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Review

A broad look into the future of systemic sclerosis

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***Abstract*:** Systemic sclerosis (SSc) is a systemic autoimmune disease with the key features of inflammation, vasculopathy and fibrosis. This article focussed on emerging fields based on the authors' current work and expertise. The authors provide a hierarchical structure into the studies of the pathogenesis of SSc starting with the contribution of environmental factors. Regulatory autoantibodies (abs) are discussed, which are parts of the human physiology and are specifically dysregulated in SSc. Abs against the angiotensin II receptor subtype 1 (AT1R) and the endothelin receptor type A (ETAR) are discussed in more detail. Extracellular vesicles are another novel player to possess disease processes. Fibroblasts are a key effector cell in SSc. Therefore, the current review will provide an overview about their plasticity in the phenotype and function. Promising nuclear receptors as key regulators of transcriptional programmes will be introduced as well as epigenetic modifications, which are pivotal to maintain the profibrotic fibroblast phenotype independent of external stimuli. Fibroblasts from SSc patients exhibit a specific signalling and reactivate developmental pathways and stem cell maintenance such as by employing hedgehog and WNT, which promote fibroblast-to-myofibroblast transition and extracellular matrix generation. Pharmacological interventions, although for other indications, are already in clinical use to address pathologic signalling.

Keywords: autoantibodies, fibrosis, signalling, systemic sclerosis

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Introduction

Systemic sclerosis (SSc) belongs to the group of systemic autoimmune diseases of the connective tissue. This disease remains one of the most severe and complex diseases in the field of rheumatology with a high disease-related mortality. Interstitial lung disease (ILD), pulmonary arterial hypertension (PAH), cardiac involvement, gastrointestinal (GI) involvement and, rarely, renal failure represent the main causes for mortality. In addition, SSc severely impairs quality of life.

SSc is characterized by three main clinical features, namely, vasculopathy, inflammation and fibrosis. An early manifestation of vasculopathy is increased vascular contraction, evident in Raynaud's phenomenon. Ischaemia and reperfusion injury are associated with the development of obliterative vasculopathy causing digital ulcers, loss of acral tissue or PAH. The second typical feature is inflammation, which often results in fibrosis as third clinical feature and present at various degrees. Fibrosis often starts at the skin of the fingers and involves the proximal skin to varying degrees. To date, the pathogenesis of SSc has not been fully elucidated. In this manuscript, as broad look into the future, we describe the scientific rationale for a novel SSc concept that has the potential to identify and develop new therapeutic approaches. This concept determines the structure of the review and is illustrated in Figure 1.

Environmental factors as important contributors for SSc

Since the description of toxic oil syndrome resembling some of the clinical features of SSc, there is increasing evidence of the role of environment and exposure to toxic agents in SSc. Several environmental factors (summarized as exposome in Figure 1) have been shown to promote or trigger the development of SSc such as exposure to epoxy

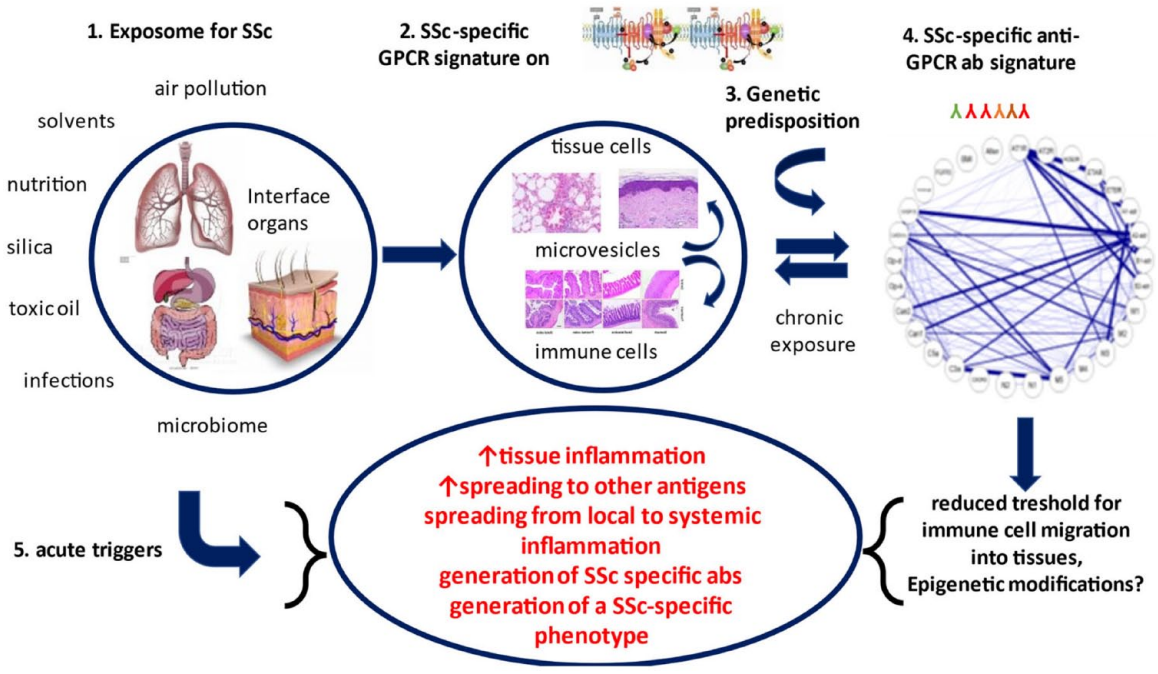

Figure 1. Proposed concept for the development of SSc. Briefly, environmental factors determine the GPCR signature and, under the influence of genetic background and if chronically present, also the anti-GPCR signature. The anti-GPCR signature affects the function of abs from regulatory to disease-driving effector molecules. Specifically, the abs determine the threshold for and direction of immune cell migration, the place and severity of inflammation, and ab-mediated signalling. In severe inflammation, this will lead to loss of tolerance towards other autoantigens, epitope spreading and the development of SSc.

resins, asbestos, silica, silicone or particulate air pollution.[1] Epidemiological studies of SSc identified strong associations with occupational factors such as exposure to silica and solvents with odds ratios (ORs) > 2 and weaker associations with epoxy resins and welding fumes.[2] Interestingly, sex is an influencing factor to be considered in the increase of risk for SSc. For example, exposure to silica increased the risk of SSc by an OR>3 in males, while it was lower in females. Pesticides increased the risk of SSc in females by an OR>3 in contrast to males, who did not show an increased risk.[2]

Another interesting area that alters the immune response and thus influences the pathophysiology of SSc is the microbiome. As indicated recently, faecal transplantation changing the gut microbiome can improve at least some GI symptoms.[3] Further studies are currently underway to determine additional effects.

Deciphering the mechanisms of disease development requires linking these environmental factors to individual genetic risk and clinic *via* multi-OMIC approaches. Corresponding investigations have been initiated in the field of autoimmunity and helped to identify individual risk factors and to develop strategies for precision medicine.[4] Ideally, multi-OMIC approaches could be used to identify disease prevention strategies.

Interplay between environmental factors and expression of G protein-coupled receptors

G protein-coupled receptors (GPCRs) are activated by extracellular substances or signals and transmit them into the cell interior, leading to cellular responses such as cell growth, changes in gene transcription, post-translational modifications and cell–cell interactions. These processes enable adaptations of the body to changing environmental conditions.[5,6] GPCRs are highly conserved in vertebrates and have similar functions in different species. They are expressed in immune cells and also in selected tissue-resident cells, depending on the GPCR.[5] For example, the angiotensin II receptor subtype 1 (AT1R) and endothelin receptor type A (ETAR) are expressed in fibroblasts and endothelial cells of the lung and also in the skin, heart and kidneys of healthy

donors.[7,8] They are also expressed in monocytes, neutrophils, B-cells and T-cells.[8–10] Traffic-related air pollution with exposure to diesel increases the expression of ETAR, and experimental bacterial infections increase that of AT1R in the lung.[11,12] These data provide a causal relationship between predisposing conditions for SSc and AT1R/ETAR expression. In SSc, upregulated AT1R and ETAR expression has been shown in the lung, in the skin as well as in the immune cells[13–15] and was particularly identified in early disease.[5,9] Therefore, it can be assumed that environmental conditions affecting the lungs or skin could result in increased AT1R as well as ETAR expression (see Figure 1).

Autoantibodies against GPCRs are important players in SSc

Natural IgG autoantibodies (abs) against GPCRs can be detected in all individuals and represent an emerging field for understanding of chronic diseases, including SSc. Anti-GPCR abs are thought to play an important role in immune cell homeostasis.[16] Most likely, they reflect the expression of their corresponding GPCR, which rely on environmental conditions.[5,6,16] As the foetal environment is different from those of infants and adults, autoimmunity to GPCR could escape from tolerance. Changes in the GPCR expression, in their conformation and complex formation with other receptors (dimerization) or proteins could result in tolerance break most likely by epitope spreading.[6] In line with this, epitopes of a specific anti-GPCR ab differ in SSc and healthy donors.[17] In addition, disease-specific ab cross-reactivities and receptor activations have been identified, which were different from the signalling induced by activation of just one receptor. It is likely that disease-specific heterodimerizations of different GPCRs or proteins occur, and current studies support this hypothesis. In the future, we expect more evidence for the hypothesis that phenotypic variabilities in the GPCR abs and cross-reactivities to other receptors result in different ab function and contribute to the development and to the phenotype of SSc.

Among anti-GPCR abs, those abs targeting the AT1R as well as the ETAR could be centrally involved in the pathogenesis of SSc.[18,19] They have the potential to modulate the function of AT1R and ETAR in resident tissue cells such as in endothelial cells, fibroblasts as well as in innate and adaptive immune cells.

Antibodies directed to AT1R and ETAR induce SSc pathways

Although present in physiological levels in all individuals,[16] patients with SSc often show increased levels of anti-AT1R and anti-ETAR abs.[8,18] However, low levels of anti-ETAR abs have been described particularly in acute vascular diseases such as in giant cell vasculitis. This may reflect increased ab binding to endothelial cells and consequently, a reduction in the circulatory ab levels.[20] Our recent data indicate that long-term use of endothelin receptor blockers is accompanied by low anti-AT1R ab levels.

In addition to quantitative differences of anti-GPCR abs to healthy controls, anti-AT1R abs or anti-ETAR abs show disease-specific correlations with other abs and form a disease-specific functional network. As shown before by using receptor blockers, these abs exhibit other functions than those from healthy donors.[16]

As shown *in vitro* and *ex vivo*, anti-AT1R and anti-ETAR abs derived from SSc patients are cross-reactive and stimulate both receptors.[7] In endothelial cells, anti-AT1R abs stimulate the expression of adhesion molecules, cytokines and chemokines.[10,18] In fibroblasts, they also induce collagen expression.[10] Our current data indicate that they contribute to lung and skin inflammation, skin fibrosis and obliterative vasculopathy. As suggested by our *in vitro* studies, the abs could exhibit independent, synergistic and different effects on the cells as the natural ligands.[5,10,21] In addition, they act agonistic and as allosteric ligands. However, further studies on purified anti-AT1R and ETAR abs are necessary to verify the role of quantitative and qualitative differences of these abs, which is still a challenge for the future.

The antibody network summarized as 'antibodiom' is a possible contributor to SSc

In addition to anti-AT1R and anti-ETAR abs, further antibodies are detected in patients with SSc, which form a specific network.[16] So far, the biological function of other abs, which target, for example, fibroblast growth factors, the chemokine receptors CXCR3 and 4, Platelet-derived growth factor (PDGF) receptors or adrenergic receptors, is unknown or at least only partially understood. Artificial intelligence such as machine learning linking the different abs to clinical data will provide hypotheses about the potential role of individual abs in the development of SSc. This will

require validation in different patient cohorts and mechanistic studies. By analysing multiple abs in different SSc symptoms, we expect several novel biomarkers for SSc as well as pathways, which will hopefully lead to the development of novel therapies.

The presence of physiological levels of anti-GPCR abs and their high plasticity in the interactions to other GPCRs as well as to intracellular proteins could provide a rationale for the development of an autoimmune response to intracellular antigens and for disease-specific autoimmune responses.[6] Anti-GPCR abs could be a weak link for the development of classical abs to intracellular antigens. This hypothesis needs to be proven in the future. Furthermore, linking specific GPCR signatures to specific ab signatures and to environmental factors could help to discover mechanisms of how environmental factors are translated into the development of SSc and its phenotype.

However, the regulatory effects of anti-GPCR abs and of immunoglobulins in general are a novel fascinating field. Injection of IgG from SSc patients into mice induced signs of vasculopathy and inflammation, not present by application of IgG from healthy donors.[7] Transfer of peripheral blood mononuclear cells from SSc patients induced antinuclear antibodies, anti-AT1R abs, severe inflammation in the lung, in the muscles as well as in the kidneys, which was diminished upon B-cell depletion.[22] These data indicate a causal role of B-cells and of abs as their main effector molecules at least for some of the SSc features such as for ILD. As reflected by the use of intravenous immunoglobulins (IVIGs) in various autoimmune diseases, the sum of all individual antibodies, the 'antibodiom', exhibits regulatory effects. Accordingly, IVIGs from healthy donors induced several cytokines and regulatory proteins in monocytic THP1 cell lines.[23] In contrast, IgG from SSc patients transferred individual and disease-specific pathways into monocytes and induced an inflammatory and profibrotic cytokine milieu in the supernatants.[23] Machine learning was able to link this ab-induced proteome to disease symptoms and pathways present in the corresponding donors. This suggests that the induced proteins reflect disease processes and could thus provide a window to identify pathways.[23] For those analyses, monocytes and monocyte-derived cells are very interesting immune cells. They show a broad functional and morphologic variability, which is also accompanied by a high variability in the expression of GPCR and other proteins. Their stimulation could help to identify novel biomarkers and proteins involved in individual disease processes. In a recent study, purified IgG from diffuse SSc patients was shown to modify the phenotype of healthy donor-derived dermal fibroblasts and induced profibrotic properties.[24] The ab-induced transcriptome and protein profile was particularly more pronounced in patients positive for topoisomerase I antibodies. These data show that SSc patient-derived abs could induce a fibrotic fibroblast phenotype. Whether abs also induce epigenomic changes remains to be studied.

Recently, novel technologies such as single-cell RNA sequencing have emerged allowing to globally study effects of antibodies. By this method, a specific signallome induced by a single pathogenic factor can be measured in various rare and frequent cells, which could help to understand diseases and their underlining mechanisms. This method can also allow to study the possible contribution of a specific antibody in SSc pathogenesis. This hypothesis is currently investigated.

Extracellular vesicles: another interesting player in SSc pathogenesis

Extracellular vesicles (EVs) are further potentially important players in disease-associated processes and in the regulation of the intercellular communication. EVs are phospholipid bilayer particles that comprise exosomes with a size of 40–150 nm and micro-vesicles with a size of >150 nm.[25] Micro-vesicles are generated by shedding of the plasma membrane, while exosomes are released into the extracellular space after fusion of multivesicular bodies (MVBs) with the plasma membrane. Therefore, exosomes have an intraluminal origin and contain membrane embedded proteins such as GPCRs, cytoplasmic proteins, receptors, cytokines, chemokines, major histocompatibility complexes, enzymes, chaperones, lipids like cholesterol, ceramides, different nucleic acids (mRNA, miRNA, dsDNA), tetraspanins and, importantly, disease-related autogenic peptides.[26,27]

The levels of micro-vesicles are increased in SSc and show associations with inflammation and fibrosis.[28] They are also capable of inducing apoptosis in circulating angiogenic cells and may thus contribute to impaired vasculogenesis in SSc.[29] The function of exosomes varies depending on

the cell of origin, which determines their size, membrane structure and activation status.

Initially thought to be waste products of cells, it is now widely accepted that EVs play an important role in intercellular and paracrine communication and thus contribute to immune regulation. EVs can be internalized by other cells or bind to other cells *via* their surface receptors.[30] For example, lung endothelial cells take up EVs from vascular smooth muscle cells of SSc, which promotes endothelial cell migration and angiogenesis.[30] In SSc, exosomes contain micro-RNAs. These EVs induce a profibrotic phenotype in cultured fibroblasts.[31] Moreover, EVs from human endothelial cells derived from PAH patients administered proliferation of human smooth muscle cells and thus can contribute to the development of PAH.[32,33] These data indicate a paracrine way to spread pathogenic factors from tissues, for example, to endothelial cells and from these to the blood stream. The lung as barrier organ with a low number of tissue cells seem to be ideal for the spreading of information. Taken together, EVs can induce paracrine and systemic disease mechanisms and profibrotic properties. They also can affect the cellular composition in tissues. In SSc, the interaction of EVs with abs is a novel emerging field to investigate.

Mechanisms of fibrosis and the role of fibroblasts

Fibroblasts are a key effector cell in SSc and as discussed before, various factors such as abs and exosomes can alter their phenotype.[24,31] To determine the contribution of a particular factor to the alteration of the fibroblast phenotype, it is crucial to characterize the fibroblast population of patients. There is increasing evidence that fibroblasts are a heterogeneous cell population with distinct, sometimes opposing functions. These functional differences are particularly overt in disease context. In fibrotic diseases such as SSc, the fibroblast pool is dominated by fibroblast subpopulations that synthesize large amounts of extracellular matrix (ECM). In contrast, in rheumatoid arthritis, a prototypical chronic inflammatory disease, fibroblast populations proliferate promoting matrix degradation and inflammation through the release of matrix degrading enzymes and proinflammatory mediators. The increasing availability of single-cell OMIC techniques enabled the identification of individual subpopulations based on transcriptional differences. In SSc, recent seminal work from the Lafyatis laboratory identified 10 subpopulations of fibroblasts in skin from SSc patients.[34] Characterizing functionally distinct subpopulations of fibroblasts may have therapeutic implications. An individualized targeting of pathogenic subpopulations may not only provide increased efficacy but also limit adverse events by sparing subpopulations required to maintain tissue homeostasis. However, the factors that contribute to the differentiation and persistence of these subpopulations often remain unclear. We are just beginning to understand these regulatory mechanisms.

The transcription factor PU.1, a member of the Erythroblast Transformation Specific (ETS) family of transcription factors encoded by the *SPI1* gene, serves as an essential orchestrator of profibrotic gene expression programmes in fibroblasts and may thus be required for differentiation into profibrotic fibroblast subsets.[35] The expression of PU.1 is upregulated in a subset of profibrotic fibroblasts in SSc and in other fibrotic diseases. PU.1 induces the expression of fibrosis-associated gene signatures and can convert proinflammatory and resting fibroblasts into profibrotic fibroblasts with increased expression of contractile proteins and ECM components such as type I collagen. Pharmacological or genetic inactivation of PU.1 blocks profibrotic transcriptional networks and enables re-programming of fibrotic fibroblasts into homeostatic fibroblasts with antifibrotic effects across different organs.[35]

Another transcription factor involved into the differentiation of resting fibroblasts into profibrotic fibroblast subsets is Engrailed 1 (EN1). EN1 is a member of the family of homeodomain-containing transcription factors. During the developing murine dermis, EN1 positive fibroblasts gradually replace EN1 negative fibroblasts in earlier stages of skin development, but the expression decreases before birth.[36] However, former EN1-positive cells can give rise to a subpopulation of fibroblasts that has a high capacity for ECM production and is required for skin scaring in adult mice.[36,37] Györfi and coworkers characterized EN1 as a molecular amplifier of TGF-β signalling in myofibroblast differentiation in the context of SSc. They demonstrated that EN1 is induced in some subsets of fibroblasts in a TGF-β/SMAD3-dependent manner and that EN1 in turn facilitates the transcription of a subset of profibrotic TGF-β target genes to promote cytoskeleton organization and

ROCK activation required for fibroblast-to-myofibroblast differentiation. Mechanistically, EN1 does not directly bind to the promoters of profibrotic target genes, but predominantly signals *in trans* by modulating the activity of the specificity protein (SP) family members. Knockdown of EN1 prevented cytoskeletal reorganization, inhibited fibroblast-to-myofibroblast transition and ameliorated experimental skin fibrosis in mouse models and human skin models.

Nuclear receptors – key regulators of critical transcriptional programmes

Nuclear receptors form a superfamily of transcriptional regulators with 48 members identified to date. Several members of the nuclear receptor family are associated with the pathogenesis of SSc and other fibrotic diseases.

PPARγ (NR1C3) was the first nuclear receptor implicated in fibroblast activation. The expression of PPARγ is downregulated in SSc skin and also in cultured SSc fibroblasts by TGF-β–SMAD-dependent pathways.[38] The downregulation of PPARγ, in turn, promotes TGF-β signalling as PPARγ competes with SMAD3 for the transcriptional coactivator histone acetyltransferase p300.[39,40] Activation of PPARγ, for example, with pharmaceutical agonists, inhibits TGF-β-induced fibroblast-to-myofibroblast differentiation and ameliorates experimental fibrosis.[39,41–43] However, the pan-PPAR agonist lanifibranor failed to demonstrate antifibrotic efficacy in a randomized, controlled, phase II trial in patients with dc SSc. Selective PPARγ agonists have been withdrawn from the market due to cardiovascular adverse events and are thus currently not available for clinical studies.

NR4A1 (also known as Nur77 or TR3) is also downregulated in SSc as well as in other fibrotic diseases.[44] Under physiologic conditions, NR4A1 inhibits the expression of profibrotic genes downstream of TGF-β by transrepression of SP1.[44] However, in SSc and other fibrotic diseases, persistently high levels of TGF-β represses the antifibrotic effects of NR4A1 by histone deacetylase-induced silencing of the gene encoding NR4A1 and phosphorylation of NR4A1, which promotes its degradation.[44,45] NR4A1 agonists exert antifibrotic effects in multiple rodent models of fibrosis including common mouse models of SSc.[44]

VDR (vitamin D receptor, NR1I1) has also been shown to exert antifibrotic effects in models of SSc and other fibrotic disorder. The expression of VDR is decreased in the skin of patients with SSc,[46] and vitamin D deficiency is common in SSc and other chronic diseases.[47–52] Activated VDR binds to phosphorylated SMAD3 to inhibit canonical TGF-β–SMAD-signalling.[46] Treatment with VDR agonists ameliorates fibrosis in murine models of SSc and other fibrotic diseases.[53–57]

Epigenetic modifications – tissue memory as a target for therapeutic intervention

Fibroblasts explanted from SSc skin display a profibrotic phenotype with increased expression of contractile proteins and ECM. As mentioned above, abs from SSc patients as well as EVs could continuously activate fibroblasts. However, this activated phenotype persists for several passages *in vitro*. The persistence of the profibrotic phenotype is caused by epigenetic modifications in SSc fibroblasts that establish a profibrotic tissue memory. Prolonged exposure of fibroblasts to a profibrotic environment induces a complex pattern of different epigenetic alterations. These epigenetic changes render SSc fibroblasts partially independent of external stimuli and maintain the profibrotic myofibroblast phenotype.[58,59] So far, the role of abs to induce epigenetic modifications remains to be studied. Epigenetic alterations including DNA methylation, histone acetylation, histone methylation and non-coding RNAs such as microRNAs (miRNAs) or long non-coding RNAs (lncRNAs) are increasingly recognized as drivers of progressive fibrotic tissue remodelling.[58,60–66] Selected examples and potential approaches for novel therapeutic strategies are discussed below.

DNA methylation

DNA can be methylated at position C5 of the pyrimidine ring of cytosine residues by three DNA methyltransferases (DNMTs): DNMT1, DNMT3A and DNMT3B.[67] The interaction of methyl-CpG-binding domain (MBD) proteins with these methylated cytosine residues promotes the recruitment of repressor complexes to silence transcription.[68] Several studies demonstrated a role of altered DNA methylation in fibrotic diseases including SSc.[58,69–72] The best studied gene regulated by DNA methylation in SSc is Friend leukaemia integration factor 1 (FLI1), a transcription factor of the ETS family.[58,73,74] FLI1

exerts antifibrotic effects and limits TGF-β-induced fibroblast activation under homeostatic conditions.[75] In fibrotic conditions with chronic activation of TGF-β signalling, however, FLI1 expression and activity are repressed by epigenetic and post-translational mechanisms. TGF-β induces DNMT-dependent methylation of the FLI1 promoter to silence its expression[62,76] as well as PKCδ-mediated phosphorylation of FLI1 to foster its degradation.[77] Moreover, DNMT-induced silencing of the suppressor of cytokine signalling 3 (SOCS3) facilitates prolonged activation of JAK2/STAT3 signalling and may thus sensitize SSc fibroblasts to TGF-β (Dees et al.[70]). DNA methylation also promotes activation of canonical WNT signalling by silencing of the endogenous WNT antagonists DKK1 and SFRP1. Treatment with the DNMT inhibitor 5-Aza-2′-deoxycytidine, which is in clinical use for myelodysplastic syndromes, has consistently been shown to exert antifibrotic effects in murine models of SSc and other fibrotic diseases.[62,70,78]

Histone modifications

Histone modifications include acetylation and methylation at various sites. First evidence for a role of histone modulations in the pathogenesis of SSc was provided by the observation that treatment with histone deacetylation inhibitors reduced the activation of SSc fibroblasts and ameliorated bleomycin-induced skin fibrosis,[79] providing evidence that histone acetylation regulates the expression of profibrotic genes in fibroblasts.

Histone acetylation also modulates the outcome of fibrotic diseases by fine-tuning autophagy. Autophagy describes the catabolic cellular process of degradation of unnecessary or dysfunctional cellular organelles in particular during starvation or in response to cellular stress.[80] However, components of the autophagy machinery are involved in unconventional secretion of proteins.[81–83] Aberrant activation of autophagy has been implicated into the pathogenesis of fibrotic diseases with cell type and context-dependent outcomes.[84–92] Zehender *et al.* demonstrated that autophagy is activated in a TGF-β-dependent manner in SSc fibroblasts. TGF-β represses the expression of the H4K16 histone acetyltransferase MYST1 *via* SMAD3 to promote the expression of core components of the autophagy machinery. The resulting activation of autophagy induces fibroblast activation and tissue fibrosis. Re-establishment of the epigenetic control of autophagy by forced expression of MYST1 in fibroblasts impairs myofibroblast differentiation and ameliorates experimental dermal and pulmonary fibrosis.

The expression of the profibrotic transcription factor PU.1 is controlled by a complex network of epigenetic effector mechanisms involving histone modifications.[35] In resting fibroblasts, PU.1 expression is silenced, and the promoter and the –17 kb upstream regulatory element of the PU.1 locus are dominated by the presence of repressive H3K9me3 and H3K27me3 marks. In fibrotic or inflammatory environments, the –17 kb upstream regulatory element of the PU.1 locus becomes active as shown by H3K27 acetylation and loss of H3K9me3 and H3K27me3. These epigenetic alterations at the PU.1 locus promote expression of PU.1 protein in fibrotic fibroblasts. However, they are not sufficient to induce PU.1 protein in inflammatory fibroblasts due to post-transcriptional block of PU.1 translation by miR-155.

Reactivation of developmental pathways in SSc–hedgehog and WNT signalling as targets for therapeutic intervention

Several lines of evidence from different groups in complementary model systems demonstrate that hedgehog signalling and WNT signalling are central pathways of fibroblast activation in SSc and other fibrotic diseases.[93–101] As both of these pathways are essentially required for embryonic development, they are often referred to as developmental pathways. In most cell types in adults except stem cells, these pathways are inactive under homeostatic conditions. However, they can be reactivated upon injury to promote proliferation and differentiation of target cells. The persistent activation of these developmental pathways in fibrotic diseases indicates a failure of appropriate termination of these pathways in SSc; indeed, at least for canonical WNT signalling, epigenetic alterations may interfere with termination of WNT signalling by repression of endogenous WNT antagonists.

The expression of the ligand sonic hedgehog (SHH) and of the downstream transcription factor GLI2 is upregulated in the skin of patients with SSc (Horn *et al.*, 2012).[102] Moreover, SHH concentrations are elevated in the blood of patients with SSc and correlate with the extent of fibrosis.[102] Hedgehog signalling is highly interlinked with TGF-β signalling. TGF-β induces the expression of SHH and of GLI2 in fibroblasts (Horn *et al.*,[97,98]). Activation of hedgehog

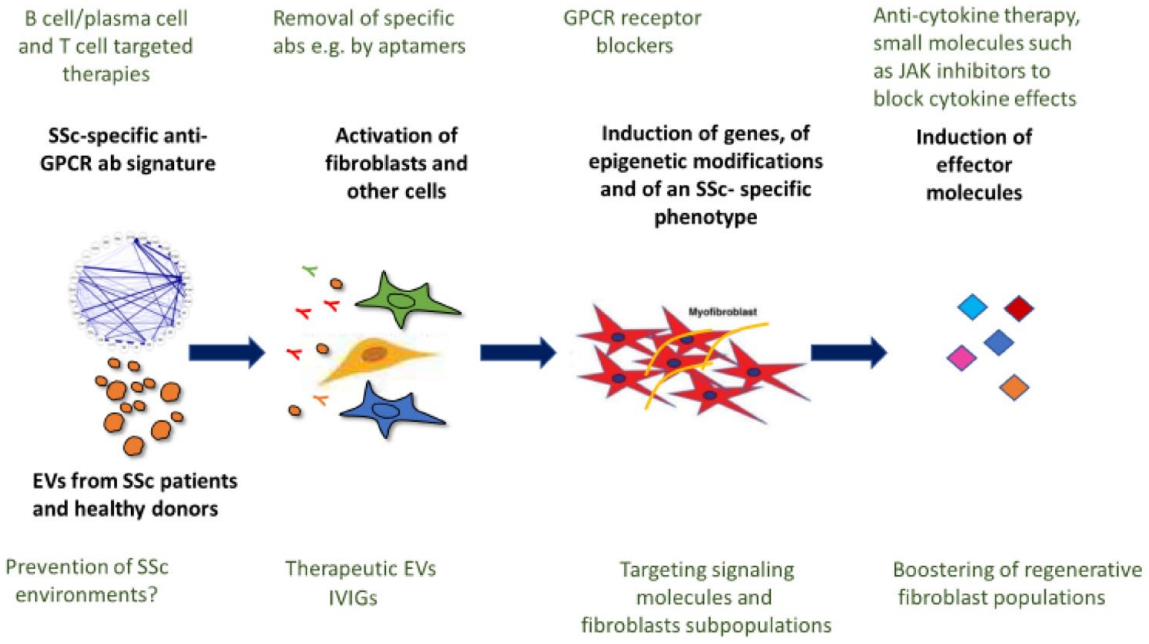

Figure 2. Concept of cell activation by abs and EVs and of therapeutic targets derived from this concept.

signalling stimulates fibroblast-to-myofibroblast transition and promotes experimental skin fibrosis (Horn *et al.*,[97,98]), whereas pharmacologic or genetic inactivation of hedgehog signalling ameliorates experimental fibrosis in murine models of SSc and other fibrotic diseases.[103–106]

β-catenin-dependent WNT signalling, also referred to as 'canonical' WNT signalling, is active in SSc as well as multiple fibrotic conditions. Activation of canonical WNT signalling occurs at different levels with upregulation of WNT proteins, downregulation of endogenous WNT inhibitors and by transcriptional synergism with other transcription factors and cofactors.[93,94,107–111] As for hedgehog signalling, multiple interactions of canonical WNT signalling with TGF-β have been unravelled in SSc. TGF-β can activate canonical WNT signalling in fibroblasts. This regulation occurs in particular at the level of endogenous WNT antagonists such as dickkopf 1 (DKK1) or secreted frizzled-related protein 1 (SFRP1).[112–114] Canonical WNT signalling is sufficient and required for fibrotic tissue remodelling, and targeted inhibition of WNT signalling exerts potent antifibrotic effects in various preclinical models of SSc and other fibrotic diseases.[35,70,93,101,107,111,114–120]

Despite their crucial role in embryonic development and stem cell maintenance, hedgehog and WNT signalling are both assessable for pharmacologic intervention. For hedgehog signalling, smoothened inhibitors are already in clinical use for neoplastic diseases, and GLI2 inhibitors are used in clinical development.[121] Compounds with WNT inhibitory activity such as pyrvinium are also in clinical use and more selective and potent WNT inhibitors such as porcupine or tankyrase inhibitors are in use in clinical development. However, fibrotic diseases such as SSc require long-term treatment; given the roles of WNT and hedgehog signalling in stem cell regeneration, specific strategies such as intermittent dosing or low-dose combination therapies will be required to minimize the effects on the stem cell compartment.

Therapeutic implications

At the present time, immunosuppressive drugs represent the key drug strategy for the treatment of inflammation and of inflammation-mediated fibrotic changes in SSc. However, immunosuppressive drugs often do not achieve sufficient therapeutic efficacy regarding fibrotic changes. Therefore, there is a high unmet medical need for the identification of new therapeutic strategies. The induction of profibrotic pathways by regulatory abs or EVs and the characterization of key mechanism of fibrosis offer several therapeutic opportunities as illustrated in Figure 2.

A causal treatment strategy to prevent formation of abs supports the use of B-cell- and plasma cell-targeting therapies. As a proof of concept, autologous stem cell transplantation reduces the levels of anti-AT1R and anti-ETAR abs.[21] This could be a rationale for the use of B-cell-targeting therapies, which show promising results in recent clinical studies particularly for the therapy of ILD and inflammatory skin fibrosis in SSc.[122] Whether the levels of ati-AT1R abs could predict the response to rituximab remained to be studied. Current studies are ongoing to support the use of B-cell- and plasma cell-targeting therapies. In addition, recent studies revealed an important role of T-cells in the generation of anti-GPCR abs (Yue et al.[123]). Therefore, particularly CD4+ T-cells might become an important target for the treatment of SSc. Recently, aptamers have been introduced to remove anti-GPCR abs with first successful applications. Their potential role in the treatment of SSc is currently studied in preclinical models.

A further therapeutic strategy is to block the receptor targeted by abs. An established approach in treatment of SSc is the use of endothelin receptor blockers and drugs affecting the renin-angiotensin system. These are particularly used for treatment of vascular complications such as PAH, digital ulcers and renal crisis.[124,125] Cross-reactivities of the abs may indicate insufficient blockade by only one receptor blocker and therefore, combination of receptor blockers or the generation of blockers targeting several receptors might be reasonable. The presence of stimulating abs also indicates the necessity to continuously block the receptors to achieve clinical effects. In the future, more mechanistic studies are required to distinguish ab effects from those of the natural ligands.

Specific blockade of ab-mediated signalling remains a future target. Proteins, also induced by abs, emerged as therapeutic targets. As example, anti-cytokine therapy targeting interleukin-6 has recently been approved by the Food and Drug Administration (FDA) and is now under consideration by the European authorities. Other cytokines such as IL-8, MCP-1, TGFß or CCL18 could also be interesting targets in SSc, which need to be explored in the future. In addition; IVIGs are successfully used in severe autoimmune diseases including in SSc patients.[126] As shown by our group,[23] IVIGs exhibit regulatory function by induction of several proteins, for example, in monocytes. They could compete with the IgGs from SSc patients.

In addition to the potential role of EVs to spread information and to induce disease pathways, EVs can also be applied therapeutically. Thus, EVs from mesenchymal stem cells exhibit anti-inflammatory and protective effects, for example, in vascular remodelling.[31] Remarkably, neutrophil-derived EVs demonstrate the ability to bind specifically to local sites of inflammation, release bioactive payloads and abrogate inflammation.[127] For future therapy strategies, EVs can serve as potential biological scaffolds able to deliver anti-inflammatory treatments target-specific when administered systemically as well as exhibit immune-regulatory effects that resemble the cells from which they originated.[128,129]

So far, data providing evidence that anti-GPCR abs reflect pathologic environmental factors are missing. However, anti-GPCR abs could be crucial in the identification of patients at risk for severe infections as currently shown in COVID-19 infection, a disease with some similarities to SSc.[130] Here, AT1R abs were among the best to discriminate severe from mild COVID-19 infection. The identification of vulnerable persons could help to avoid harm at an individual basis.[131]

Targeting processes in main effector cells such as in fibroblasts, epigenetic drugs and small molecule agonists/antagonists of nuclear receptors are addressed in clinical studies to translate preclinical findings on antifibrotic effects into the clinic. Targeting pathogenic fibroblast subpopulations and boostering regenerative subpopulations could provide another novel concept for precision medicine.

Taken together, in this article, we present both a holistic approach and a focused approach on a specific cell type, which are both complementary and indispensable to identify mechanisms of SSc and of novel future targets for therapies. However, this article is driven by the perspectives of the authors. Abs and EVs are an upcoming field, and we are still in the beginning of mechanistic studies to decipher the alphabet of GPCR ab functions. Novel technologies emerged to identify novel phenotypes and pathways. Table 1 summarized some prospects for the future.

Table 1. Example for research questions and prospects for the future.

Future tasks	Prospects for the future
Linking specific environmental factors to the anti-GPCR ab signature and the ab function	Prevention and modulation of SSc
Linking anti-GPCR abs with disease-specific antibodies	Prevention and modulation of SSc
Linking specific ab effects to the clinic of SSc patients	Risk assessment and precision medicine
Mechanistic studies for single and specific anti-GPCR abs on key cells	Identification of therapeutic targets. Drug discovery
Discover the pathogenic and therapeutic potential of EVs	Drug development
Compare the SSc-specific cell phenotype (e.g. of fibroblasts) with ab and EV-induced phenotype	Risk assessment, biomarker development
Identification of key mechanism in fibrosis	Drug discovery
Translation of preclinical findings on antifibrotic effects with epigenetic drugs and small molecule agonists/antagonists of nuclear receptors in clinical studies	Novel antifibrotic therapies
Identification and selective targeting of pathogenic fibroblast subpopulations and boostering of regenerative subpopulations	Precision medicine

ab, autoantibody; EV, extracellular vesicle; GPCR, G protein-coupled receptor; SSc, systemic sclerosis.

Declarations

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Author contributions
Gabriela Riemekasten: Conceptualization, Data curation, Formal analysis, Project administration, Resources, Validation, Writing – original draft.

Jörg H.W. Distler: Conceptualization, Data curation, Methodology, Resources, Software, Supervision, Writing – original draft, Writing – review & editing.

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