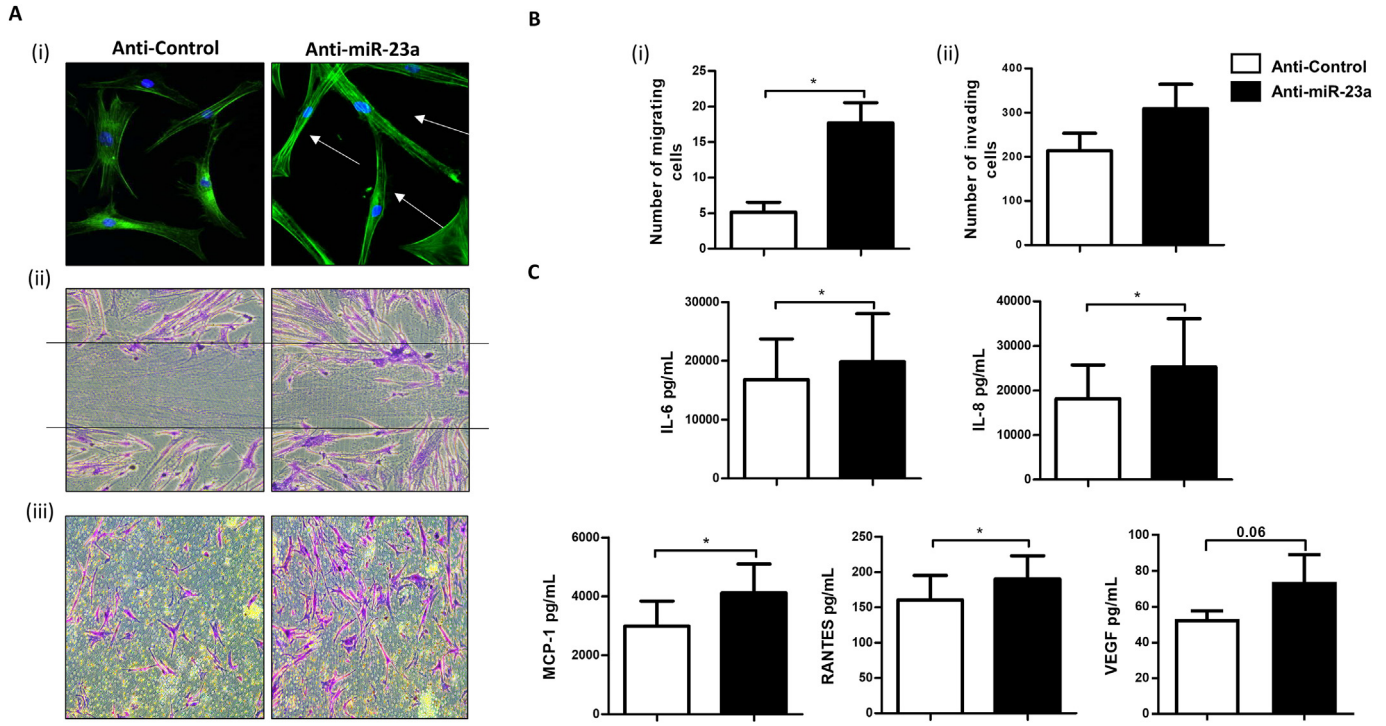


**Fig. 3. Reduced expression of miR-23a promotes elongation, migration, invasion and secretion of pro-inflammatory mediators in PsA SFC.** PsA SFC were transfected with control anti-miR (anti-Control) and anti-miR-23a for 48hr and cultured for a further 24hr. (A) Cells stained for F-actin (green), nuclei were counterstained with DAPI (Blue) (i), representative photomicrographs demonstrating wound repopulation (ii) and invasion (iii) in response to anti-miR-23a transfection versus control. (B) Bar graph quantifying the number of migrating (i) and invading (ii) per experiment (n = 5). (C) Bar graphs demonstrating secretion of IL-6, IL-8, MCP-1, RANTES and VEGF in PsA SFC (n = 6) transfected with anti-miR-23a (100 μM) or anti-Control for 48hr and 24hr full RPMI. Data are represented as Mean ± SEM, $*p < 0.05$ significantly different to control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

with anti-miR-23a. Decreased expression of miR-23a induced a diffuse enrichment of F-actin expression and cell elongation (white arrows), indicative of cell movement. Consistent with this observation, decreased expression of miR-23a also increased the migrative capacity of PsA SFC as assessed by wound repair assays ($p < 0.05$; Fig. 3B and C (ii)). To further investigate the effect of miR-23a on PsA SFC invasiveness, two components of cellular invasion - matrix degradation and migration - were assessed by matrigel transwell assay and expression of matrix metalloproteinase. Decreased expression of miR-23a induced PsA SFC invasion (Fig. 3C).

To further elucidate the role of miR-23a in regulating the pro-inflammatory phenotype of PsA SFC, the secretion of key pro-inflammatory mediators was quantified by ELISA. In PsA SFC transfected with the anti-miR-23a, levels of IL-6, IL-8, MCP-1, RANTES (all $p < 0.05$) and VEGF ($p = 0.06$) were increased compared to control-transfected cells (Fig. 3D), with concomitant increased expression of MMP1 and MMP3 (Supplementary Fig. 1B).

### 3.5. PDE4B is a direct target of miR-23a in PsA SFC and is elevated in PsA synovial tissue

To identify novel candidate targets of miR-23a which may be involved in mediating the effect on PsA SFC activation, we utilised two different miR target prediction algorithms – TargetScan [15] and

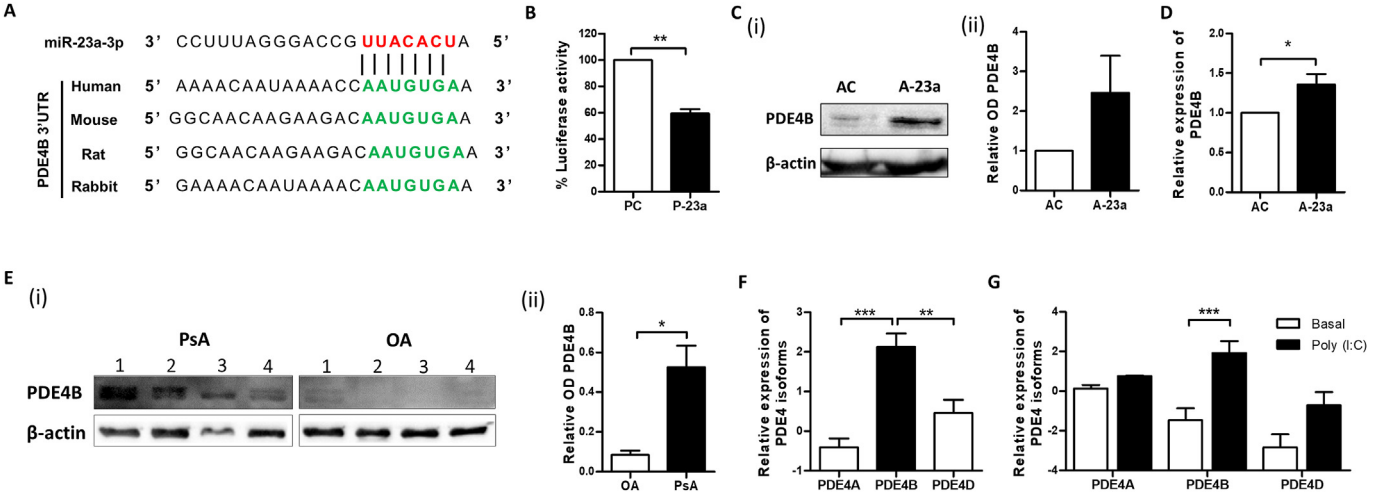


**Fig. 4. PDE4B is directly regulated by miR-23a in PsA SFC.** (A) The predicted interaction of miR-23a with the conserved miR-23a binding sites within the 3′UTR region of the PDE4B mRNA. (B) Luciferase reporter activity from PDE4B-3′UTR constructs with over expression of miR-23a (P-23a) compared to scrambled pre-miR control (PC) (n = 3). Anti-miR-23a transfected PsA SFC (72hr) analysed for (C) protein levels by immunoblot (n = 4) and (D) mRNA expression of PDE4B (n = 6). (D) PsA and OA synovial tissue homogenates immunoblotted for PDE4B and β-actin as control and densitometry values for relative band intensity of PDE4B/β-actin. (F) Bar graph quantifying the mRNA levels of PDE4 isoforms, PDE4A, PDE4B, PDE4C (not detectable) and PDE4D in PsA ST (n = 6) (G) Bar graph demonstrating the expression of PDE4A, PDE4B and PDE4D in PsA SFC following PIC stimulation in PsA SFC. Data are expressed as Mean ± SEM, $*p < 0.05$, $**p < 0.01$, $***p < 0.005$ significantly different to control.

Dianna Micro T [16], yielding a list of 763 common target predictions. Among these, phosphodiesterase 4B (PDE4B) was of particular interest given the approved treatment of PDE4 blockade in PsA [11]. The predicted miR-23a binding site within the 3′UTR of PDE4B showed a high degree of conservation across different mammalian species (Fig. 4A).

To validate the direct regulation of PDE4B by miR-23a, a luciferase reporter gene assay was performed. The 3′UTR of the PDE4B was cloned downstream of the luciferase gene in a dual-luciferase reporter vector (Supplementary Fig. 2). miRNA inhibit protein translation and/or destabilize the mRNA, so direct binding of miR-23a to the 3′UTR of PDE4B would result in decreased luciferase activity. Co-transfection of pre-miR-23a with the PDE4B 3′UTR luciferase reporter vector into HEK 293 cells reduced the luciferase activity ($p < 0.01$; Fig. 4B), indicating that miR-23a directly regulates PDE4B expression.

To validate miR-23a-regulated PDE4B in PsA SFC, the effect of reduced expression of miR-23a in PsA SFC on PDE4B protein levels was examined. Transfection of PsA SFC with anti-miR-23a strongly induced PDE4B protein expression (Fig. 4C(i)). Furthermore, a significant increase in PDE4B mRNA expression was demonstrated following anti-miR-23a transfection ($p < 0.05$; Fig. 4D).

The expression of PDE4B was then examined in PsA synovial tissue in which miR-23a expression was repressed. PDE4B protein expression was significantly increased in PsA synovial tissue compared to OA controls ($p < 0.05$; Fig. 4E). To further dissect the potential pathogenic function of PDE4B in PsA, the expression of PDE4 isoforms; PDE4A, PDE4B, PDE4C and PDE4D were examined in PsA synovial tissue and SFC in response to pro-inflammatory stimuli. Interestingly, PDE4C expression was not detectable in PsA ST or PsA SFC. Comparison between PDE4A, PDE4B and PDE4D demonstrated that PDE4B expression maintains the highest level of PDE4 isoforms in PsA ST ($p < 0.01$ and $p < 0.005$; Fig. 4F). To further examine the regulation of PDE4B, PsA SFC were stimulated with up-stream regulator of miR-23a; Poly(I:C), and PDE4 expression analysed. TLR-3 activation in response to Poly (I:C) significantly increased the mRNA expression of PDE4B in PsA SFC ($p < 0.05$; Fig. 4G).

### *3.6. Blockade of PDE4 inhibits the effect of Poly(I:C) stimulation on PsA SFC migration, cell polarisation and expression of pro-inflammatory mediators*

Following demonstration of the concomitant regulation of miR-23a and PDE4B in Poly(I:C)-stimulated PsA SFC, we sought to investigate whether PDE4 blockade could influence Poly(I:C) induced PsA SFC pro-inflammatory phenotype. Therefore, PsA SFC were stimulated with Poly (I:C) in the presence or absence of a PDE4 specific inhibitor, rolipram, and cell migration, and cytokine expression analysed. PDE4 blockade significantly reduced Poly(I:C)-induced PsA SFC migration in a wound-repair assay ($p < 0.05$; Fig. 5A and B), paralleled with decreased Poly (I:C)-induced MMP1 and MMP3 expression (Supplementary Fig. 3A). In line with this, following the blockade of PDE4, Poly(I:C)-stimulated PsA SFC lost their elongated shape and instead formed a more rounded and less invasive phenotype that better resembled DMSO control treated cells (Supplementary Fig. 3B). Furthermore, Poly(I:C)-induced expression of MCP-1 and RANTES were significantly decreased in the presence of PDE4 inhibitor (Fig. 5C). IL-8 and IL-6 secretion was also decreased but this did not reach significance.

### *3.7. Inhibition of PDE4 blocks mir-23a-mediated effects on PsA SFC*

To assess whether targeting of PDE4 could directly influence miR-23a-mediated pathogenic mechanisms in PsA SFC, the effect of the PDE4 inhibitor, rolipram, on miR-23a expression, cell migration, MMP and cytokine expression was assessed. Under DMSO control conditions, anti-miR-23a transfected PsA SFC showed a significant reduction in miR-23a expression ($p < 0.001$, Fig. 5D). Interestingly, transfection with anti-miR-23a followed by 24hr treatment with rolipram did not result in a significant decrease in miR-23a expression. In anti-miR-23a transfected PsA SFC treated with rolipram, a significant decrease in anti-miR-23a induced migration was observed ($p < 0.05$, Fig. 5E). Similar to our results in PDE4 blockade on Poly(I:C)-stimulated PsA SFCs, rolipram treatment in anti-miR-23a transfected cells resulted in a significant decrease in the mRNA levels of MMP1 ($p < 0.05$), MMP3, MCP-1 ($p < 0.01$) and RANTES (Fig. 5F), with no difference observed for under anti-control conditions (data not shown).

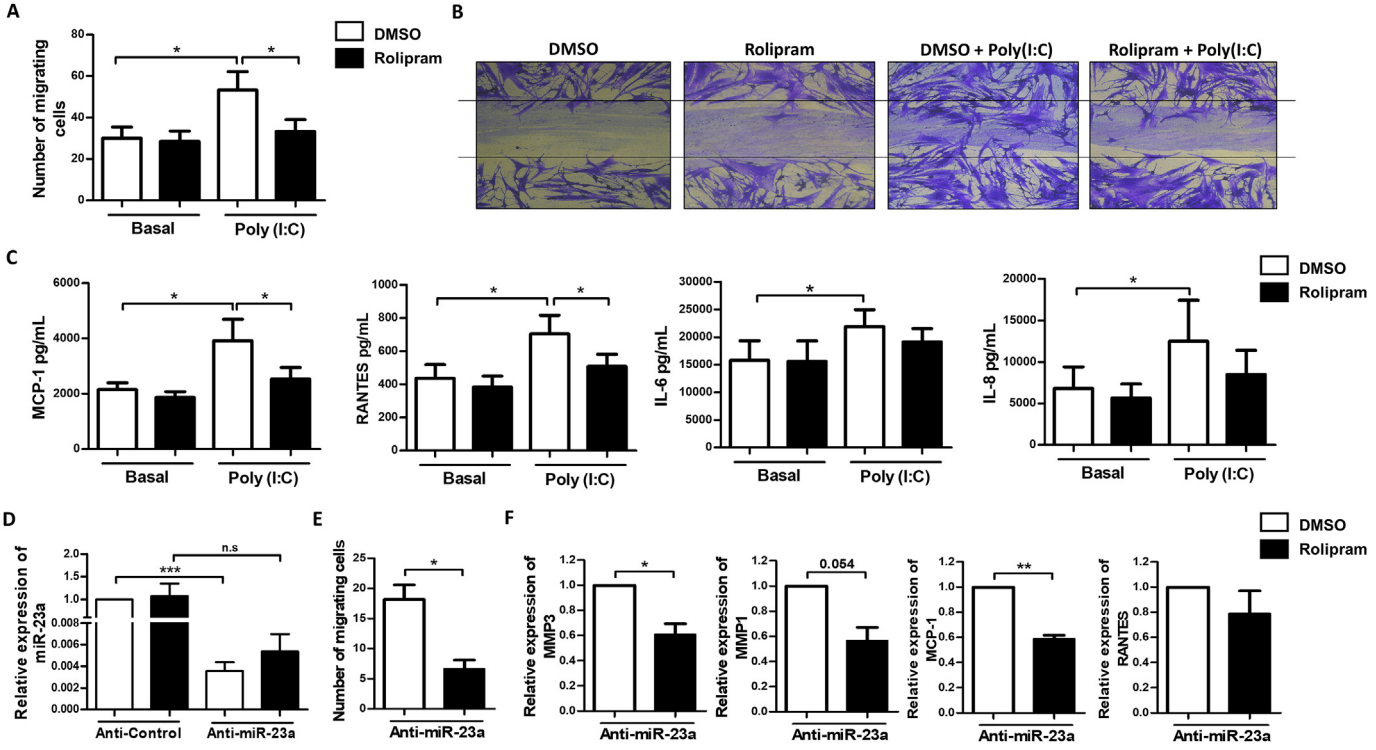


**Fig. 5. Blockade of PDE4 inhibits Poly(I:C) and anti-miR-23a-induced PsA SFC pro-inflammatory mechanism.** (A) Bar graph and (B) representative images of PsA SFC migration in response to 24hr treatment with Poly(I:C) (10 μg/ml) in the presence of PDE4 inhibitor Rolipram (10 μM) or DMSO control (10 μM). (C) Bar graphs showing the measured secretion of MCP-1, RANTES, IL-6 and IL-8 from PsA SFC following the outlined treatments by ELISA (n = 6). (D) Expression levels of miR-23a following anti-miR-23a transfection in the presence or absence of Rolipram (10 μM) or DMSO (10 μM) with appropriate transfection controls. (E) Bar graph quantifying PsA SFC (n = 5) migrating cell number in response to miR-23a in the presence or absence of Rolipram versus DMSO control. (F) mRNA levels of MMP-1, MMP-3, RANTES and MCP-1 in miR-23a deficient PsA SFC in the presence of Rolipram (n = 3). Data are represented as Mean ± SEM. Paired *t*-test and Wilcoxon signed rank were used appropriately. *p < 0.05, significantly different to control.

## 4. Discussion

Changes in the inflamed synovium accompanied by the destruction of adjacent cartilage and bone as observed in inflammatory arthritis invariably requires an activated synovial fibroblasts phenotype. The role of the miR-23a family in the transition from indolent to metastatic malignancies has been extensively reported in cancer studies [9,17–20], however their potential role in SFC activation in synovial inflammation has not been fully elucidated. In the present study, we demonstrate for the first time decreased miR-23a expression in PsA synovial tissue which inversely correlated with disease activity. The clinical observations were supported by *in vitro* studies which demonstrated increased PsA SFC migration, invasion and secretion of key pro-inflammatory mediators following suppression of miR-23a. Using a luciferase reporter gene assay, we identified PDE4B as a novel direct target of miR-23a, and demonstrated that repression of miR-23a in PsA SFC resulted in increased PDE4B mRNA and protein expression. Increased PDE4B expression was observed in PsA compared to OA synovial tissue. TLR-3 induced PsA SFC migration and secretion of pro-inflammatory cytokines, effects of which were inhibited by PDE4 inhibition. Moreover, rolipram significantly inhibited miR-23a-induced SFC migration, MMP levels and pro-inflammatory cytokine expression. These data suggest a model in which, repressed expression of miR-23a in response to TLR3 activation mediates PsA SFC pro-inflammatory mechanisms via the direct targeting of PDE4B. These effects can, at least in part, be inhibited by PDE4 blockade, thus our results demonstrate a potential novel anti-inflammatory mechanism of PDE4 targeted therapies in PsA SFC (Fig. 6).

Initially, we observed that the expression levels of miR-23a were significantly lower in synovial tissue from PsA compared to OA patients, with lower levels associated with increased synovial inflammation. The differential expression profiles of miR-23a in PBMC and synovial tissue samples from PsA patients highlights the significance of studying miRNA at the site of inflammation where site-specific microenvironments may affect both miRNA expression and function. Increasing evidence supports the potential pathogenic role of miR-23a in T cell immunity, B cell development and macrophage activation in chronic inflammation [21–23]. A significant reduction in cytotoxic activity was observed in activated $CD8^+$ T cells with over expressed miR-23a, an effect mediated through repressed expression of lysosomal-associated membrane protein-1 (LAMP-1), a key component of the cytotoxic granule membrane [24]. Similarly, elevated levels of miR-23a in hematopoietic progenitor cells inhibited B-cell development both *in vitro* and *in vivo* [22]. Furthermore, decreased expression of miR-23a was observed in TLR3, TLR4 and TLR9 activated RAW264.7 macrophages cells, suggesting TLR signalling as key regulator influencing miR-23a expression in inflammation [25]. This is consistent with our results in which a decrease in miR-23a expression in PsA SFC was demonstrated in response to TLR-3 and TLR-4 activation. While IL-17A is significantly implicated in the pathogenesis of PsA, and previous studies have shown that IL-17A regulates synovial fibroblast invasive functions [26,27], in this study IL-17A had no significant effect on the miR-23a cluster. Numerous studies support TLR activation as a key pathogenic mechanism in synovial inflammation, with several DAMP ligands being identified in the synovial joint, including heat shock proteins, self-nucleic acid structures, serum amyloid A, fibrinogen, ECM components and ROS [28]. In previous studies, RA synovial fluid derived double strand RNA activated TLR-3 in cultured RA SFC to promote the secretion of IL-6, CXCL10 and CCL5 [29]. More recently, Cavassani et al. demonstrated the *in vivo* responsiveness of TLR-3 receptors in response to necrotic cell derived RNA in a mouse model of sepsis and demonstrated that TLR-3 knockout or administration of TLR-

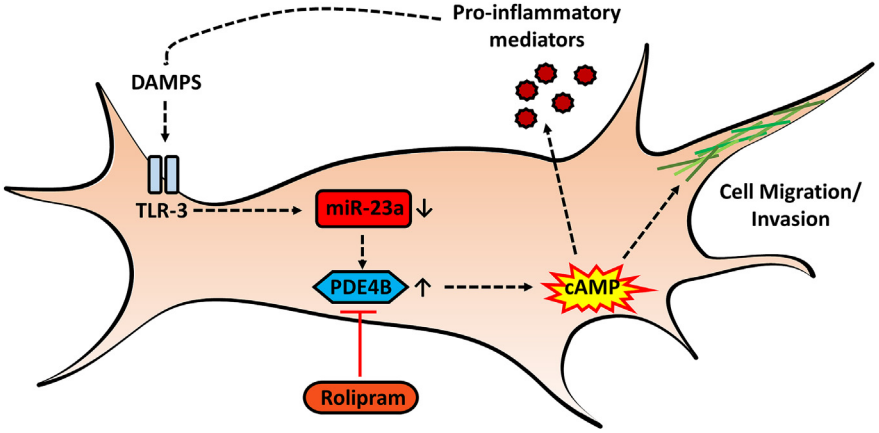


**Fig. 6. miR-23a/PDE4B axis in PsA SFC pathology.** TLR3 activation by endogenous DAMPs which decreases the expression of miR-23a, which in turn induces PDE4B resulting in decreased cAMP and enhanced pro-inflammatory PsA SFC mechanisms including expression of pro-inflammatory mediators and cell motility, all of which are key process is synovial inflammation. These alterations can further promote joint destruction and generate further DAMP-mediated activity resulting in a positive feedback loop. Rolipram treatment interferes with this positive feedback loop by targeting PDE4B activity and thereby potentially decreasing the incidence of local joint destruction and DAMP generation resulting in elevated expression of miR-23a and repressed mechanisms of SFC activation.

3 neutralizing antibodies could circumvent injury-induced inflammation and sepsis-induced mortality [30].

Despite the recent awareness of the imprinted SFC phenotypes based on their anatomical location [31,32], the molecular mechanisms involved in the intrinsic changes in SFC behavior during synovial inflammation remains poorly understood, particularly in PsA. In this study, we demonstrated that following transfection with anti-miR-23a, PsA SFC acquired an increased migratory/invasive phenotype accompanied by up-regulation of matrix degrading enzymes MMP1 and MMP3, key processes involved in mediating joint destruction [33]. Additionally, repression of miR-23a in PsA SFC also promoted the secretion pro-inflammatory mediators; IL-6, IL-8, MCP-1 and RANTES and pro-angiogenic factor VEGF. Consistent with our data, a study performed in dermal fibroblasts demonstrated elevated proliferation and migration in response to decreased miR-23b expression while repressed expression of miR-23a in osteosarcoma and prostate cancer has been shown to promote enhanced cell migration and invasion [34,35]. Furthermore in RA chondrocytes, decreased expression of IL-6, MCP-1 and MMP3 following elevated expression of miR-23a through the targeted inhibition of IKKα, a key regulator of NF-kB signalling has been demonstrated [36]. In other cells types, miR-23a mediates TNF-α-induced endothelial cell function, an effect mediated through caspase-7/caspase-3 pathways [37] and can alter the migratory potential of hematopoietic progenitor cells through enhanced CXCL12 activity [38].

Investigation of the molecular mechanisms revealed PDE4B as a direct target and mediator of miR-23a-mediated SFC activation. PDE4B has been previously described as the main pro-inflammatory isoform of PDE4 [39,40], with a recent study demonstrating elevated expression of PDE4B in PsO dermal fibroblasts compared to relative controls [41]. In this study, we extend these observations and show that PDE4B is the most highly expressed isoform in PsA synovial tissue and SFC in response to miR-23a regulator, Poly(I:C). We observed that PDE4 blockade was effective in inhibiting both Poly(I:C) and anti-miR-23a-induced migration and in reducing pro-inflammatory mediators, demonstrating a novel anti-inflammatory mechanisms in PsA SFC pathology. Therefore, we hypothesise that miR-23a/PDE4B axis blockade may offer beneficial anti-inflammatory properties to PsA synovial inflammation and disease progression. In support of this hypothesis, PDE4 inhibition in RA synovial explant cultures reduced TNFα, MCP-1, RANTES, IL-1β, and macrophage inflammatory protein (MIP) 1α and 1β expression [42,43] and decreased severity of inflammation in mice models of arthritis [43,44]. Furthermore, reduced TGF-β-induced dermal fibroblast migration was observed in response to PDE4 blockade [41], while TLR-3-induced expression of IL-6 and IL-8 in airway smooth muscle cells was significantly decreased following inhibition of PDE4 [45]. Furthermore, our findings also prompt additional studies to investigate miR-23a phenotyping as favourable response predictor to PDE4 blockade in PsA.

In conclusion, these data identify miR-23a as an important regulator of PsA SFC activation through the previously unidentified regulation of PDE4B in the PsA synovial tissue. While further studies are required to validate these pro-inflammatory mechanisms *in vivo*, the functional assays and clinical associations shown in these studies already provide insight into molecular mechanism controlling SFC activation during synovial inflammation and support the potential therapeutic benefit of targeting miR-23a regulated pathways in patients with PsA.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jaut.2018.08.008.

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