Objectives: The aim of this study is to characterise the crosstalk between AP1- and hedgehog signalling in fibrotic tissue disease in SSc.

Methods: Co-localization of cJUN and GLI2 in fibrotic skin was analysed by confocal microscopy and interaction was shown by Co-IP. cJUN/AP1 signalling and GLI2 signalling were inhibited *in vitro* and *in vivo* using the pharmacological inhibitors T5224 and GANT61. Hedgehog signalling was activated in mice by fibroblast-specific overexpression of constitutively active Smoothened (Smo^{ACT} mice).

Results: Expression profiling of all AP1 family members revealed most pronounced differences for cJUN in Smo^{ACT} mice. The expression of cJUN colocalized with the hedgehog transcription factor GLI2. Overexpression and colocalization of cJUN and GLI2 were also observed in fibroblasts in the skin of SSc patients. The number of GLI2- and cJUN double-positive fibroblasts was strongly increased in involved SSc skin compared to healthy controls and was particularly high in samples of SSc patients with diffuse and progressive disease. Based on the upregulation of both cJUN and GLI2 and their colocalization, we hypothesised that cJUN and GLI2 might interact with each other to amplify fibroblast activation and tissue fibrosis. Stimulation of resting fibroblasts with TGFβ induces both cJUN and GLI2 mRNA and protein in a time-dependent manner. cJUN and GLI2 are also induced upon stimulation with Sonic hedgehog (SHH), demonstrating that TGFβ and SHH are both capable to activate cJUN and GLI2-dependent transcription. This crosstalk occurs by direct interaction of cJUN and GLI2, which amplifies the transactivation potential of both transcription factors. Co-immunoprecipitation demonstrated that stimulation of fibroblasts with TGFβ or SHH induces direct interaction of cJUN and GLI2. Overexpression of cJUN and GLI2 resulted in activation in both AP1 and Hedgehog target genes. The central role of the crosstalk between cJUN and GLI2 for tissue fibrosis was further highlighted by the finding that hedgehog-induced fibrosis was strongly reduced by AP1 inhibition. Smo^{ACT} mice developed extensive skin fibrosis. Treatment with a cJUN/AP1 inhibitor T5224, however, strongly ameliorated hedgehog-induced fibrosis in Smo^{ACT} mice.

Conclusions: We demonstrate in the present study that the concomitant activation of AP1- and hedgehog signalling amplifies signalling through both cascades to promote excessive fibroblast activation and tissue fibrosis. This finding may open venues for combined inhibition of AP1- and hedgehog signalling for the treatment of fibrosis.

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FRI0401

THE TYROSINE PHOSPHATASE SHP2 CONTROLS TGFβ-INDUCED STAT3 SIGNALLING TO REGULATE FIBROBLAST ACTIVATION AND FIBROSIS

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Background: SHP2, encoded by the PTPN11 gene, is a ubiquitously expressed non-receptor tyrosine phosphatase (PTP). Altered expression and activity levels of SHP2 have also been implicated into the pathogenesis of autoimmune diseases.

Objectives: In our study, we aim to characterise the role of SHP2 as a possible novel molecular checkpoint of transforming growth factor-β (TGFβ)-induced JAK2/STAT3 signalling in fibrotic diseases.

Methods: Mice carrying *Shp2^{fl/y} × Col1a2-CreER* were generated to specifically knockout *Shp2* in fibroblasts. SHP2 was inhibited using NSC-87877 inhibitor. The role of Shp2 was investigated in three different mouse models: Bleomycin- and TBRI^{CA} induced dermal fibrosis as well as TSK1 genetic model of skin fibrosis. The construct pCMV3-Flag-JAK2 was used for overexpression and for *in vitro* mutagenesis of human JAK2. Co-immunoprecipitation was performed to confirm the interaction between SHP2 and JAK2. STAT3 activation was analysed by reporter assay. Protein and mRNA expression levels were analysed by Western Blot and qPCR.

Results: Our data characterise SHP2 as an important regulator of TGFβ signalling in fibroblasts. Although SHP2 expression is decreased in systemic sclerosis (SSc), the moderate downregulation of SHP2 expression is not sufficient to counterbalance the hyper-activation of TGFβ signalling in SSc. However, *Shp2*-deficient murine fibroblasts are less responsive to TGFβ. An impaired response to the profibrotic effects of TGFβ was also observed in human SSc fibroblasts with

siRNA-mediated knockdown of *SHP2* and upon pharmacological inhibition of SHP2. We show that SHP2 can dephosphorylate JAK2 at the inhibitory Y570 site to promote TGFβ-dependent activation of JAK2 and its downstream mediator STAT3. In contrast, pharmacologic inhibition of SHP2, overexpression of a catalytically inactive SHP2 mutant or knockout of *SHP2* inhibits dephosphorylation of pJAK2^{Y570} preventing phosphorylation of JAK2 at the activation site Y1007/Y1008 and subsequent activation of STAT3 in TGFβ-stimulated fibroblasts and in experimental fibrosis. Fibroblasts overexpressing SHP2 are more susceptible to the profibrotic effects of TGFβ, whereas the stimulatory effects of TGFβ on myofibroblast differentiation and collagen release are reduced in *Shp2* knockout fibroblasts. In addition, fibroblast-specific inactivation of *Shp2* *in vivo* protected from experimental fibrosis induced by overexpression of a constitutively active TGFβ receptor type I, by bleomycin injection and ameliorated fibrosis in TSK1 mice.

Conclusions: The present study describes for the first time a role of SHP2 in the pathogenesis of SSc. SHP2 is required for the activation of JAK2 and STAT3 by TGFβ. Inactivation of SHP2 prevents fibroblast activation and tissue fibrosis, providing evidence that SHP2 may be a potential target for the treatment of fibrosis.

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FRI0402

MICRORNA-125B REGULATES APOPTOSIS IN SYSTEMIC SCLEROSIS

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Background: The role of apoptosis in systemic sclerosis (SSc) is an emerging topic. MicroRNAs (miRs) are a class of small noncoding RNAs that regulate many important biological processes including apoptosis.

Objectives: To analyse the differential expression, regulation and pathophysiological role of miR-125b in systemic sclerosis.

Methods: MiR-125b expression was assessed by qPCR on RNA derived from cultured fibroblasts, whole skin biopsies and paraffin embedded dermis and epidermis from healthy controls (HC) and SSc patients. Fibroblasts were stimulated with pro-inflammatory and/or pro-fibrotic cytokines and with different epigenetic stimuli. Knockdown with anti-miR-125b (or scrambled controls) in HC fibroblasts was performed to identify downstream effects. RNA from healthy fibroblasts (n=4) after knockdown was proceeded to RNAseq using Illumina HiSeq2000. Bioinformatics analysis included differential gene expression and pathway analysis. Sequencing data were validated using qPCR (on HC and SSc fibroblasts). Apoptosis was assessed by Caspase-Glo 3/7 assay, Western Blot of cleaved caspase 3 and Annexin V live assay on cultured fibroblasts.

Results: MiR-125b was downregulated in SSc skin biopsies (n=10, median 47%, Q₁,35%, 69%; p<0.01) in comparison with HC skin (n=10). To localise its expression in the skin, we separately analysed miR-125b expression in dermis and epidermis of paraffin embedded skin where it was downregulated in both compartments (n=3 each). The expression of miR-125b in primary dermal fibroblasts was also downregulated (median 53%, Q₁,36%, 58%; n=10, p<0.01) compared to HC fibroblasts (n=10).

MiR-125b expression was not affected by major cytokines operative in SSc such as TGFβ or PDGF. However, TSA (histone deacetylase pan-inhibitor) downregulated miR-125b expression in a time- and dose-dependent manner (n=5, median 23%, Q₁,19%, 48%; p<0.01).

RNAseq identified >3500 differentially expressed genes with p<0.05. More than half of the differentially expressed genes with at least 15% change were predicted targets of miR-125b by TargetScan and MiRWalk, indicating successful functional

inhibition of miR-125b. Gene ontology revealed apoptosis regulation as the main activated pathway. Apoptotic genes included BAK1, BMF and BBC3, which are part of the BCL2 apoptosis pathway and predicted targets of miR-125b. Consistent with the sequencing results, qPCR confirmed that miR-125b knockdown upregulated these genes 24, 48 and 72 hours after transfection ($n=12$, $p<0.01$ for each). BAK1 showed the strongest induction, that was also confirmed on the protein level by Western blot. Accordingly, miR-125b knockdown resulted in an increased apoptosis (at least 1.5-fold, $n=10$, $p<0.01$) compared to scrambled controls, measured by Caspase-Glo 3/7 assay 24, 48 or 72 hours post-transfection. Consistently, miR-125b overexpression decreased apoptosis (by at least 50%, $n=10$, $p<0.01$) at these time points. Cleaved Caspase 3 was upregulated in anti-miR-125b transfected cells (median 2.3 fold, $Q_{1,3}$ 1.6, 4; $n=10$, $p<0.01$) confirmed by Western Blot. Annexin V live assay showed prevailing of apoptosis after miR-125b downregulation.

Conclusions: MiR-125b is downregulated in SSc skin and primary SSc dermal fibroblasts. MiR-125b downregulation increases apoptosis in dermal fibroblasts that might be a compensatory strategy against excessive fibrosis that could be used for therapeutic purposes.

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FRI0403 MITOCHONDRIAL DYSFUNCTION AND OXIDATIVE STRESS IN MYOSITIS: A CENTRAL PATHOGENIC PATHWAY FROM MOUSE TO MAN

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Background: Myositides are severe diseases leading to a bedridden state and possibly death. The lack of animal model with spontaneously-occurring autoimmune myositis has hampered pathophysiological and therapeutic research. Auto-immune-prone NOD mice represent an invaluable model of type 1 diabetes (T1D). Inducible T cell co-stimulator (ICOS) is involved in germinal centre reaction and induction of helper T cell responses. We developed a unique model of myositis by invalidating the ICOS pathway in NOD mice¹. Muscle holoproteome analysis in diseased mice together with observations of mitochondrial dysfunction in patients with dermatomyositis² suggest a main role of oxidative stress in disease pathogenesis.

Objectives: To determine the pathogenic role of oxidative stress and evaluate the effect of antioxidant therapy on *Icos*^{-/-} NOD myositis.

Methods: Disease course was studied in *Icos*^{-/-} NOD mice by grip strength, locomotor analysis and MRI. Muscle pathology was evaluated after conventional stainings, or by immuno-enzymology or immuno-histochemistry. Muscle-infiltrating cells were characterised by flow cytometry. New autoantibodies were identified in mice by serum proteomic analysis and sought for in myositis patients by ALBIA. Mouse muscle proteomic and transcriptomic analyses were performed by LC-MS/MS (Orbitrap) and RT-PCR, respectively. Muscle free radical production was assessed by EPR. Mice were treated by prednisolone (10 mg/kg/day) or N-acetylcysteine (2 g/L) in drinking water.

Results: *Icos*^{-/-} NOD mice did not develop diabetes. Instead, myositis spontaneously occurred with decreased grip strength, impaired cadence, reduced print area and death around 40 wks. Pathological muscle analysis revealed necrotic myofibers and important inflammatory infiltrates (CD4⁺ T cells, macrophages). Muscle lesions yielded MRI T2 hypersignals that regressed under steroids. CD4⁺ T cells transferred disease to NOD.scid recipients. Serum proteomic analysis revealed a new autoantibody directed against a mitochondrial antigen. It was found present in ~1% individuals from a ~700 myositis patient cohort. Oxidative stress was manifest in *Icos*^{-/-} NOD muscle by augmented free radical production, H₂O₂ production-related atrophy, altered O₂ consumption and dysregulation of several mitochondrial genes and proteins. N-acetylcysteine partially prevented myositis or ameliorated established disease.

Conclusions: This work establishes *Icos*^{-/-} NOD mice as a unique paradigm of myositis. A new autoantibody was discovered. Oxidative stress is present in diseased muscle. Antioxidant therapy is effective in a preventive or curative fashion. Together with our previous data in dermatomyositis patients², this work indicates the central role of mitochondrial dysfunction and oxidative stress in myositis pathogenesis and opens new perspectives for therapy.

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FRI0404 CHARACTERISATION OF A MONOCYTES/MACROPHAGES CELL SUBSET CO-EXPRESSING BOTH M1 AND M2 PHENOTYPE MARKERS IN SYSTEMIC SCLEROSIS PATIENTS

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Background: In the pathogenesis of systemic sclerosis (SSc), the immune cell activation is an important event that includes alteration in the macrophage polarisation.¹

Macrophages may polarise into classically activated (M1), which are characterised by the expression of specific markers such as Toll-like receptors (TLR2 and 4) and costimulatory molecules (CD80 and CD86), or alternatively activated (M2), which are characterised by the expression of specific phenotype markers such as mannose receptor-1 (CD206) and macrophage scavenger receptors (CD204 and CD163). M2 are present in the circulation and in the skin infiltrate of SSc patients (pts), where they seem to contribute to fibrosis.²⁻⁴

Objectives: To characterise circulating M2 monocytes/macrophages from SSc pts and healthy subjects (HSs) by their co-expression of CD204, CD163 and CD206, as well as cells expressing both M1 and M2 phenotype markers.

Methods: Fifty-eight SSc pts (54 females/4 males, mean age 63±13 years), fulfilling the new EULAR/ACR criteria for SSc, and 27 age-matched HSs were consecutively enrolled after Informed Consent was obtained. Peripheral blood was collected and the antibodies CD14-APC-Vio770 and CD45-VioGreen were used to identify the monocyte/macrophage lineage; CD204-VioBright-FITC, CD163-PE-Vio770 and CD206-PeerCP-Vio700 were used to characterise the M2 phenotype, whereas CD80-APC, CD86-VioBlue, TLR4-PE and TLR2-PE-Vio615 were used to characterise the M1 phenotype (Miltenij Biotech). Flow Cytometry analysis was performed using Navios Flow Cytometer and the related Navios analysis software (Beckman Coulter).

Results: In the CD14⁺cell subset (monocytes), the CD14⁺CD163⁺CD206⁺CD204⁺cell percentage was significantly increased in SSc pts compared to HSs ($p=0.02$). Inside the CD14⁺CD163⁺CD206⁺CD204⁺monocytes/macrophages a subset of cells co-expressing also TLR4, CD80 and CD86 was detected. This mixed population (M2/M1) of cells was significantly increased in SSc pts compared to HSs ($p=0.003$).

At the same time, circulating monocytes/macrophages showing a full M2 phenotype and characterised as CD204⁺CD163⁺CD206⁺cells were investigated independently of the expression of CD14, and they also resulted significantly increased in SSc pts compared to HSs ($p<0.0001$).

In the CD204⁺CD163⁺CD206⁺cell subset (M2), the percentage of cells expressing also TLR4, CD80 and CD86 (M1) was significantly increased in SSc pts compared to HSs ($p<0.0001$).

Conclusions: These results describe for the first time a subset of circulating cells belonging to the monocyte/macrophage lineage with a mixed phenotype, which are characterised by the expression of both M1 and M2 surface markers. These