ARTICLE IN PRESS

Journal of Autoimmunity xxx (2018) 1–9

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

Serum microRNA screening and functional studies reveal miR-483-5p as a potential driver of fibrosis in systemic sclerosis

Eleni Chouri [a, b], Nila H. Servaas [a, b], Cornelis P.J. Bekker [a, b], Alsya J. Affandi [a, b], Marta Cossu [a, b], Maarten R. Hillen [a, b], Chiara Angiolilli [a, b], Jorre S. Mertens [a, b], Lucas L. van den Hoogen [a, b], Sandra Silva-Cardoso [a, b], Maarten van der Kroef [a, b], Nadia Vazirpanah [a, b], Catharina G.K. Wichers [a, b], Tiago Carvalheiro [a, b], Sofie L.M. Blokland [a, b], Barbara Giovannone [b, c], Laura Porretti [d], Wioleta Marut [a, b], Barbara Vigone [e], Joel A.G. van Roon [a, b], Lorenzo Beretta [e], Marzia Rossato [a, b, f, 1], Timothy R.D.J. Radstake [a, b, *, 1]

[a] Department of Rheumatology & Clinical Immunology, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands
[b] Laboratory of Translational Immunology, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands
[c] Department of Dermatology/Allergology, University Medical Center Utrecht, Utrecht, The Netherlands
[d] Flow Cytometry Service, Clinical Chemistry and Microbiology Laboratory, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico di Milano, Milan, Italy
[e] Scleroderma Unit, Referral Center for Systemic Autoimmune Diseases, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico di Milano, Milan, Italy
[f] Department of Biotechnology, University of Verona, Verona, Italy

A R T I C L E I N F O

Article history:
Received 9 October 2017
Received in revised form
21 December 2017
Accepted 21 December 2017
Available online xxx

Keywords:
Systemic sclerosis
Circulating microRNAs
miR-483-5p
Fibrosis

A B S T R A C T

Objective: MicroRNAs (miRNAs) are regulatory molecules, which have been addressed as potential biomarkers and therapeutic targets in rheumatic diseases. Here, we investigated the miRNA signature in the serum of systemic sclerosis (SSc) patients and we further assessed their expression in early stages of the disease.
Methods: The levels of 758 miRNAs were evaluated in the serum of 26 SSc patients as compared to 9 healthy controls by using an Openarray platform. Three miRNAs were examined in an additional cohort of 107 SSc patients and 24 healthy donors by single qPCR. MiR-483-5p expression was further analysed in the serum of patients with localized scleroderma (LoS) (n = 22), systemic lupus erythematosus (SLE) (n = 33) and primary Sjögren's syndrome (pSS) (n = 23). The function of miR-483-5p was examined by transfecting miR-483-5p into primary human dermal fibroblasts and pulmonary endothelial cells.
Results: 30 miRNAs were significantly increased in patients with SSc. Of these, miR-483-5p showed reproducibly higher levels in an independent SSc cohort and was also elevated in patients with preclinical-SSc symptoms (early SSc). Notably, miR-483-5p was not differentially expressed in patients with SLE or pSS, whereas it was up-regulated in LoS, indicating that this miRNA could be involved in the development of skin fibrosis. Consistently, miR-483-5p overexpression in fibroblasts and endothelial cells modulated the expression of fibrosis-related genes.
Conclusions: Our findings showed that miR-483-5p is up-regulated in the serum of SSc patients, from the early stages of the disease onwards, and indicated its potential function as a fine regulator of fibrosis in SSc.

© 2018 Elsevier Ltd. All rights reserved.

1. Introduction

Systemic sclerosis (SSc) is a rare, systemic autoimmune disorder characterized by vascular damage, immune activation and fibrosis of the skin and/or internal organs [1]. Even if the pathogenesis of

* Corresponding author. University Medical Centre Utrecht, Heidelberglaan 100, 3584 CX, Utrecht, The Netherlands.
E-mail address: t.r.d.j.radstake@umcutrecht.nl (T.R.D.J. Radstake).
[1] These authors contributed equally.

https://doi.org/10.1016/j.jaut.2017.12.015
0896-8411/© 2018 Elsevier Ltd. All rights reserved.

Please cite this article in press as: E. Chouri, et al., Serum microRNA screening and functional studies reveal miR-483-5p as a potential driver of fibrosis in systemic sclerosis, Journal of Autoimmunity (2018), https://doi.org/10.1016/j.jaut.2017.12.015

ARTICLE IN PRESS

SSc still remains elusive, increasing evidence suggests that microvascular injury and endothelial cell activation are the earliest events in the evolution of the disease [2]. The complex interplay between altered endothelial cells and immune cells infiltrating the tissues results in the secretion of inflammatory cytokines and profibrotic mediators, leading to myofibroblast transition and culminating in excessive deposition of extracellular matrix (ECM), defined as fibrosis, the hallmark of SSc [3–5]. Activated myofibroblasts in fibrotic tissue can derive from different precursors including activated fibroblasts, pericytes, fibrocytes, epithelial cells and endothelial cells, and are characterized by *de novo* expression of α-smooth muscle actin (αSMA) [6].

Recently, microRNAs (miRNAs) were proposed as possible novel players in SSc fibrosis, being capable of modulating several fibrotic-related genes [7,8]. MiRNAs constitute a class of short non-coding RNAs (19–24 nucleotides) that regulate gene expression at the post-transcriptional level. Beside their intracellular function, miRNAs are also present in biological fluids, where they circulate bound to protein complexes or enclosed in extracellular vesicles, including exosomes [9]. It has been demonstrated that miRNAs embedded in exosomes are involved in cell-to-cell communication by transferring their content into the recipient cells [9,10]. Furthermore, circulating miRNAs are often indicated as potential biomarkers and therapeutic targets in several diseases, since they are highly stable molecules and their aberrant expression frequently reflects the pathophysiology of diseases [11,12]. MiRNAs have been shown to be altered in the serum of SSc patients, implicating them as possible diagnostic biomarkers for SSc and potential players in the disease pathogenesis [7,13,14].

Hitherto, the knowledge on circulating miRNAs in SSc is limited and a broad profiling of circulating miRNAs is lacking. Moreover, the expression of secretory miRNAs in early SSc patients, who do not yet present with skin fibrosis, has not been explored thus far. Identifying molecular changes at the early stages is of utmost importance, as an understanding of the pathophysiological pathways leading up to disease could open novel avenues for therapeutic intervention before the actual onset of fibrosis.

Here, we present a comprehensive profiling of 758 miRNAs in the serum of SSc patients. MiR-483-5p was demonstrated to be consistently upregulated in two independent SSc cohorts and showed high expression before the onset of fibrosis. Moreover, functional experiments indicated the potential implication of miR-483-5p in the modulation of fibrosis.

2. Material & methods

2.1. Patients

Blood from patients and sex- and age-matched healthy controls (HC) was obtained from the University Medical Center Utrecht in the Netherlands and the Scleroderma Unit of the Fondazione IRCCS Policlinico of Milan in Italy. All patients signed a consent form approved by the local institutional review boards, prior to participation in the study. Samples and clinical information were treated anonymously immediately after collection. Patients fulfilling the ACR/EULAR 2013 classification criteria [15] were classified in relation to the extent of skin fibrosis as limited cutaneous (lcSSc) or diffuse cutaneous SSc (dcSSc) [16]; patients fulfilling the classification criteria without skin fibrosis will be referred to as non-cutaneous SSc (ncSSc) throughout the manuscript. Additionally, early SSc (eaSSc) subjects were defined as patients presenting with Raynaud's phenomenon (RP) and SSc-specific autoantibodies and/or typical nailfold videocapillaroscopy (NVC) abnormalities [17]. The presence of interstitial lung disease (ILD) was identified as typical involvement of the lung parenchyma >5% on high resolution CT [18] accompanied by a reduced forced vital capacity (FVC) or a diffusing capacity for carbon monoxide (DLco) < 80% of predicted values [19].

Two separate cohorts were recruited for the current study (Discovery and Validation Cohort) at the University Medical Center Utrecht and the Scleroderma Unit of Fondazione IRCCS Policlinico of Milan. Early SSc patients were also included in the validation cohort. Demographics and clinical characteristics of the patients are depicted in Table 1.

2.2. Blood collection and RNA extraction

Blood was collected in a Vacutainer® SST II tube (BD Vacutainer) and serum was isolated after centrifugation at 1500g for 10 min at room temperature and stored at −80 °C prior to use. Serum RNA was extracted from 200 μl of serum using the miRcury RNA Isolation kit for Biofluids (Exiqon), according to the manufacturer's instructions. During extraction, 300 pg of a non-human synthetic miRNA (Arabidopsis thaliana ath-miR-159a) was added to each sample as a spike-in to monitor the technical variability during the isolation and for subsequent data normalization.

RNA from cells was isolated using the Allprep Universal Kit (Qiagen), according to the manufacturer's instructions. The concentration of RNA was determined using a Qubit 2.0 fluorimeter (Invitrogen) and the Qubit RNA HS Assay Kit (Molecular Probes, Life Technologies).

2.3. MiRNA profiling

The screening of 758 miRNAs in the Discovery cohort was performed by TaqMan RT-qPCR on an OpenArray platform (LifeTechnologies) according to manufacturer's instructions with minor adjustments. 2.5 μl of isolated serum RNA was reverse-transcribed by using the miRNA megaplex RT primer pools (LifeTechnologies). cDNA was pre-amplified using the Megaplex PreAmp Primer pools in the presence of the TaqMan PreAmp Master mix (Life Technologies), by using the following thermal cycler conditions: 95 °C for 10 min, 55 °C for 2 min, 72 °C for 2 min and 16 cycles of 95 °C for 15 s and 60 °C for 4 min and one single cycle of 96 °C for 10 min. The miRNA OpenArray profiling was performed on the amplified cDNA diluted 1:40 in 0.1x TE (pH8.0), by using the Taqman OpenArray master mix on the QuantStudio 12k flex Real-Time PCR system (LifeTechnologies). The Relative Quantification method of the GeneExpression Suite Software (ThermoFisher) was applied to analyze the data by using the comparative threshold cycle method (Crt). Low expressed miRNAs were excluded from the analysis: Crt was set at 27 maximum and only miRNAs with high amplification quality (>1.24) were taken into consideration. The expression of each miRNA was calculated after normalization on the levels of the exogenous spike-in ath-miR-159a and expressed as Fold Change (FC) as compared to the control samples. MiRNA profiling data have been deposited in NCBI's Gene Expression Omnibus (GSE) and are accessible through GEO Series accession number GSE108918.

2.4. MiRNA and gene expression analysis

The validation of miRNA expression in serum was performed by single Taqman miRNA RealTime-quantitative PCR (RT-qPCR) assays (LifeTechnologies). Briefly, 2.5 μl of serum RNA was reverse-transcribed using the specific microRNA assays (ath-miR159a; ID 000338, has-miR-501-3p; ID 002435, has-miR-483-5p; ID 002338, U6 snRNA; ID 001973, Life Technologies) and measured with the specific TaqMan assay on the QuantStudio 12k flex System, in the presence of the TaqMan Fast Advanced Master mix (LifeTechnologies), following manufacturer's instructions. MiRNA

Please cite this article in press as: E. Chouri, et al., Serum microRNA screening and functional studies reveal miR-483-5p as a potential driver of fibrosis in systemic sclerosis, Journal of Autoimmunity (2018), https://doi.org/10.1016/j.jaut.2017.12.015

ARTICLE IN PRESS

Table 1
Demographics and clinical characteristics of the donors included in the study.

	Discovery Cohort				Validation Cohort				
Patient group (n)	HC(9)	ncSSc(8)	lcSSc(9)	dcSSc(9)	HC (24)	ncSSC(26)	lcSSc(35)	dcSSc(29)	eaSSc(17)
Age (yr.)	53(49–55)	49(37–52)	63(54–67)	63(54–66)	56(46–62)	58(49–66)	60(49–69)	50(45–57)	48(37–72)
Female (n, %)	6 (67%)	8 (100%)	6 (67%)	5 (56%)	22 (92%)	25 (96%)	33 (94%)	20 (69%)	16 (94%)
ANA (n pos, %)	–	6 (75%)	9 (100%)	8 (89%)	–	25 (96%)	32 (91%)	27 (93%)	16 (94%)
ACA (n pos, %)	–	3 (38%)	2 (22%)	3 (33%)	–	16 (62%)	17 (49%)	2 (6.9%)	10 (59%)
Scl70 (n pos, %)	–	2 (25%)	4 (44%)	2 (22%)	–	3 (12%)	12 (34%)	14 (48%)	3 (18%)
mRSS	–	0	7 (5–8)	12 (8–15)	–	0	4 (2–5)	11 (6–18)	0
ILD	–	0	2 (22%)	7 (78%)	–	4 (15%)	10 (29%)	15 (52%)	0
Disease Duration	–	3 (2–7)	4 (2–5)	12 (7–20)	–	7 (2–15)	16 (5–23)	8 (2–16)	–

Values reported indicate the number (n) of patients and the median for each parameter (Interquartile Range (IQR)), if not otherwise indicated. Yr., years; ANA, antinuclear antibodies; pos, positivity; ACA, anticentromere antibodies; Scl70, antitopoisomerase antibodies; mRSS, modified Rodnan Skin score; ILD, Interstitial Lung disease; HC, healthy controls; ncSSc, non-cutaneous SSc; lcSSc, limited cutaneous SSc; dcSSc, diffuse cutaneous SSc; eaSSc, early SSc.

expression values were calculated according to the comparative threshold cycle method (Ct) after normalization to the spike-in ath-miR-159a. The relative fold change (FC) of the control samples was set at 1 and was used to calculate the fold change of the target miRNAs in patients or treated cells ($FC = 2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct$ sample - ΔCt reference).

Expression of protein-coding genes was analyzed by real-time quantitative-PCR (RT-qPCR) using a 4 ng retrotranscribed RNA with iScript reverse transcriptase kit (Bio-Rad). Reactions were conducted using the SybrSelect mastermix with 500 nM specific primer pairs on a QuantStudio 12k flex (Life Technologies). RT-qPCR data were normalized to the expression of GUSB and analyzed using the comparative threshold cycle method as described for miRNAs. All primers used are listed in the Supplementary Table 1.

2.5. Exosome isolation and exosomal RNA extraction

Serum was centrifuged at 3000g for 15 min at 4 °C. Exosomes were isolated from 250 μl serum by precipitation using the Exoquick solution (Systems Biosciences), according to the manufacturer's protocol. After exosome precipitation, 300 pg of the spike-in, ath-miR-159a, was added to each sample during the exosomal RNA extraction (exoRNA). ExoRNA was isolated according to the manufacturer's protocol (Systems Biosciences).

2.6. Fibroblast and HPAEC culture and transfection

Primary dermal fibroblasts were obtained as discarded material after cosmetic surgery from anonymous donors who gave prior informed consent for the use of material in research. Dermal fibroblasts were isolated using the Whole Skin Dissociation Kit (Miltenyi Biotech), following the manufacturer protocol. Briefly, biopsies with diameter of 3 × 4mm were digested overnight at 37 °C and processed with the gentleMACS Dissociator (Miltenyi Biotech) to obtain a single cell suspension. Fibroblasts were cultured in DMEM medium (Life Technologies) containing 10% heat-inactivated fetal bovine serum (FBS, Biowest) and used in passages between 3 and 5. Human pulmonary artery endothelial cells (HPAEC, ThermoFisher) were cultured in endothelial basal medium (EBM-2 Basal Medium, Lonza) containing manufacturer-recommended supplemental growth factors (EGM-2 Bullet-kit) supplemented with 10% FBS, and used in passages 3–4.

50.000 fibroblasts or HPAEC were seeded in 12-well plates in their respective culture medium and after 24 h culture medium was replaced with Opti-MEM I Reduced Serum Medium (Life-Technologies). Fibroblasts were transfected with 100 nM miR-483-5p mimic or miR-scramble control (Ambion) using the transfection reagent DharmaFECT-1 (Dharmacon) for 24 h, while HPAECs were transfected with 20 nM miR-483-5p mimic or miR-scramble control for 6 h. After removal of transfection medium, cells were recovered overnight in complete medium. Before stimulation, cells were pre-starved for 24 h in DMEM with 1% FBS or for 3 h in EBM-2 with 0.1% FBS and subsequently were either left untreated or stimulated respectively either with 10 ng/ml TGFb1 (Biolegend) or TGFb2 (R&D systems) for 24 or 48 h. Cells were lysed in RLTplus buffer (Qiagen) supplemented with beta-mercaptoethanol for RNA isolation.

2.7. Statistical analysis

ExpressionSuite Software (Life Technologies) was used for the analysis of miRNA profiling data. Independent *t*-test on the comparative Ct method (ΔΔCt) was performed on ExpressionSuite software and differences were considered significant at the level of $p < .05$ (uncorrected p-value). GraphPad Prism 6.0 Software or IBM SPSS 21 Software was used for the subsequent analysis in the study. Kruskal-Wallis and Dunn's multiple comparison post hoc tests, Mann-Whitney *U* test and Wilcoxon signed rank test were applied to compare data that were not normally distributed as indicated. Spearman's rho was used to assess correlations. Differences were considered statistically significant at $p < .05$.

The miRWalk 2.0 database (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/custom.html) was used to retrieve the miRNA targets and pathway enrichment analysis was performed on the ToppGene Suite website (https://toppgene.cchmc.org/enrichment.jsp). Pathways were considered significantly enriched at $p < .05$ adjusting the false discovery rate (FDR) using Benjamini-Hochberg.

3. Results

3.1. The profile of circulating miRNAs is altered in the serum of SSc patients

The expression level of 758 miRNAs and 3 small-nuclear RNAs was evaluated in the serum of healthy donors ($n = 10$) and SSc patients, either without any evident skin involvement (ncSSc, $n = 8$), or with limited cutaneous fibrosis (lcSSc, $n = 9$), or diffuse cutaneous SSc (dcSSc, $n = 9$). Twenty-nine serum miRNAs and one small nuclear RNA (snRNA-U6) were differentially expressed in at least one group of patients as compared to the healthy controls in the discovery cohort (Table 2).

We then sought to validate the three highly dysregulated short-RNAs that showed a significant change in all the three SSc subsets, namely miR-483-5p, miR-501-3p and snRNA-U6 (Fig. 1A). We evaluated their expression in an independent SSc validation cohort, encompassing 107 SSc patients and 24 healthy donors. Here, the elevated expression of miR-483-5p and snRNA-U6 was replicated in SSc patients, while the levels of miR-501-3p were not

Please cite this article in press as: E. Chouri, et al., Serum microRNA screening and functional studies reveal miR-483-5p as a potential driver of fibrosis in systemic sclerosis, Journal of Autoimmunity (2018), https://doi.org/10.1016/j.jaut.2017.12.015

Table 2
MiRNAs differentially expressed in the serum of SSc patients.

miRNA	ncSSc		lcSSc		dcSSc	
	FC	p value	FC	p value	FC	p value
snRNA-U6	**4.968**	**.030**	**5.160**	**.014**	**5.496**	**.003**
miR-483-5p	**2.087**	**.022**	**2.730**	**.041**	**3.145**	**.009**
miR-501-3p	**1.994**	**.021**	**3.401**	**.000**	**2.377**	**.001**
miR-423-5p	1.100	.715	**2.685**	**.011**	1.952	.097
miR-223*	1.936	.140	**2.290**	**.031**	1.753	.222
miR-320b	1.875	.023	**2.422**	**.005**	1.670	.121
miR-23a	1.642	.124	**2.137**	**.012**	1.562	.071
miR-324-3p	1.758	.033	**2.048**	**.006**	1.428	.105
miR-491	1.639	.081	**2.165**	**.001**	1.484	.086
miR-532-3p	1.811	.080	**2.151**	**.002**	1.422	.187
miR-345	0.995	.680	**2.199**	**.010**	1.398	.220
miR-133a	1.832	.044	**2.051**	**.033**	0.973	.943
miR-22*	1.496	.143	**2.028**	**.012**	0.985	.785
miR-410	**0.473**	**.041**	0.914	.579	0.718	.379
miR-15b	0.806	.681	1.048	.539	**0.485**	**.013**
miR-331	0.665	.224	0.872	.591	**0.480**	**.018**
miR-103	0.717	.383	0.779	.174	**0.460**	**.003**
miR-30b	0.719	.304	0.374	.149	**0.452**	**.043**
let-7d	0.646	.227	0.744	.596	**0.448**	**.006**
miR-26b	0.876	.762	0.811	.123	**0.444**	**.003**
miR-181a	0.854	.666	1.011	.985	**0.444**	**.017**
miR-28	0.773	.488	0.742	.667	**0.418**	**.014**
miR-30c	0.282	.053	0.789	.259	**0.409**	**.005**
miR-144*	0.816	.311	0.624	.071	**0.402**	**.006**
miR-374	0.815	.721	0.653	.048	**0.352**	**.002**
miR-374-5p	0.638	.376	0.680	.089	**0.329**	**.007**
miR-191	0.941	.581	0.981	.773	**0.297**	**.006**
miR-142-3p	0.689	.087	0.948	.759	**0.257**	**.003**
miR-26a	0.613	.135	0.736	.094	**0.211**	**.001**
miR-409-3p	**0.300**	**.040**	0.540	.508	**0.188**	**.025**

The expression of 758 miRNAs was analyzed in RNA extracted from the serum of SSc patients and healthy controls in the discovery cohort, using the OpenArray platform. The expression of each miRNA was calculated as Fold Changes (FC) as compared to the mean of the healthy control group. MiRNAs were considered differentially expressed with a FC of ≥ 2 or $\leq 0{,}5$ and a p-value of <0,05 in at least one patient-group vs healthy controls (indicated in bold); ncSSc, non-cutaneous SSc; lcSSc, limited cutaneous SSc; dcSSc, diffuse cutaneous SSc.

significantly different (Fig. 1B). Interestingly, the increase in miR-483-5p and snRNA-U6 was also present in eaSSc patients (Fig. 1C), demonstrating that these short-RNAs are altered before the appearance of evident fibrosis.

3.2. MiR-483-5p is specifically up-regulated in scleroderma patients

In order to verify whether the increased levels of miR-483-5p and snRNA-U6 were specific for SSc patients, their expression was evaluated in the serum of patients with other autoimmune diseases, either presenting fibrotic features, such as localized scleroderma (LoS) ($n = 22$), or systemic non-fibrotic diseases, such as systemic lupus erythematosus (SLE) ($n = 33$) and primary Sjögren's Syndrome (pSS) ($n = 23$) (Supplementary Table 2).

Similar to what we observed in SSc patients, miR-483-5p was also significantly increased in the serum of LoS patients (Fig. 2A) whereas the increase of snRNA-U6 was specific to SSc (Fig. 2B). These results suggest that miR-483-5p is specific for conditions marked by skin fibrosis. In addition, both SSc cohorts showed that miR-483-5p levels were the highest in dcSSc patients, i.e. the group with the most extensive skin fibrosis. In agreement with this, we also observed that in the validation cohort miR-483-5p expression correlated with the modified Rodnan Skin Score (mRSS) in dcSSc particularly ($\rho = 0.405$ $p = .029$, Supplementary Fig. 1). Furthermore, miR-483-5p is predicted to regulate target genes enriched in pathways related to SSc pathogenesis and linked to fibrosis, such as PDGF signaling or insulin growth factor signaling (Supplementary Table 3). In the light of these observations as well as the fact that miR-483-5p has been associated with fibrosis previously [20], we further investigated its potential role in SSc pathogenesis.

3.3. Circulating miR-483-5p is embedded in serum exosomes and stable over time

To investigate whether miR-483-5p could be implicated in cell-to-cell communication during SSc pathogenesis - frequently orchestrated by exosomal trafficking - we analyzed whether the pool of circulating miR-483-5p in the serum of SSc patients was stored in exosomes. MiR-483-5p was highly enriched in the exosome fraction compared to the exosome-depleted serum ($FC > 30$, $p < .0001$) (Fig. 3A). Similar to the results obtained in total serum, the levels of miR-483-5p were significantly higher in the exosomes of both eaSSc ($FC = 3.8$ $p = .020$) and SSc patients ($FC = 3.7$ $p = .004$) compared to healthy controls (Fig. 3B). Furthermore, the treatment of serum with RNase, prior to RNA isolation, did not affect the levels of miR-483-5p detected in either patients or controls, thus confirming that the circulating miR-483-5p pool is protected inside extracellular vesicles (Fig. 3C). In addition, miR-483-5p showed stable expression in patients and healthy controls over multiple samplings of the same individuals over a period of 15 months (Fig. 3D). All these observations indicated that miR-483-5p is stably embedded in exosomes, suggesting that miR-483-5p might play a role in SSc pathogenesis by affecting target cells implicated in the fibrotic process, e.g. fibroblasts and endothelial cells.

3.4. MiR-483-5p modulates the expression of fibrosis-related genes in fibroblasts and endothelial cells

As a proof of concept to investigate the possible role of miR-483-5p in fibrosis development, we mimicked the uptake of miR-483-5p by transfecting it into fibroblasts and endothelial cells, as potential recipient cells, and evaluated the expression of genes implicated in myofibroblast transition and collagen production.

Upon TGFbeta (TGFb) stimulation, a known fibrotic mediator in SSc, overexpression of miR-483-5p in fibroblasts (Fig. 4A) led to the up-regulation of Collagen-type IV Alpha 1 and Alpha 2 Chain (Col4A1 and Col4A2) and down-regulation of Collagen-type I Alpha 2 Chain (Col1A2) (Fig. 4B). In line with what was observed in fibroblasts, Col4A1 and Col4A2 expression were also induced in TGFb-stimulated endothelial cells upon miR-483-5p transfection, while Col1A2 was reduced (Fig. 4D). Additionally, the expression of αSMA and SM22A, two known myofibroblast differentiation markers, were both induced transcriptionally in miR-483-5p transfected endothelial cells (Fig. 4D). Noteworthy, miR-483-5p uptake significantly elevated the transcriptional levels of αSMA, SM22A, Col4A1 and Col4A2 also in unstimulated endothelial cells (Supplementary Fig. 2A). Moreover, miR-483-5p overexpression significantly lowered the expression of Fli-1, a suppressor of collagen transcription (Supplementary Fig. 2A). On the contrary Col5A1, Col5A2 and two genes not primarily involved in extracellular matrix deposition, namely beta-2-microglobulin (B2M) and Interleukin-6 (IL6) were not significantly changed either in fibroblasts or in endothelial cells (Supplementary Figs. 2B and C). Of note, the upregulatory effect of miR-483-5p on Col4A1 and SM22A was evident even at low doses, at overexpression levels (0.02 nM, FC~20) within the range observed in the circulation of SSc patients (Supplementary Fig. 3). Moreover, the modulations observed could be specifically attributed to the biological effect of miR-483-5p, as they were not evident when overexpressing at the same dose a non-targeting microRNA (miR-Scr) or miR-618, a miRNA previously

Please cite this article in press as: E. Chouri, et al., Serum microRNA screening and functional studies reveal miR-483-5p as a potential driver of fibrosis in systemic sclerosis, Journal of Autoimmunity (2018), https://doi.org/10.1016/j.jaut.2017.12.015

ARTICLE IN PRESS

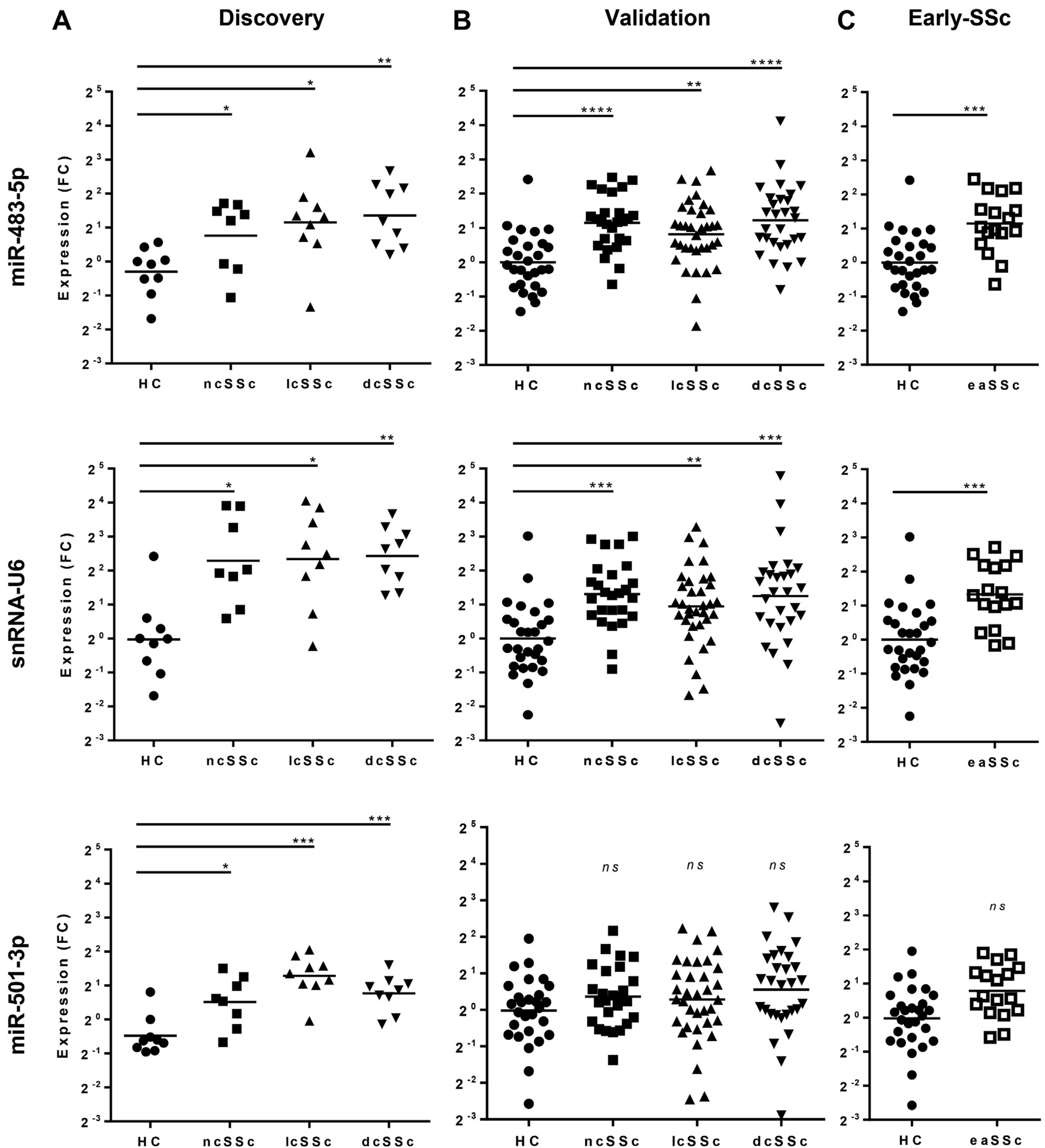

Fig. 1. MiR-483-5p and snRNA-U6 are higher expressed in SSc serum. The expression of miR-483-5p, snRNA-U6 and miR-501-3p were analyzed in RNA extracted from the serum of donors included in the discovery cohort using the OpenArray platform (A) and in the validation cohort by quantitative RT-qPCR (B). The expression of the miRNAs was also assessed in the serum of early SSc patients collected simultaneously with the validation cohort (C). The expression is presented as fold change (FC) as compared to the mean expression of healthy donors. HC, Healthy controls; eaSSc, early SSc; ncSSc, non-cutaneous SSc; dcSSc, difusse cutaneous SSc; lcSSc, limited cutaneous SSc. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

shown to be dysregulated in the immune compartment of SSc patients [21], but not reported to be altered in their circulation or in fibroblast and endothelial cells (Supplementary Fig. 4). Taken together, these findings suggest that miR-483-5p can have an impact on fibrosis development by inducing myofibroblast differentiation and an imbalance in collagen production.

Please cite this article in press as: E. Chouri, et al., Serum microRNA screening and functional studies reveal miR-483-5p as a potential driver of fibrosis in systemic sclerosis, Journal of Autoimmunity (2018), https://doi.org/10.1016/j.jaut.2017.12.015

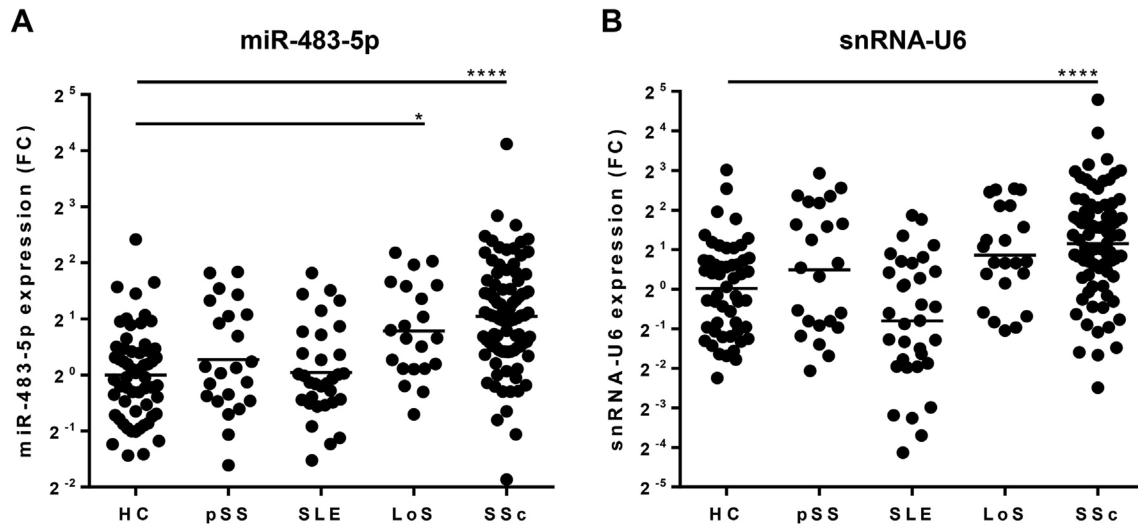

Fig. 2. MiR-483-5p is up-regulated specifically in scleroderma patients (SSc and LoS) among other autoimmune diseases. The expression of miR-483-5p (A) and snRNA-U6 (B) were analyzed in the serum of healthy controls (HC), Sjögren's syndrome (pSS), systemic lupus erythematosus (SLE), localized scleroderma (LoS) and systemic sclerosis (SSc) patients by single-assay RT-qPCR. The expression is presented as fold change (FC) as compared to the mean expression of the HC group. ****p < .0001.

Fig. 3. MiR-483-5p is embedded in circulating serum exosomes. The expression of miR-483-5p was assessed in exosomes compared to serum-depleted exosomes (A) and in the exosomes of SSc patients compared to healthy controls (selected from the validation cohort) after exosome precipitation by Exoquick solution (B). The levels of miR-483-5p were assessed in the serum of patients and healthy controls after the treatment of serum with RNAse (C). The expression of miR-483-5p was examined in the serum of HC and SSc patients in 2 different time points of the same donors over a period of 15 months (D). The expression of miR-483-5p is presented as fold change (FC). *p < .05, **p < .01, ****p < .0001.

Please cite this article in press as: E. Chouri, et al., Serum microRNA screening and functional studies reveal miR-483-5p as a potential driver of fibrosis in systemic sclerosis, Journal of Autoimmunity (2018), https://doi.org/10.1016/j.jaut.2017.12.015

ARTICLE IN PRESS

4. Discussion

The aetiopathology of systemic sclerosis is poorly understood and the molecular mechanisms that underlie the excessive ECM deposition have not been truly identified yet. Representing a new class of regulatory molecules, miRNAs have recently been investigated as potential key factors in the pathogenesis of SSc [7,14]. Moreover, the fact that circulating miRNAs can reflect the pathogenic states in autoimmunity suggests these molecules as promising disease markers and potential therapeutic targets. In the present study we examined the miRNA signature in the serum of SSc patients. To our knowledge this is the first study investigating a comprehensive miRNA profiling in the serum of SSc patients, including subjects with or without clinically overt skin fibrosis.

Our profiling analysis highlighted that the serum of SSc patients is markedly different than healthy controls in terms of miRNA expression, with 30 miRNAs altered in one or more SSc clinical subtypes. Among them, some miRNAs previously reported in other studies were included (Supplementary Table 4). Consistent with this literature, circulating miR-30b, miR-142-3p and miR-150 were differentially expressed in SSc patients [22–24]. However, miR-142-3p and miR-150 showed the opposite trend of expression in SSc patients in our study [23,24]. Furthermore, in our discovery cohort miR-92a was not differentially expressed and let-7a did not reach statistical significance, despite being identified as dysregulated in SSc previously [25,26]. One possible explanation for the altered expression pattern of the aforementioned miRNAs may lie in ethnic differences, as demonstrated in previous studies [27]. Another plausible reason is the lack of appropriate validation in additional groups of patients or the variability in the clinical features of the participants [28], especially when analysing patients with a high degree of heterogeneity as in SSc.

In our study, the elevated expression of circulating miR-483-5p and snRNA-U6 was reproducible in an independent cohort of SSc patients in all the disease subsets, confirming that these two molecules are robustly dysregulated in SSc patients regardless of the genetic background and the disease heterogeneity. In addition, miR-483-5p demonstrated stable expression levels over time in multiple samplings of patients and healthy controls, suggesting the validated miR-483-5p as a potential disease marker. Preliminary analysis based on the Receiver Operating Characteristic (ROC) Analysis indicated a high predictive ability of miR-483-5p as SSc

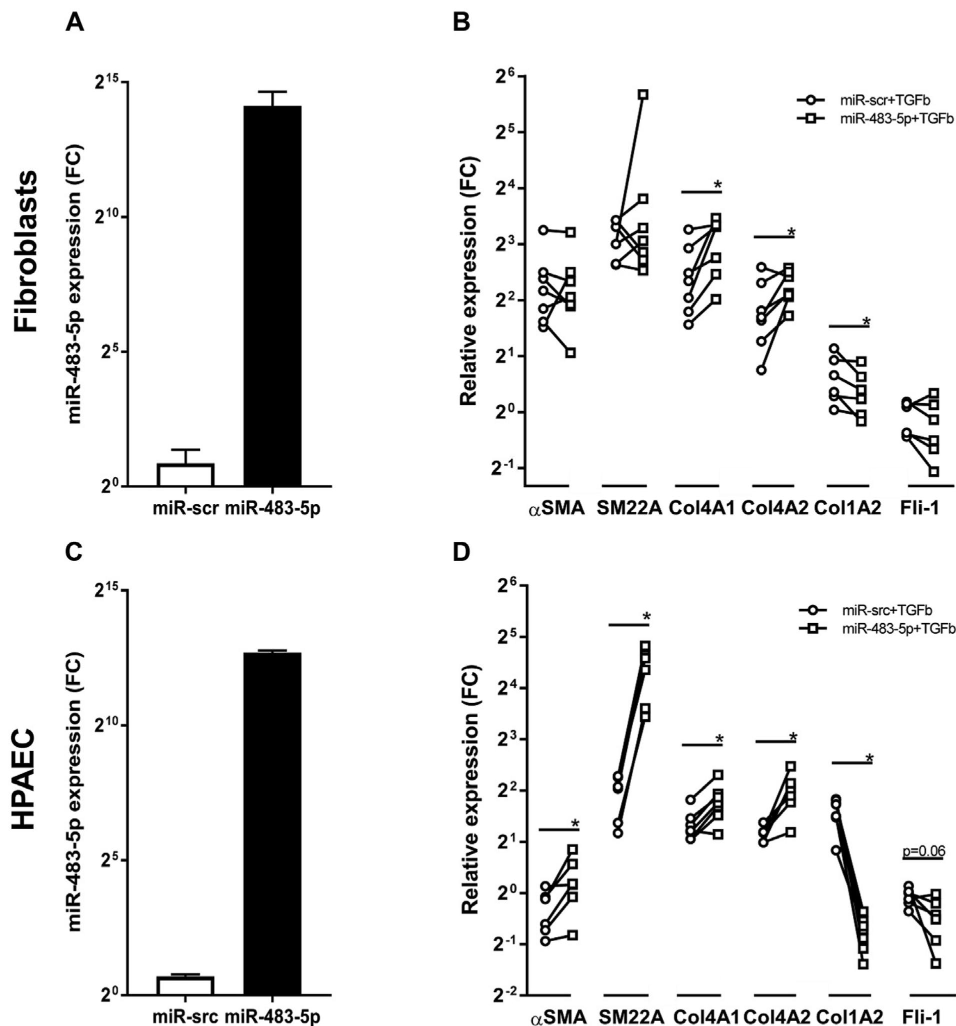

Fig. 4. miR-483-5p modulates the expression of fibrotic-related genes in human dermal fibroblasts and endothelial cells. Human dermal fibroblasts or pulmonary artery endothelial cells (HPAEC) were transfected with control non-targeting miRNA (miR-Scr) or miR-483-5p mimic. (A, C) miR-483-5p transfection efficiency was measured by quantitative RT-qPCR (N = 3). (B, D) The impact of miR-483-5p overexpression on the mRNA expression of fibrotic genes and myofibroblast-related markers was assessed by RT-qPCR after 24 h of stimulation with TGFb1 (fibroblasts) or TGFb2 (HPAEC). Results are presented as the fold change (FC) compared to the control miR-src transfections of 6–7 independent experiments on distinct fibroblast donors or HPAEC. *p < 0,5.

Please cite this article in press as: E. Chouri, et al., Serum microRNA screening and functional studies reveal miR-483-5p as a potential driver of fibrosis in systemic sclerosis, Journal of Autoimmunity (2018), https://doi.org/10.1016/j.jaut.2017.12.015

biomarker, with area under the curve (AUC) of 0.868 and more than 60% of SSc patients exceeding a potential optimal cut-off of normal levels (Supplementary Fig. 5). However, the high variability of miR-483-5p among individuals or the methodological standardization issues in using miRNAs for clinical diagnosis, might limit the utility of miR-483-5p as a biomarker. Adequate studies should be conducted to verify the potential of miR-483-5p as a (early) marker for SSc, as well as its prognostic value, also in combination with other clinical parameters.

Noteworthy, miR-483-5p was specifically up-regulated both in localized scleroderma and systemic sclerosis, but not in other systemic autoimmune diseases like systemic lupus erythematosus and primary Sjögren's syndrome, indicating that this miRNA could be implicated in the fibrosis characterizing scleroderma patients. Additionally, as previously reported in patients with adenocarcinoma [29], our study demonstrated that miR-483-5p is circulating in SSc serum enclosed in exosomes. Interestingly, miR-483-5p is encoded in an intron of the imprinted insulin-like growth factor (IGF2) gene [30], which is increased in SSc-lung fibroblasts [31]. IGF2 is associated with SSc-related pulmonary fibrosis and triggers an increase in ECM production in SSc fibroblasts as compared to normal fibroblasts [31]. Since miR-483-5p is co-expressed together with its host gene IGF2 [30], is embedded in exosomes and is predicted to regulate fibrosis-related signalling, we hypothesize that it may be involved in a feed-forward loop that promotes ECM deposition and hence the development of fibrosis in SSc.

Supporting our hypothesis, the overexpression of miR-483-5p in fibroblasts and endothelial cells regulated the expression of fibrosis-related genes. Notably, high levels of miR-483-5p induced collagen IV (Col4A1 and Col4A2) production in fibroblasts and endothelial cells. Col4, which is a primary collagen in the basement membranes around blood vessels and in the dermoepidermal junction in the skin, is reported to be dysregulated in SSc. More specifically, Col4 is up-regulated in the serum of SSc patients and positively correlates with mRSS [32] and it is also higher expressed in endothelial cells obtained from SSc-lungs compared to control lungs [33]. Together with our data, this suggests that miR-483-5p could play a pro-fibrotic role in SSc by inducing Col4.

Confirming the role of miR-483-5p as a potential fibrotic driver, miR-483-5p overexpression in endothelial cells enhanced the transcriptional levels of αSMA and SM22A, two known myofibroblast differentiation markers, indicating that the cells undergo myofibroblast transition. In addition, miR-483-5p lowered the expression of Fli-1, a negative regulator of ECM that is known to be suppressed in SSc skin [34]. In contrast, high levels of miR-483-5p inhibited collagen 1 (Col1) production. This suggests a more complex molecular mechanism underlying the role of miR-483-5p in SSc, which may neglect the machinery maintaining collagen homeostasis. In view of these findings, we propose that miR-483-5p may act as a regulator of fibrogenesis, which enhances the levels of Col4 and promotes myofibroblast differentiation, whereas it inhibits Col1. Due to its dual activity, one can speculate that altered levels of miR-483-5p result in an imbalance of the fibrotic processes in fibroblasts and endothelial cells and possibly affect the physiological equilibrium in ECM deposition, thus favoring the transition to the disease state.

Previous literature demonstrates that miRNA-enriched exosomes are involved in crosstalk between cells and might act as signaling mediators in physiological and pathological processes [9,35]. As an example, cardiac fibroblast-derived exosomal miRNAs can be transported to cardiomyocytes, leading to cellular hypertrophy [34]. Thus based on these evidences, we propose that the altered levels of circulating exosomal miR-483-5p may be taken up by activated fibroblasts and endothelial cells, already in the early disease stages. Our results showed that the uptake of the miRNA in these cells may affect the physiological equilibrium in ECM deposition and contribute to the establishment of the fibrotic phase of the disease. These observations suggest the need for additional investigations focused on the possible transfer of miR-483-5p from circulating vesicles to affected cells and its impact in the evolution of the disease.

5. Conclusions

Comprehensive profiling demonstrated that circulating miRNAs are significantly altered in the serum of SSc patients compared to healthy individuals. Of note, miR-483-5p was up-regulated in the serum of SSc patients, either presenting with or without evident skin fibrosis. This study proposes that miR-483-5p serves as a new molecular marker for early stages of the disease that is implicated in the molecular circuitry leading to fibrosis in SSc.

Conflicts of interest disclosure

The authors reported no conflict of interests for the work described here.

Funding

This work was supported by Reumafonds (Project 13-2-304). MR was funded by IEF Marie Curie fellowship (622811, MicroSCAP). TR was funded by ERC Proof of Concept grant(678426).

Acknowledgments

We would like to thank Emmerik F. Leijten, Aike A. Kruize and Evelien Ton from the Department of Rheumatology & Clinical Immunology in University Medical Centre Utrecht for patient inclusion. We are grateful to all the patients and healthy donors who have participated in this study.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jaut.2017.12.015.

References

[1] T.R. Katsumoto, M.L. Whitfield, M.K. Connolly, The pathogenesis of systemic sclerosis.,, Annu. Rev. Pathol. 6 (2011) 509–537, https://doi.org/10.1146/annurev-pathol-011110-130312.
[2] A. Gabrielli, E.V. Avvedimento, T. Krieg, Scleroderma, N. Engl. J. Med. 360 (2009) 1989–2003, https://doi.org/10.1056/NEJMra0806188.
[3] D.J. Abraham, J. Varga, Scleroderma: from cell and molecular mechanisms to disease models, Trends Immunol. 26 (2005) 587–595, https://doi.org/10.1016/j.it.2005.09.004.
[4] J. Varga, D. Abraham, Systemic sclerosis: a prototypic multisystem fibrotic disorder, J. Clin. Invest. 117 (2007) 557–567, https://doi.org/10.1172/JCI31139.
[5] C. Chizzolini, N.C. Brembilla, E. Montanari, M.E. Truchetet, Fibrosis and immune dysregulation in systemic sclerosis, Autoimmun. Rev. 10 (2011) 276–281, https://doi.org/10.1016/j.autrev.2010.09.016.
[6] R.T. Kendall, C.A. Feghali-Bostwick, Fibroblasts in fibrosis: novel roles and mediators, Front. Pharmacol. 5 (2014), https://doi.org/10.3389/fphar.2014.00123.
[7] J.C.A. Broen, T.R.D.J. Radstake, M. Rossato, The role of genetics and epigenetics in the pathogenesis of systemic sclerosis, Nat. Rev. Rheumatol. 10 (2014) 671–681, https://doi.org/10.1038/nrrheum.2014.128.
[8] N. Altorok, B. Kahaleh, Epigenetics and systemic sclerosis, Semin. Immunopathol. 37 (2015) 453–462, https://doi.org/10.1007/s00281-015-0504-6.
[9] X. Chen, H. Liang, J. Zhang, K. Zen, C.Y. Zhang, Secreted microRNAs: a new form of intercellular communication, Trends Cell Biol. 22 (2012) 125–132, https://doi.org/10.1016/j.tcb.2011.12.001.
[10] H. Valadi, K. Ekström, A. Bossios, M. Sjöstrand, J.J. Lee, J.O. Lötvall, Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells, Nat. Cell Biol. 9 (2007) 654–659, https://doi.org/

ARTICLE IN PRESS

10.1038/ncb1596.
[11] I. Alevizos, G.G. Illei, MicroRNAs as biomarkers in rheumatic diseases, Nat. Rev. Rheumatol. 6 (2010) 391–398, https://doi.org/10.1038/nrrheum.2010.81.
[12] A. Gallo, M. Tandon, I. Alevizos, G.G. Illei, The majority of microRNAs detectable in serum and saliva is concentrated in exosomes, PLos One 7 (2012), e30679, https://doi.org/10.1371/journal.pone.0030679.
[13] A.J. Affandi, T.R.D.J. Radstake, W. Marut, Update on biomarkers in systemic sclerosis: tools for diagnosis and treatment, Semin. Immunopathol. 37 (2015) 475–487, https://doi.org/10.1007/s00281-015-0506-4.
[14] H. Zhu, H. Luo, X. Zuo, MicroRNAs: their involvement in fibrosis pathogenesis and use as diagnostic biomarkers in scleroderma, Exp. Mol. Med. 45 (2013) e41, https://doi.org/10.1038/emm.2013.71.
[15] F. van den Hoogen, D. Khanna, J. Fransen, S.R. Johnson, M. Baron, A. Tyndall, M. Matucci-Cerinic, R.P. Naden, T.A. Medsger, P.E. Carreira, G. Riemekasten, P.J. Clements, C.P. Denton, O. Distler, Y. Allanore, D.E. Furst, A. Gabrielli, M.D. Mayes, J.M. van Laar, J.R. Seibold, L. Czirjak, V.D. Steen, M. Inanc, O. Kowal-Bielecka, U. Müller-Ladner, G. Valentini, D.J. Veale, M.C. Vonk, U.A. Walker, L. Chung, D.H. Collier, M.E. Csuka, B.J. Fessler, S. Guiducci, A. Herrick, V.M. Hsu, S. Jimenez, B. Kahaleh, P.A. Merkel, S. Sierakowski, R.M. Silver, R.W. Simms, J. Varga, J.E. Pope, 2013 Classification criteria for systemic sclerosis: an american college of rheumatology/european league against rheumatism collaborative initiative, Arthritis Rheum. 65 (2013) 2737–2747, https://doi.org/10.1002/art.38098.
[16] E. Carwile LeRoy, C. Black, R. Fleischmajer, S. Jablonska, T. Krieg, T.A. Medsger, F. Wollheim, Scleroderma (systemic sclerosis): classification, subsets and pathogenesis, J. Rheumatol. 15 (1988) 202–205.
[17] E.C. LeRoy, J. Medsger, Criteria for the classification of early systemic sclerosis, J. Rheumatol. 28 (2001) 1573–1576.
[18] N.S.L. Goh, S.R. Desai, S. Veeraraghavan, D.M. Hansell, S.J. Copley, T.M. Maher, T.J. Corte, C.R. Sander, J. Ratoff, A. Devaraj, G. Bozovic, C.P. Denton, C.M. Black, R.M. du Bois, A.U. Wells, Interstitial lung disease in systemic sclerosis: a simple staging system, Am. J. Respir. Crit. Care Med. 177 (2008) 1248–1254, https://doi.org/10.1164/rccm.200706-877OC.
[19] B. Vigone, A. Santaniello, M. Marchini, G. Montanelli, M. Caronni, A. Severino, L. Beretta, Role of class II human leucocyte antigens in the progression from early to definite systemic sclerosis, Rheumatol. (Oxford) 54 (2014) 707–711, https://doi.org/10.1093/rheumatology/keu381.
[20] F. Li, N. Ma, R. Zhao, G. Wu, Y. Zhang, Y. Qiao, D. Han, Y. Xu, Y. Xiang, B. Yan, J. Jin, G. Lv, L. Wang, C. Xu, X. Gao, S. Luo, Overexpression of miR-483-5p/3p cooperate to inhibit mouse liver fibrosis by suppressing the TGF-β stimulated HSCs in transgenic mice, J. Cell Mol. Med. 18 (2014) 966–974, https://doi.org/10.1111/jcmm.12293.
[21] M. Rossato, A.J. Affandi, S. Thordardottir, C.G.K. Wichers, M. Cossu, J.C.A. Broen, F.M. Moret, L. Bossini-Castillo, E. Chouri, L. van Bon, F. Wolters, W. Marut, M. van der Kroef, S. Silva-Cardoso, C.P.J. Bekker, H. Dolstra, J.M. van Laar, J. Martin, J.A.G. van Roon, K.A. Reedquist, L. Beretta, T.R.D.J. Radstake, Association of MicroRNA-618 expression with altered frequency and activation of plasmacytoid dendritic cells in patients with systemic sclerosis, Arthritis Rheumatol. 69 (2017) 1891–1902, https://doi.org/10.1002/art.40163.
[22] S. Tanaka, A. Suto, K. Ikeda, Y. Sanayama, D. Nakagomi, T. Iwamoto, K. Suzuki, N. Kambe, H. Matsue, R. Matsumura, D. Kashiwakuma, I. Iwamoto, H. Nakajima, Alteration of circulating miRNAs in SSc: MiR-30b regulates the expression of PDGF receptor β, Rheumatol. (Oxford) 52 (2013) 1963–1972, https://doi.org/10.1093/rheumatology/ket254.
[23] K. Makino, M. Jinnin, I. Kajihara, N. Honda, K. Sakai, S. Masuguchi, S. Fukushima, Y. Inoue, H. Ihn, Circulating miR-142-3p levels in patients with systemic sclerosis, Clin. Exp. Dermatol. 37 (2012) 34–39, https://doi.org/10.1111/j.1365-2230.2011.04158.x.
[24] N. Honda, M. Jinnin, T. Kira-Etoh, K. Makino, I. Kajihara, T. Makino, S. Fukushima, Y. Inoue, Y. Okamoto, M. Hasegawa, M. Fujimoto, H. Ihn, MiR-150 down-regulation contributes to the constitutive type i collagen overexpression in scleroderma dermal fibroblasts via the induction of integrin β3, Am. J. Pathol. 182 (2013) 206–216, https://doi.org/10.1016/j.ajpath.2012.09.023.
[25] T. Sing, M. Jinnin, K. Yamane, N. Honda, K. Makino, I. Kajihara, T. Makino, K. Sakai, S. Masuguchi, S. Fukushima, H. Ihn, MicroRNA-92a expression in the sera and dermal fibroblasts increases in patients with scleroderma, Rheumatol. (Oxford) 51 (2012) 1550–1556, https://doi.org/10.1093/rheumatology/kes120.
[26] K. Makino, M. Jinnin, A. Hirano, K. Yamane, M. Eto, T. Kusano, N. Honda, I. Kajihara, T. Makino, K. Sakai, S. Masuguchi, S. Fukushima, H. Ihn, The downregulation of microRNA let-7a contributes to the excessive expression of type I collagen in systemic and localized scleroderma, J. Immunol. 190 (2013) 3905–3915, https://doi.org/10.4049/jimmunol.1200822.
[27] R.S. Huang, E.R. Gamazon, D. Ziliak, Y. Wen, H.K. Im, W. Zhang, C. Wing, S. Duan, W.K. Bleibel, N.J. Cox, M.E. Dolan, Population differences in microRNA expression and biological implications, RNA Biol. 8 (2011) 692–701, https://doi.org/10.4161/rna.8.4.16029.
[28] B.V.J. Cuppen, M. Rossato, R.D.E. Fritsch-Stork, A.N. Concepcion, Y. Schenk, J.W.J. Bijlsma, T.R.D.J. Radstake, F.P.J.G. Lafeber, On behalf of all S. all SRU investigators, Can baseline serum microRNAs predict response to TNF-alpha inhibitors in rheumatoid arthritis? Arthritis Res. Ther. 18 (2016) 189, https://doi.org/10.1186/s13075-016-1085-z.
[29] U. Warnecke-Eberz, S.H. Chon, A.H. Hölscher, U. Drebber, E. Bollschweiler, Exosomal onco-miRs from serum of patients with adenocarcinoma of the esophagus: comparison of miRNA profiles of exosomes and matching tumor, Tumor Biol. 36 (2015) 4643–4653, https://doi.org/10.1007/s13277-015-3112-0.
[30] N. Ma, X. Wang, Y. Qiao, F. Li, Y. Hui, C. Zou, J. Jin, G. Lv, Y. Peng, L. Wang, H. Huang, L. Zhou, X. Zheng, X. Gao, Coexpression of an intronic microRNA and its host gene reveals a potential role for miR-483-5p as an IGF2 partner, Mol. Cell. Endocrinol. 333 (2011) 96–101, https://doi.org/10.1016/j.mce.2010.11.027.
[31] E. Hsu, C.A. Feghali-Bostwick, Insulin-like growth factor-II is increased in systemic sclerosis-associated pulmonary fibrosis and contributes to the fibrotic process via Jun N-terminal kinase- and phosphatidylinositol-3 kinase-dependent pathways, Am. J. Pathol. 172 (2008) 1580–1590, https://doi.org/10.2353/ajpath.2008.071021.
[32] S. Motegi, A. Sekiguchi, C. Fujiwara, S. Toki, O. Ishikawa, Possible association of elevated serum collagen type IV level with skin sclerosis in systemic sclerosis, J. Dermatol. 44 (2017) 167–172, https://doi.org/10.1111/1346-8138.13564.
[33] F.A. Mendoza, S. Piera-Velazquez, J.L. Farber, C. Feghali-Bostwick, S.A. Jiménez, Endothelial cells expressing endothelial and mesenchymal cell gene products in lung tissue from patients with systemic sclerosis-associated interstitial lung disease, Arthritis Rheumatol. 68 (2016) 210–217, https://doi.org/10.1002/art.39421.
[34] M. Kubo, J. Czuwara-Ladykowska, O. Moussa, M. Markiewicz, E. Smith, R.M. Silver, S. Jablonska, M. Blaszczyk, D.K. Watson, M. Trojanowska, Persistent down-regulation of Fli1, a suppressor of collagen transcription, in fibrotic scleroderma skin, Am. J. Pathol. 163 (2003) 571–581, https://doi.org/10.1016/S0002-9440(10)63685-1.
[35] C. Bang, S. Batkai, S. Dangwal, S.K. Gupta, A. Foinquinos, A. Holzmann, A. Just, J. Remke, K. Zimmer, A. Zeug, E. Ponimaskin, A. Schmiedl, X. Yin, M. Mayr, R. Halder, A. Fischer, S. Engelhardt, Y. Wei, A. Schober, J. Fiedler, T. Thum, Cardiac fibroblast-derived microRNA passenger strand-enriched exosomes mediate cardiomyocyte hypertrophy, J. Clin. Invest. 124 (2014) 2136–2146, https://doi.org/10.1172/JCI70577.

Please cite this article in press as: E. Chouri, et al., Serum microRNA screening and functional studies reveal miR-483-5p as a potential driver of fibrosis in systemic sclerosis, Journal of Autoimmunity (2018), https://doi.org/10.1016/j.jaut.2017.12.015