


## Original article

**Dysregulated miRNome of plasmacytoid dendritic cells from patients with Sjögren's syndrome is associated with processes at the centre of their function****Maarten R. Hillen** <sup>1,2,\*</sup>, **Eleni Chouri**<sup>1,2,\*</sup>, **Maojie Wang**<sup>3</sup>, **Sofie L. M. Blokland**<sup>1,2</sup>, **Sarita A. Y. Hartgring**<sup>1,2</sup>, **Arno N. Concepcion**<sup>2</sup>, **Aike A. Kruize**<sup>2</sup>, **Boudewijn M. T. Burgering**<sup>3</sup>, **Marzia Rossato**<sup>1,2,4</sup>, **Joel A. G. van Roon**<sup>1,2,\*</sup> and **Timothy R. D. J. Radstake**<sup>1,2,\*</sup>**Abstract**

**Objective.** A considerable body of evidence supports a role for type-I IFN in the pathogenesis of primary SS (pSS). As plasmacytoid dendritic cells (pDCs) are a major source of type-I IFN, we investigated their molecular regulation by measuring expression of a large set of miRNAs.

**Methods.** pDCs were isolated from peripheral blood of pSS patients ( $n=30$ ) and healthy controls ( $n=16$ ) divided into two independent cohorts (discovery and replication). Screening of 758 miRNAs was assessed by an OpenArray quantitative PCR-based technique; replication of a set of identified miRNAs was performed by custom array. Functional annotation of miRNA targets was performed using pathway enrichment. Novel targets of miR-29a and miR-29c were identified using a proteomic approach (stable isotope labelling with amino acids in cell culture).

**Results.** In the discovery cohort, 20 miRNAs were differentially expressed in pSS pDCs compared with healthy control pDCs. Of these, differential expression of 10 miRNAs was confirmed in the replication cohort. The dysregulated miRNAs were involved in phosphoinositide 3-kinase-Ak strain transforming and mammalian target of rapamycin signalling, as well as regulation of cell death. In addition, a set of novel protein targets of miR-29a and miR-29c were identified, including five targets that were regulated by both miRNAs.

**Conclusion.** The dysregulated miRNome in pDCs of patients with pSS is associated with aberrant regulation of processes at the centre of pDC function, including type-I IFN production and cell death. As miR-29a and miR-29c are pro-apoptotic factors and several of the novel targets identified here are regulators of apoptosis, their downregulation in patients with pSS is associated with enhanced pDC survival.

**Key words:** Sjögren's syndrome, plasmacytoid dendritic cells, microRNAs, type-I interferon

**Rheumatology key messages**

- We identify 10 miRNAs consistently dysregulated in primary SS plasmacytoid dendritic cells in two independent cohorts.
- The dysregulated miRNome of primary SS plasmacytoid dendritic cells is associated with dysregulation of phosphoinositide 3-kinase-Ak strain transforming-mammalian target of rapamycin signalling and survival.
- Novel (shared) protein targets of miR-29a and miR-29c are identified, suggesting improved pSS plasmacytoid dendritic cells survival.

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## Introduction

Primary SS (pSS) is a systemic autoimmune disease characterized by mononuclear infiltration of the exocrine glands [1]. In a majority of patients, increased expression of genes that are downstream of the type-I IFN receptor is observed in circulating immune cells, referred to as the IFN signature. The presence of this signature is associated with increased B cell-hyperactivity and disease activity [2]. In addition, deficiency for the type-I IFN receptor prevents Sjögren's-like symptoms in a mouse model [3].

Plasmacytoid dendritic cells (pDCs) comprise a rare immune cell subset (<0.5% of leukocytes) that is capable of rapidly producing large amounts of type-I IFN upon activation on recognition of viral nucleic acids by endosomal Toll-like receptors (TLR) 7 and 9 [4]. pDC numbers are decreased in the circulation of patients with pSS [5] and are increased in the salivary glands correlating with the number of IFN- $\alpha$ -producing cells [5–7]. In addition, circulating pDCs from patients with an IFN signature have increased expression of genes encoding receptors via which pDCs can be activated, including TLR7 [8]. Importantly, pDCs can also be activated by endogenous nucleic acids such as those contained in immune complexes [9]. As such, pDCs are perceived to be major contributors to type-I IFN production in pSS and crucial mediators of immunopathology.

miRNAs are single-stranded, small non-coding (snc) RNAs of 19–25 nucleotides in length that regulate gene expression at the post-transcriptional level. miRNAs account for 1–5% of the human genome and can negatively regulate expression of at least 30% of the protein-coding genes [10], making them crucial regulators of cellular function. In patients with pSS, changes in the expression of miRNAs have been shown for a range of compartments and cell types and their expression was previously associated with disease activity, the IFN signature and lymphoma development [11–17]. Considering the relevance of type-I IFN and pDCs in pSS and the lack of knowledge on pDC function in these patients, molecular analysis of pSS pDCs should provide a better understanding of pathogenesis. Since miRNAs play a crucial role in the regulation of cellular and immunological processes, we investigated the expression of a large set of miRNAs in purified pDCs from patients with pSS. In addition, we identified novel targets of the robustly dysregulated family members miR-29a and miR-29c using a proteomic approach.

## Methods

### Patients and controls

Two independent cohorts of patients and controls (discovery and replication) were established. A discovery cohort was used to screen the expression of a large panel of 758 miRNAs and other sncRNAs, and a replication cohort was used to test the reproducibility of the results. pSS patients ( $n=30$ ) were classified according to the American-European consensus group criteria [18]. Sixteen healthy donors were included as the control

group (healthy controls, HC). There were no significant differences in age and gender between the groups in either cohort. This research was performed in accordance with the medical ethical regulations of the University Medical Center Utrecht and the declaration of Helsinki; all patients gave their written informed consent. The characteristics of the individuals included in the study are depicted in Table 1. An overview of the approach used in the discovery and replication phases is depicted in Fig. 1A.

### Cell isolation

Blood dendritic cell antigen (BDCA)-4<sup>+</sup> pDCs were isolated from peripheral blood mononuclear cells (PBMCs) by magnetic-activated cell sorting using the BDCA-4<sup>+</sup> isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. After pDC isolation, monocytes were isolated from the pDC-depleted cell fraction of 21 pSS patients (randomly selected) using the CD14<sup>+</sup> isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

For cell culture experiments,  $5 \times 10^4$  magnetic-activated cell-sorted pDCs were seeded in 96-well round-bottom plates in RPMI Glutamax culture medium (Gibco, Dublin, Ireland) supplemented with 10% fetal bovine serum (Biowest, Nuaille, France), penicillin, streptomycin (both Gibco) and 10 ng/ml IL-3 (ImmunoTools, Friesoythe, Germany). After 2 h of resting, pDCs were left untreated or TLR agonists were added: 100 ng/ml of lipopolysaccharide, 10  $\mu$ g/ml of R848 or 1  $\mu$ M CPG-C (all Invivogen, San Diego, CA, USA). After 24 h of culture, cells were harvested and lysed in RLTPlus supplemented with beta-mercapthoethanol and stored at  $-80^\circ\text{C}$ .

### RNA extraction and IFN-score quantification

Total RNA containing the small-RNA fraction was purified from lysates of isolated cells using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To determine the IFN-score in the monocyte samples, expression of five IFN-induced genes (*IFI44L*, *IFI44*, *IFIT3*, *Ly6E* and *MX1*) was quantified as previously described [2].

### miRNA quantification

miRNA profiling was performed in the discovery cohort as previously described by our group [19]. Data were normalized using the global normalization approach. Data points with low expression (relative threshold cycle <27) were set at 27 and data points with poor amplification (Ampscore <1.24) were excluded from the analysis. To ensure only robust data were considered, miRNAs expressed in <80% of donors were excluded from the discovery phase.

To confirm the differential expression of 14 selected miRNAs in the replication cohort, quantification was performed using a custom panel of these miRNAs and four reference sncRNAs (miR-17, miR-191, RNU48, snc-U6) that all showed good abundance and stable expression between patients and controls. Data were analysed according to the comparative threshold cycle method, after normalization by the mean Ct of the reference sncRNAs. Expression levels were depicted as relative

TABLE 1 Donor characteristics

	Discovery cohort		Replication cohort	
	HC	pSS	HC	pSS
N (male/female)	5 (0/5)	14 (1/13)	11 (1/10)	16 (0/16)
Age (years)	58 (54–67)	56 (29–70)	53 (29–64)	52 (26–70)
LFS (foci/4 mm <sup>2</sup> )	–	2.1 (1.0–5.0)	–	2.0 (1.0–4.0)
ESSDAI	–	2 (0–19)	–	5 (0–13)
ESSPRI	–	5 (2–9)	–	5 (1–8)
Schirmer (mm/5 min)	–	5 (0–15)	–	7 (0–30)
ANA [no. positive (%)]	–	11 (79)	–	14 (93)
SSA [no. positive (%)]	–	8 (57)	–	13 (82)
SSB [no. positive (%)]	–	4 (29)	–	10 (63)
Serum IgG (g/l)	–	14 (8–30)	–	18 (9–33)

Values are median (range) unless stated otherwise. HC: healthy control; pSS: primary SS; LFS: lymphocytic focus score; ESSDAI: EULAR SS disease activity index; ESSPRI: EULAR SS patient reported index; SSA: anti-SSA/Ro; SSB: anti-SSB/La.

fold-change compared with one HC sample, which was set at 1.

For analysis of cell culture experiments, the expression of miR-29c (hsa-miR-29c-3p, ID: 479229) was measured by specific Taqman Real Time quantitative PCR (qPCR) assays (Life Technologies, Carlsbad, CA, USA) using the Quantstudio 12k Flex System (Life Technologies). Expression of target miRNAs was normalized to the expression of snc-U6 (U6-snrRNA, ID: 001973) and the relative expression was normalized to the medium condition, which was set at 1.

### Statistics

Differences in miRNA expression between pSS patients and HC in the discovery cohort were analysed using ThermoFisher Cloud software, which is *t*-test based. For analysis of the replication cohort data, statistical analysis was performed with GraphPad Prism and SPSS. Differences in sncRNA expression between pSS and HC were assessed using the Mann–Whitney *U* test (two-sided). For correlations with disease parameters and IFN-score, Spearman's rho was used. Differences were considered to be statistically significant at  $P < 0.05$ .

More detailed description of the materials and methods can be found in the supplementary material, section Methods, available at *Rheumatology* online.

## Results

### Expression of 10 miRNAs is consistently decreased in pSS pDCs compared with HC

Expression of 758 miRNAs was assessed in pDCs obtained from peripheral blood of pSS patients ( $n = 14$ ) and HC ( $n = 5$ ) from the discovery cohort using OpenArray qPCR-based screening (see Fig. 1A for an overview of the approach used). We detected expression of 250 miRNAs in pDCs. Of these, miRNAs with a relative expression of 2-fold higher or lower in pSS patients compared with HC (at  $P < 0.05$ ) were selected; 26 miRNAs met these

criteria (Fig. 1B). To ensure that all miRNAs selected for further analysis were expressed in the large majority of samples, six miRNAs that were expressed in  $<80\%$  of the samples were excluded. The 20 selected differentially expressed miRNAs were all expressed at a lower level in patients compared with controls (Table 2).

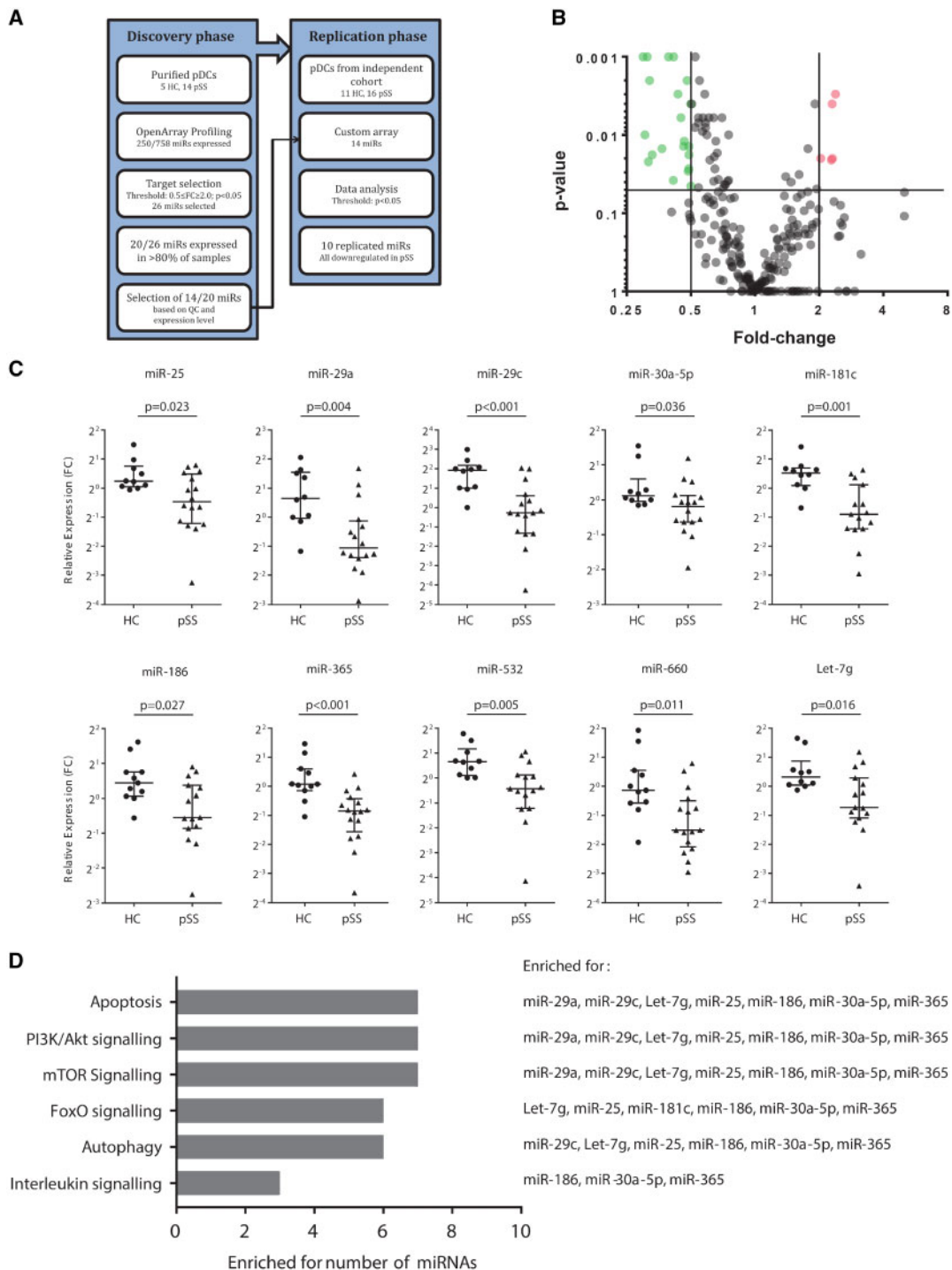
For replication we selected 14 of these miRNAs based on the robustness of the difference between patients and controls, their level of expression and the quality of amplification in the array. The expression of these 14 selected miRNAs was quantified using a custom qPCR-based array in purified pDCs from an independent replication cohort of pSS patients ( $n = 16$ ) and HC ( $n = 11$ ) using a custom qPCR-based array. For 10 of these miRNAs differential expression ( $P < 0.05$ ) was replicated in the pSS group. Consistent with the results from the discovery analysis, all 10 of the replicated miRNAs were downregulated in patients compared with HC (Fig. 1C, Table 2). Of these, miR-29a and miR-29c consistently showed the most robust fold-change difference between pSS and HC.

As expression of miRNAs was associated with the IFN-score in pDCs from patients with SLE [20], we used qPCR to quantify the IFN-score in monocytes (as previously described [2]) isolated from a subset of the pSS donors included in the study ( $n = 21$ , randomly selected). The expression of two replicated miRNAs modestly correlated with the IFN-score (miR-25:  $r = 0.45$ ,  $P = 0.049$ ; miR-186:  $r = 0.53$ ,  $P = 0.014$ ). There were no statistically significant correlations between miRNA expression and lymphocytic focus score (LFS), EULAR SS disease activity index (ESSDAI), EULAR SS patient reported index (ESSPRI), Schirmer or serum IgG (all  $P > 0.15$ , not shown).

### Replicated miRNAs regulate processes at the centre of pDC function and cell death

To better understand the consequence of the dysregulated miRNome in pSS pDCs, we functionally annotated the *in silico* predicted and experimentally supported targets for the identified miRNAs using pathway enrichment.

**Fig. 1** miRNAs consistently dysregulated in pSS pDCs target genes in pathways crucial for pDC function



Overview of the discovery and validation approach (A). Expression of 758 miRNA was measured in isolated pDCs from donors in the discovery cohort. We selected miRNAs different between pSS and HC with a FC of  $0.5 < FC < 2.0$  and a *P*-value of  $< 0.05$  for potential follow-up, which included 26 miRNAs (B). miRNAs that met the set thresholds are coloured in green for those expressed at lower level in patients compared with controls; red dots represent miRNAs expressed at a higher level in patients. Based on quality-control and expression level, 14 miRNAs were selected to be measured in the replication cohort using a custom quantitative PCR-based array. Of these, 10 miRNAs were significantly differentially expressed. Data from the replication cohort are depicted in (C). Functional annotation of the targets of the replicated miRNAs indicates their regulation of relevant pathways in pDCs, including apoptosis and PI3K-AKT-mTOR signalling (D). Medians  $\pm$  IQR are shown. \* indicates  $P < 0.05$ . Akt: Ak strain transforming; pDC: plasmacytoid dendritic cells; pSS: primary SS; HC: healthy control; FC: fold change; PI3K: phosphatidylinositol 3-kinase; mTOR: mammalian target of rapamycin.

**TABLE 2** miRNAs differentially expressed in purified pDCs from patients with pSS

Target	Discovery		Replication	
	Fold-change	P-value	Fold-change	P-value
<b>miR-29c</b>	<b>0.42</b>	<b>0.001</b>	<b>0.26</b>	<b>&lt;0.001</b>
<b>miR-29a</b>	<b>0.39</b>	<b>0.001</b>	<b>0.38</b>	<b>0.004</b>
<b>miR-181c</b>	<b>0.49</b>	<b>0.029</b>	<b>0.41</b>	<b>0.001</b>
<b>miR-660</b>	<b>0.50</b>	<b>0.045</b>	<b>0.42</b>	<b>0.011</b>
<b>miR-365</b>	<b>0.33</b>	<b>0.018</b>	<b>0.43</b>	<b>&lt;0.001</b>
<b>miR-532</b>	<b>0.45</b>	<b>0.006</b>	<b>0.43</b>	<b>0.005</b>
<b>Let-7g</b>	<b>0.49</b>	<b>0.027</b>	<b>0.49</b>	<b>0.016</b>
<b>miR-25</b>	<b>0.47</b>	<b>0.012</b>	<b>0.53</b>	<b>0.023</b>
<b>miR-186</b>	<b>0.49</b>	<b>0.014</b>	<b>0.55</b>	<b>0.027</b>
<b>miR-30a-5p</b>	<b>0.32</b>	<b>0.002</b>	<b>0.66</b>	<b>0.036</b>
miR-340#	0.32	0.022	0.45	0.071
miR-29b	0.50	0.004	0.51	0.134
miR-30e-3p	0.30	0.010	0.65	0.057
miR-30d	0.30	0.001	0.73	0.318
miR-362-3p	0.31	0.001	-	-
miR-181a-2#	0.37	0.015	-	-
miR-502-3p	0.44	0.003	-	-
miR-125b	0.46	0.014	-	-
miR-16	0.48	0.002	-	-
miR-15a	0.49	0.018	-	-

Expression of 758 miRNAs was measured in the discovery cohort using the OpenArray platform. Twenty miRNAs were differentially expressed in pSS patients as compared with healthy donors with a relative fold-change of  $\leq 0.5$  or  $\geq 2.0$  and a  $P$ -value of  $P < 0.05$ . Expression of 14 miRNAs was measured in the independent replication cohort using a custom Taqman-based array. Ten miRNAs were significantly different between patients and controls ( $P < 0.05$ ) in the replication cohort and are shown in bold. pDCs: plasmacytoid dendritic cells; pSS: primary SS.

Of the 10 replicated miRNAs identified in our analysis, seven regulate Phosphatidylinositol 3-kinase (PI3K)-AKT signalling and mammalian target of rapamycin (mTOR) signalling, and six are involved in FoxO signalling, which are pathways known to be crucial for key functions of pDCs including cytokine production and T cell stimulation [21]. In addition, seven and six of these miRNAs are involved in regulation of apoptosis and autophagy, respectively (Fig. 1D). Interestingly, target genes of family members miR-29a and miR-29c were annotated in both the PI3K-AKT-mTOR and apoptosis pathways.

Endosomal TLR7 and TLR9 represent crucial pathways via which pDCs are activated in systemic autoimmune diseases, as they can bind endogenous nucleic acids such as those present in immune complexes [4]. In addition, TLR triggering can regulate cell death in immune cells, while previous data indicate that miR-29c can promote apoptosis in pDCs [22]. As such, TLR triggering can constitute a possible pathway through which miR-29c is downregulated. To examine this, the expression of miR-29c was quantified in pDCs purified from HC and stimulated with the TLR4 ligand lipopolysaccharide; R848, an imidazoquinoline that ligates TLR7 and -8; or the TLR9 ligand CPG-C for 24 h. Indeed, both endosomal TLR ligands R848 and CPG-C caused downregulation of miR-29c (supplementary Fig. S1, available at *Rheumatology* online), while lipopolysaccharide did not affect miR-29c expression.

#### Identification of novel targets regulated by both miR-29a and miR-29c

Because of their predicted important role in pDC function and the consistently robust expression differences between pSS and HC, miR-29a and miR-29c were further studied. Interestingly, miR-29a and 29c are members of the same family with almost identical sequences and the same seed regions. To gain a deeper understanding of the consequence of the combined downregulation of miR-29a and miR-29c, we performed stable isotope labelling with amino acids in cell culture (SILAC). This mass-spectrometry-based method compares protein expression between cells cultured in the presence and absence of miRNA overexpression, thus allowing the identification of novel targets or validation of predicted targets at the protein level. We analysed the proteins that were downregulated (with  $>0.3$ -fold change between mimic and scramble control) upon transfection of cells with mimics for miR-29a or miR-29c (Fig. 2A), and then compared them to a list of *in silico*-predicted targets of each of these miRNAs. Twenty-four predicted targets of miR-29a and 13 predicted targets of miR-29c were downregulated in the SILAC analysis (Fig. 2B, Table 3). The downregulated proteins included several targets that were previously associated with autoimmunity, including CDK6, DCP2, LAMC1 and SERPINB9 [23–26]. Five of the identified targets were downregulated by both miR-29a and miR-29c, including



**TABLE 3** Protein targets downregulated by miR-29a or miR-29c in the SILAC with a seed-region for the respective miRNA

Protein	Ratio SCR/29a (Log2)	Ratio 29a/SCR (Log2)	Protein	Ratio SCR/29c (Log2)	Ratio 29c/SCR (Log2)
<b>FSTL1</b>	0.87	-1.02	<b>FSTL1</b>	0.37	-1.33
<b>CASP7</b>	0.52	-0.56	<b>CASP7</b>	0.56	-0.33
<b>CD276</b>	0.51	-1.43	<b>CD276</b>	0.89	-0.74
<b>SERPINH1</b>	0.51	-0.58	<b>SERPINH1</b>	0.43	-0.33
<b>F11R</b>	0.47	-1.78	<b>F11R</b>	0.36	-0.53
CDK6	0.69	-1.04	LAMC1	0.54	-0.53
DCP2	0.46	-0.47	ESPNL	0.49	-0.63
CTDSPL2	0.44	-0.32	EDEM3	0.47	-0.32
RNF138	0.44	-0.56	SPDL1	0.47	-0.30
LIMS1	0.43	-0.42	ABHD5	0.41	-0.47
SHPRH	0.38	-0.78	STC2	0.33	-0.32
LEMD2	0.37	-0.53	SERPINB9	0.32	-0.32
MAZ	0.36	-0.49	MLLT11	0.31	-0.49
KCTD5	0.36	-0.47			
WDFY1	0.35	-0.37			
PPT1	0.35	-0.33			
ARHGAP19	0.34	-0.35			
CCNA2	0.34	-0.44			
SLC39A10	0.34	-0.50			
CBX2	0.34	-0.63			
PAN2	0.34	-0.62			
AP3M2	0.31	-0.42			
TMTC3	0.31	-0.32			
ZBTB10	0.31	-0.40			

SILAC was performed for miR-29a and miR-29c and differences in expression between the scramble-control (SCR) and miRNA mimic are shown. Targets downregulated by the miRNA mimic compared with the SCR in both modes (miR/SCR and SCR/miR) that were also a predicted target of the respective miRNA were included. Targets with a ratio above |0.3| in both modes were selected as target and depicted here; targets common between both miRs are shown in bold. SILAC: stable isotope labelling with amino acids in cell culture.

regulators of apoptosis caspase (CASP)7, Serpin H family member 1 (SERPINH1/HSP47) and follistatin-like protein (FSTL)-1, as well as immune-checkpoint regulator CD276/B7H3, and adhesion molecule F11R (Fig. 2C, Table 3).

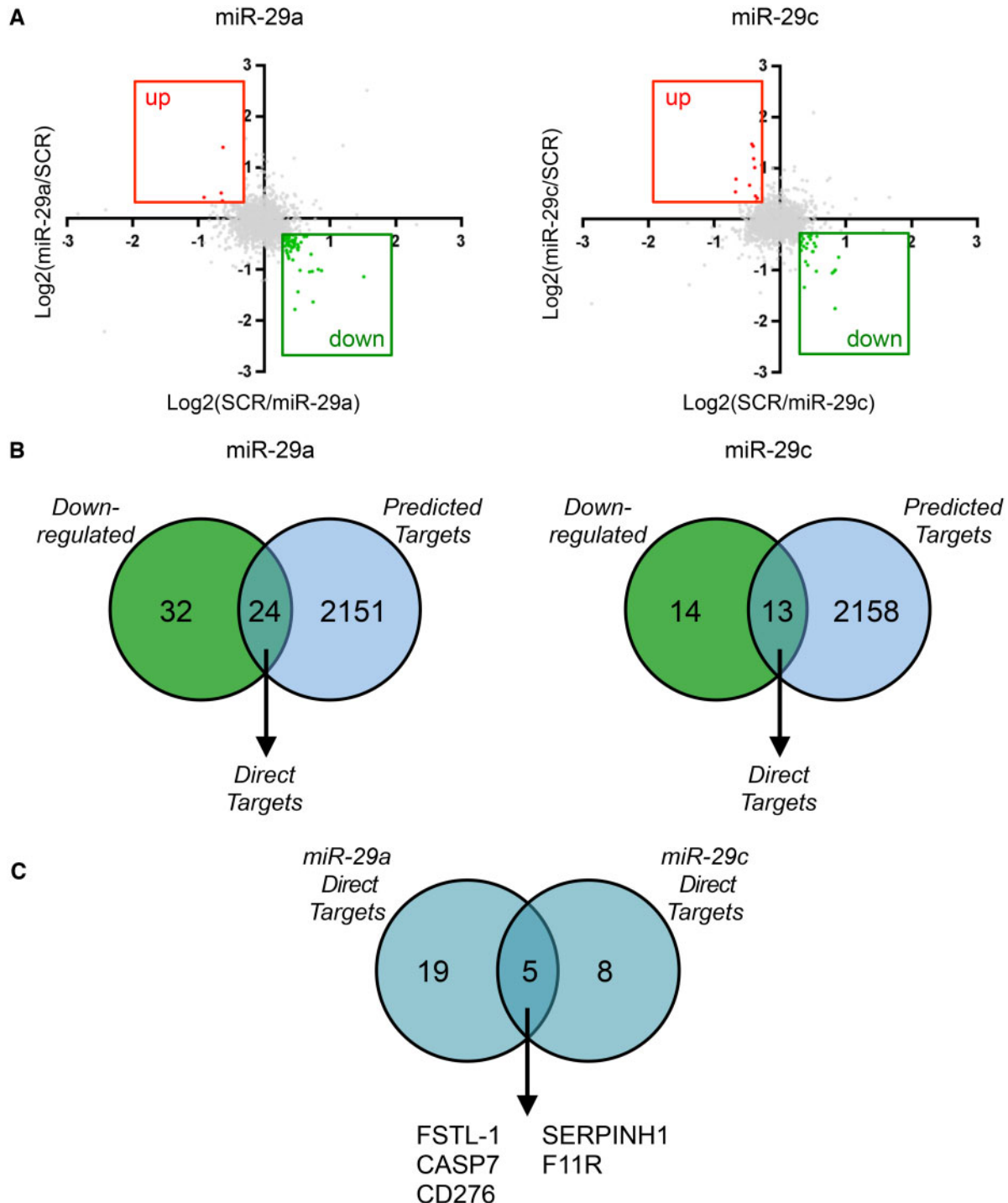
## Discussion

Using a robust approach based on replication of targets in two independent cohorts of pSS patients and controls, we identified 10 miRNAs that are consistently decreased in pDCs from patients with pSS compared with controls. These changes in the miRNome primarily seem to affect PI3K-AKT-mTOR signalling and regulation of cell death, both regulated by the two most robustly different miRNAs: miR-29a and miR-29c. For these two miRNAs, we identified a substantial set of novel targets on the protein level, five of which were regulated by both miRNAs in our analysis.

The identified dysregulated miRNAs seem to be unique for pDCs in pSS, as none of the miRNAs that are differentially expressed in pSS PBMCs or salivary gland tissue were replicated in our study [11–14, 17]. However, this was not unexpected as pDCs make up only 0.1–0.5% of PBMCs and as such differences found in PBMCs are not likely to be reflected in isolated pDCs and vice versa.

Similarly, relatively small numbers of pDCs at the site of inflammation could explain why miRNAs differentially expressed in the salivary glands were not identified here. Alternatively, the lack of overlap between miRNAs identified in circulating pDCs and salivary glands may be due to inter-donor variation in the composition of immune cells infiltrating the salivary glands. In line with this, differences in miRNA expression profiles between paired salivary gland tissue, PBMCs and salivary gland epithelial cells were previously demonstrated in patients with pSS [14]. Considering the possible contribution of pDCs to salivary gland inflammation and type-I IFN production in pSS patients, it would be worthwhile to compare miRNA expression in salivary glands and circulating cells of IFN signature-positive and -negative pSS patients in a future study.

We observed a consistent decreased expression of miRNAs in pSS pDCs, similar to data published in SLE pDCs where a broadly decreased expression of miRNAs was linked to the IFN signature [20]. Despite the fact that an IFN signature is present in a majority of patients with pSS and is associated with increased disease activity [2], we observed no clear association between the IFN-score and miRNA expression, contrary to what was observed in SLE. Only two replicated miRNAs modestly and positively

**Fig. 2** Identification of novel protein targets of miR-29a and miR-29c using SILAC

HEK-293T cells were pre-cultured in culture medium supplemented with light- or heavy-isotope labelled amino acids to achieve steady-state labelling. Then, cells were transfected with a mimic for miR-29a, a mimic for miR-29c or an SCR for 48 h. Cells were washed and lysed. Light- and heavy-isotope medium cultured cells were mixed in two combinations per mimic (mimic in light and SCR in heavy; SCR in light and SCR in heavy) and separated into 20 fractions using ultra-performance liquid chromatography. Peptides in each fraction were identified by mass spectrometry. Peptides consistently downregulated by the miRNA mimic in both mixes with a fold-change of  $>|0.3|$  (in green) were selected (**A**). We compared the selected peptides to a list of *in silico*-predicted targets to identify the likely targets of miR-29a and miR-29c (**B**). Of these targets, five were common between the two miRs (**C**). SCR: scramble control; SILAC: stable isotope labelling with amino acids in cell culture.

correlated with the IFN-score in our study. In addition, there was no overlap in identified miRNAs differentially expressed between our study and those identified in SLE [20]. As such, there does not seem to be a strong relationship between pDC miRNome and the IFN signature in pSS compared with what was published in SLE.

Pathway enrichment of the target genes of the identified miRNAs in our study indicated that a majority of these miRNAs are involved in signalling via PI3K/AKT, mTOR and FoxO. These molecules are connected in signalling pathways downstream from TLRs and growth factors, including epidermal growth factor as shown in salivary gland epithelial cells [27], and are crucial for pDC function. PI3K-AKT-mTOR signalling is critical for pDC development and is involved in the capacity of pDCs to control antigen processing, cytokine production and T cell stimulation [21]. Importantly, PI3K was shown to be crucial for production of type-I IFN by pDCs upon stimulation with endosomal TLR ligands [28]. FoxO is a downstream mediator of this pathway and also regulates TLR signalling in dendritic cells [29]. In addition, the PI3K-AKT-mTOR pathway and its downstream effectors, including FoxO, regulate cell death via both apoptosis and autophagy [30]. This is consistent with the fact that the targets of a large number of the identified miRNAs regulate apoptosis and autophagy. Interestingly, PI3K signalling was recently identified as a therapeutic target in pSS using patient material and a Sjögren's-like mouse model [31]. In addition, our group recently showed that targeting mTOR represents a possible therapeutic avenue in pSS [32]. Although pDCs were not studied in this context, our data highlight the potential of these strategies to inhibit pDC activity in addition to their previously published regulation of immunopathology.

Using a proteomic approach, we identified a set of novel targets of miR-29a and miR-29c, including several proteins previously associated with autoimmunity. Furthermore, we identified five novel common targets regulated by miR-29a and miR-29c. These proteins exert their effects in regulating processes that are relevant in pDCs: FSTL-1 is increased in the serum of patients with a range of autoimmune diseases including pSS and regulates antigen presentation in DCs [33]. The contribution of pDCs to these increased serum levels of FSTL-1 needs to be demonstrated, but this could represent a novel mechanism by which pDCs contribute to inflammation in pSS. CD276/B7-H3 is an immune-checkpoint molecule that is expressed on antigen-presenting cells and plays an important role in inhibition of T cell activation [34]. Hence, it could serve as a negative regulator of pDC-induced T cell activation. In addition, three of the identified miR-29 targets (CASP7, FSTL-1, HSP47) are regulators of apoptosis [35–37]. In line with this, miR-29a is known to be pro-apoptotic [38] and miR-29c was shown to promote pDC apoptosis and is downregulated in pDCs upon stimulation via TLR9 [22]. We replicated this latter finding in the present study and showed similar downregulation using the TLR7/8 ligand R848. As such, the decreased expression of miR-29c in pSS pDCs seems to be a response to

activation via endosomal TLRs and may contribute to enhanced survival of pDCs.

In summary, we here identify a set of 10 dysregulated miRNAs in pSS pDCs that affect relevant processes in pSS pDCs, including antigen presentation, cytokine production and cell death. The majority of the identified miRNAs regulate PI3K-AKT-mTOR signalling, which is a therapeutic avenue in pSS that is upstream of type-I IFN production in pDCs. In addition, these miRNAs target regulators of cell death and the two most robustly down-regulated miRNAs, miR-29a and miR-29c, are pro-apoptotic. Moreover, we identify several novel shared targets of the replicated miRNAs that regulate apoptosis. As such, the pSS pDC miRNome seems to confer resistance to cell death. We speculate that miRNA dysregulation in pDCs leads to enhanced activation via PI3K signalling and improved cell survival. Upon migration towards the salivary glands, this can contribute to the enhanced numbers of pDCs observed in the salivary glands and subsequently drive increased local production of type-I IFN. As type-I IFN is a strong driver of B cell hyperactivity, these molecular alterations underlying pDC dysregulation can form a critical component of local immunopathology.

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## Supplementary data

Supplementary data are available at *Rheumatology* online.

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