Rheumatoid arthritis progression mediated by activated synovial fibroblasts

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Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial hyperplasia and progressive joint destruction. Rheumatoid arthritis synovial fibroblasts (RASFs) are leading cells in joint erosion and contribute actively to inflammation. RASFs show an activated phenotype that is independent of the inflammatory environment and requires the combination of several factors. Although new aspects regarding RASF activation via matrix degradation products, epigenetic modifications, inflammatory factors, Toll-like receptor (TLR) activation and others have recently been uncovered, the primary pathophysiological processes in early arthritis leading to permanent activation are mostly unknown. Here, we review new findings regarding RASF activation and their altered behavior that contribute to matrix destruction and inflammation as well as their potential to spread RA.

Synovial fibroblasts (SFs) in RA
RA is a chronic inflammatory disease characterized by the progressive destruction of the joints. SFs in RA are one of the dominant cell types in the terminal layer of the hyperplastic rheumatoid synovium and at the sites of invasion into the adjacent cartilage and bone (Figure 1). In normal individuals, the synovial lining at the border to the joint cavity consists of 1–3 cell layers, predominantly containing RASFs and macrophages. In RA, the lining thickness increases to 10–15 cell layers [1-4]. In addition to the increased thickness of the synovial lining, the influx and proliferation of inflammatory cells as well as the increased proliferation and survival of resident cells contributes to synovial hyperplasia (Figure 1). The synovium shows an increased neoangiogenesis within the hyperplastic tissue, facilitating the influx of inflammatory cells [5]. Synovial hyperplasia also contributes to the attachment of the synovium to the adjacent cartilage and bone [1-4]. The hyperplastic tissue overgrows the underlying cartilage surface and invades into the cartilage and bone, leading to joint destruction. A central cell type of cartilage invasion is the SF, which actively contributes to matrix degradation. During subsequent joint destruction, RASFs actively contribute to inflammation, angiogenesis and matrix degradation by producing inflammatory cytokines, proangiogenic factors and matrix-degrading enzymes [1-4] (Box 1).

In healthy joints, SFs (i) provide the synovial fluid in the joint cavity and the adjacent cartilage with nutritive proteins and molecules necessary for the lubrication of the synovial fluid, (ii) facilitate matrix remodeling by producing matrix components and matrix-degrading enzymes and (iii) contribute to tissue repair and wound healing [6]. Although fibroblasts are not primarily immune cells, they develop certain immune properties during the course of RA [i.e. the expression of disease-specific HLA (human leukocyte antigen)-DR molecules and increased production of inflammatory cytokines]. RASFs show an activated destructive phenotype independent of the surrounding inflammatory environment in the synovium [2,3]. Proinflammatory factors produced by immune cells and RASFs further induce the secretion of matrix-degrading enzymes and inflammatory factors by RASF, contributing to joint erosion and enhancing the inflammatory cycle in RA [1-4]. Targeting the aggressive, joint destructive and proinflammatory properties of RASFs is, therefore, of special interest for the development of joint protective treatment strategies. Here, recent findings on RASF activation, function and therapeutic inhibition are discussed.

The activation of SFs in RA
Activated RASFs are detectable early after RA onset and erosions are visible at cartilage–bone junctions [1-4,6]. In these areas, progressing matrix destruction can contribute to fibroblast activation, which is likely to be perpetuated by (chronic) inflammation within the joints in early RA. However, knowledge about the mechanisms underlying the permanent activation of RASFs in RA is limited.

The activation of RASFs is at least in part independent of active inflammation within the synovium and the presence of inflammatory cells. This was shown in studies conducted in the severe combined immunodeficient (SCID) mouse model of RA [2] in which implanted isolated human RASFs invaded coimplanted human cartilage, leading to cartilage destruction in the absence of cellular and humoral immune responses [2]. Therefore, RASFs potentially contribute to the initiation and early perpetuation of RA.

Multiple factors contribute to fibroblast activation and enhance their destructive potential. Cartilage and bone

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invasion are early events in RA; therefore, the role of matrix proteins, matrix-associated factors as well as additional extra- and intracellular mechanisms in RASF activation have been the focus of many studies. Once activated, RASFs produce increased amounts of matrix-degrading enzymes and inflammatory cytokines. The latter could potentially contribute to the initiation of chronic inflammation in early RA. However, which factors lead to RASF activation in the early stages of RA and whether early RASF activation is an initial factor inducing matrix damage and/or inflammation and leading to visible joint erosion is still unknown.

RASF activation via matrix invasion and degradation

Direct RASF to matrix contact increases the activation and migration of RASFs. Adhesion molecules, including integrins [1–3] and cadherins such as cadherin-11 [7], are important for these interactions. Cartilage invasion and degradation, an early process visible in RA, liberates matrix components and matrix-associated factors. These fragments, including fibronectin and vitronectin, can activate RASFs by inducing inflammatory factors and matrix metalloproteinases (MMPs) [1–3]. RASFs stimulated with the carboxyl-terminal heparin-binding fibronectin fragment enhance the activation of MMPs, increasing the release of matrix-degrading factors and proinflammatory cytokines. These factors, in turn, recruit, retain, activate and promote the differentiation of multiple cell types that contribute to the inflammatory cycle in RA. RASFs secrete cytokines and chemokines, which increase the influx of immune cells into the hyperplastic synovium. ECs are activated and vessel formation is promoted through the secretion of proangiogenic factors such as VEGF.
Box 1. The role of matrix-degrading enzymes in RA

MMPs include collagenases, stromelysins, gelatinases and membrane-type (MT) MMPs. Collagenase-1 (MMP-1) is one of the main collagenases produced by RASFs alongside MMP-13 (for review see [1–4]). Gelatinases such as MMP-2 and MMP-9 and stromelysins such as MMP-3 and MMP-10 are also produced by RASFs [2]. MT-MMPs are also involved in RASF pathophysiology [82]. MT1-MMP (MMP-14) and MT3-MMP (MMP-16) cleave matrix components and activate other MMPs. Both MT-MMPs are expressed by RASFs; MT1-MMP and MT3-MMPs are more highly expressed than MT2-MMP (MMP-15) and MT4-MMP (MMP-17). The proteolytic activity at sites of synovial attachment to cartilage is mediated by a complex consisting of MT1-MMP, TIMP-2 and MMP-2, whereby TIMP-2 promotes the binding of pro-MMP-2 to MT1-MMP, by which it is subsequently activated [82]. Cathepsins, such as cathepsin K and L, also contribute to matrix degradation. Cathepsin L degrades central collagens and proteoglycans of cartilage matrix and is expressed by RASFs. The inhibition of cathepsin L alters cartilage invasion [83]. In addition to cartilage degradation, cathepsin K production by RASF also contributes to bone degradation [84].

cells expressing Robo3, decreases RASF migratory potential compared with untreated cells and at high passage numbers cultured RASFs, which downregulate Robo3, seem to be resistant to Slit3, suggesting that the upregulation of Robo3 potentially contributes to RASF activation [8].

RASF activation via growth factors

Growth factors produced by cells within the joint and the liberation of matrix-associated growth factors during matrix degradation are additional factors with a potential role in RASF activation. An important extracellular stimulus for fibroblasts is fibroblast growth factor (FGF). In addition to proliferating in response to FGF, RASFs release FGF [9]. Transforming growth factor (TGF)-β, produced for example by synovial macrophages, stimulates collagen production by RASFs as well as the release of interleukin (IL)-6, IL-1, MMP-1 and other growth factors including vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF) [2,10]. Interestingly, platelet-derived growth factor (PDGF) together with TGF-β also contributes to the induction of inflammatory cytokines in RASFs [11]. PDGF is important in angiogenesis and contributes to synovial hyperplasia by inducing the proliferation of inflammatory cells and RASFs [11,12]. The proliferative effect of PDGF on RASFs [2] can be impeded by the PDGF receptor tyrosine kinase inhibitor imatinib, showing a potential therapeutic strategy that targets inflammation and directly inhibits activated RASFs [12]. Interestingly, the increased expression of insulin-like growth factor 2 (IGF2) in RASFs associates with low-level inflammation in patients with RA, most likely owing to the abnormal biallelic expression of IGF2 in these individuals, which contributes to RASF proliferation [13].

RASF activation via microparticles

Microparticles, released as exogenous buds from various cell types, have been identified in RA joints [14,15]. Depending on the origin, microparticles transfer cell surface receptors between cells, they activate complement, activate complement and mediate proinflammatory effects but might also exert anti-inflammatory effects by inducing apoptosis or releasing anti-inflammatory factors. Microparticles contribute to indirect cell–cell interactions via their surface receptors, which originate from the parental cell. For instance, microparticles from synovial fluid can induce lymphocyte activating factors, especially B cell regulators [16]. In RA, microparticles derived from activated or apoptotic cells are most likely to perpetuate inflammation and the activation of inflammatory cells. Microparticles can be produced by virtually every cell type including lymphocytes and RASFs [15]. Microparticles from activated platelets have been detected in the synovial fluid of patients with RA but not osteoarthritis (OA) [17]. These particles amplify the inflammatory responses of RASFs especially via the collagen receptor glycoprotein VI.

RASF activation via proto-oncogenes and tumor-suppressor genes

Proto-oncogenes and tumor-suppressor genes including p33, p21 and e-Myc are important for RASF activation [2,3]. For example, phosphatase and tensin homolog (PTEN) expression is reduced in RASFs but mutations within the gene have not been described. The adenovirus-mediated gene transfer of PTEN ameliorated arthritis symptoms in a collagen-induced arthritis model in rats [18]. Furthermore, Akt phosphorylation was reduced as was VEGF production in RASFs after PTEN gene transfer. Therefore, the dysregulation of proto-oncogenes and tumor-suppressor genes resulting in a signaling imbalance within RASFs could be an important and early trigger of RASF activation.

RASF have epigenetic alterations

Although RASFs can be activated via numerous pathways, it is not clear which mechanisms maintain RASF activation. In particular, data obtained with the SCID mouse model stimulated the search for the factors responsible for the endogenous maintenance of synovial activation and the search for epigenetic modulations. Epigenetics includes all heritable and potentially reversible changes in the function of the genome that do not alter the nucleotide sequence of the DNA. Epigenetic modifications include biochemical changes (i.e. acetylation, ubiquination, methylation, phosphorylation) that mediate crucial developmental processes [19]. An extended research program has been developed to address epigenetic modifications in rheumatic diseases [20]. Interestingly, RASFs have a hyperacetylated genome, owing to the low activity levels of total histone deacetylase (HDAC) enzymes, possibly resulting from reduced levels of HDAC1 and HDAC2 [21]. Low-level HDAC activity might contribute to the activation of proinflammatory transcription factors.

Furthermore, DNA from RASFs is hypomethylated [22]; both in synovial tissues and in vitro, the DNA of RASFs had fewer 5-methylcytosine and methylated CG sites upstream of the L1 open reading frame than the DNA of SFs from patients with osteoarthritis. Moreover, proliferating RASFs have lower levels of DNA methyltransferase-1 (Dnmt1) than nonproliferating RASFs, and the demethylating agent 5-azaC reproduces the activated phenotype of RASFs in normal synovial cells [22]. These data support...
the hypothesis that DNA hypomethylation contributes to the chronicity of RA and could explain why a cure for RA remains elusive. Current therapies would not be expected to affect epigenetic programs.

RASFs are further characterized by the expression of distinct microRNAs (miRs), including miR-146a and -155 [23]. Because some of the miRNAs also seem to be induced by demethylation, there is a close connection between demethylation and miRNA expression. This observation highlights the complexity of the effects of epigenetic modifications in RASFs. Accordingly, future strategies for RA treatment should target the epigenetically activated phenotype of RASFs.

Interactions of RASF with the immune system
The chronic activation of the innate immune system is a central feature of RA, and immune system involvement in RASF activation and RA progression has been studied for many years. Various inflammatory factors can enhance the destructive properties of RASFs including tumor necrosis factor (TNF)α, IL-6 and IL-1β, which are also therapeutic targets in RA [24]. In turn, activated RASFs further enhance the inflammatory cycle by producing inflammatory factors themselves. Therefore, the interactions between immune cells and RASFs and the effects of inflammatory factors on the destructive functions of RASFs have been studied. However, once activated, the aggressive phenotype of RASFs is independent of inflammation [2]. It remains unclear whether inflammatory stimuli are necessary for the early RASF activation that produces the aggressive phenotype of these cells or whether the aggressive properties are merely increased by (chronic) inflammation.

RASF activation via TLR ligands
The activation of the innate immune system in RA begins, in part, before clinical signs are evident; subsequently, effector molecules in RASFs are upregulated. The potential triggers of early RASF activation include degradation products of infectious and noninfectious agents. These factors activate cells through highly conserved innate immune receptors such as TLRs [25]. In addition to infectious fragments, factors released during tissue destruction in arthritic joints are ligands for TLRs and include liberated cellular RNA and DNA fragments [25].

TLR2, TLR3 and TLR4 are expressed on RASFs (for review see [25,26]). TLR2 expression in RASFs is induced by proinflammatory cytokines such as IL-1β and TNFα as well as lipopolysaccharide (LPS) and lipopeptides. In turn, the effects of TLR2 can be enhanced by IL-1 and TNFα. TLR2 activation induces proinflammatory and matrix-destructive factors in RA, such as VEGF, IL-8, CXCL2 (Gro-2), CCL8 or monocyte chemoattractant protein (MCP)-2, intercellular adhesion molecule 1 (ICAM)-1, MMP-1, MMP-3 and MMP-13. TLR2 and TLR4 activation induces the synthesis of IL-15 in RASFs. The activation of TLR2, TLR3 and TLR4 on RASFs promotes osteoclastogenic activity by inducing receptor activator of nuclear factor (NF)-κB ligand (RANKL), thereby promoting bone destruction in RA. Together these data show a role of TLR activation in the inflammatory cycle of RA.

Interestingly, RNA released from necrotic cells in the synovial fluid owing to ongoing tissue destruction also activates RASFs by acting as an endogenous TLR3 ligand and inducing the release of inflammatory factors (e.g. interferon [IFN]-β, IL-6, visfatin and chemokines) [25,26]. This effect is independent of the presence of microbial TLR ligands. These data, together with the findings summarized in Table 1A, show the relevance of TLR activation in the RASF–dependent progression of RA. Interestingly, treatment with fluoxetine or citalopram, selective serotonin reuptake inhibitors (SSRIs) [27], inhibits the activation of TLR3 by polyriboinosinic: polyribocytidylic acid (poly IC), resulting in increased IL-6 in both human and murine models. Thus, SSRIs are potential therapeutics for early intervention in RA.

In summary, TLR activation by nonmicrobial or microbial factors might contribute to RASF activation and function. The byproducts of tissue breakdown during joint destruction that are present early after matrix damage could be central triggers in RA. By contrast, the detection of microbial particles, fragments or sequences in the synovial tissues of RA patients have only been confirmed in a limited number of patients and, therefore, might not be an initial cause of RASF activation in the majority of RA patients.

RASF activation via inflammatory factors
Systemic and local inflammation are central to RA and synovitis. Inflammatory cytokines (Box 2) induce the release of additional inflammatory factors by RASFs, thereby enhancing and potentially stabilizing the activated phenotype of RASFs [1–3], although the contribution of inflammation to initial RASF ‘priming’ has yet to be confirmed.

Inflammatory factors influence RASF activation and some strongly enhance the proinflammatory and matrix-destructive properties of RASFs [1–3]. For example, factors such as TNFα, IL-1 and IL-6 can induce further proinflammatory cytokines and chemokines in RASFs, amplifying inflammatory reactions and increasing the influx of additional proinflammatory cells into the synovium. These effects can be synergistic with factors such as IL-17. By contrast, IFN-β can abolish TNFα-mediated effects. Additional new findings are summarized in Table 1B. Of note, cell–cell and/or cell–matrix interactions contribute to the effects on RASFs observed for the respective factors. For example, RASFs expressing a 75 kDa form of osteopontin produce more IL-6 when cocultured with B lymphocytes than RASFs that do not express this protein [28]. Additional RASF activators include IL-18 and IL-20 [2]; proinflammatory mediators including chemokines and IL-6 can be induced by IL-20 in RASFs.

Chemokines are of special interest in RASF activation and function [1–3]. The stimulation of RASF leads to the expression of many chemokines (i.e. macrophage inflammatory protein [MIP]-1α), and often requires direct cell–cell interactions mediated by adhesion molecules such as β2-integrin and ICAM-1. A related chemokine MIP-3α is elevated in RASFs and is released after stimulation with IL-1β, IL-18 or TNFα. MIP-3α contributes to the perivascular chemoattraction of additional RASFs and mononuclear cells. Again, direct cell–cell contact promotes these
Complement factors are part of the innate immune system and are expressed by RASFs. However, anti-inflammatory complement regulators, including factor H-link protein-1 (FHL-1) and factor H, play an important role in the downregulation of complement cascades and are also produced by RASFs [2]. Several adipokines with proinflammatory potential, including adiponectin, resistin and visfatin, have been described in RA [30,31]. Adiponectin induces proinflammatory cytokines (IL-6 and VEGF) and matrix-degrading enzymes (i.e. MMPs) in RASFs as well as a variety of chemokines that attract cells, including lymphocytes and RASFs [30,32] (Table 1B). Leptin, another adipokine with immunomodulatory function, induces

**Table 1. Factors affecting RASFs.**

<table>
<thead>
<tr>
<th>(a) TLR activation</th>
<th>Source</th>
<th>Finding</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>TLR2, TLR3, and TLR4</td>
<td>Lipoproteins, glycolipids, dsRNA, LPS, heat-shock proteins (e.g. fibrinogen, heparin sulphate and hyaluronic acid fragments)</td>
<td>• Increased expression of the p19 subunit (but not p40) of IL-23 in RASFs. p19 induction might be proinflammatory in RASFs independent of heterodimerization with p40.</td>
<td>64</td>
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<tr>
<td>TLR2</td>
<td>Lipoproteins and glycolipids</td>
<td>• IL-6 release, involving the binding of c-Jun to the IL-6 promoter.</td>
<td>65</td>
</tr>
<tr>
<td>TLR3</td>
<td>dsRNA, LPS, heat-shock proteins</td>
<td>• Enhanced osteoclastogenesis via human monocytes cocultured with RASFs is mediated by increased RANKL and IL-1β expression by RASFs.</td>
<td>43</td>
</tr>
<tr>
<td>TLR3 and TLR4</td>
<td>dsRNA, LPS, heat-shock proteins</td>
<td>• TLR3 and TLR4 are highly expressed in RASFs from patients with early RA in comparison with patients with longstanding RA.</td>
<td>67</td>
</tr>
<tr>
<td>TLR3 and TLR4</td>
<td>poly IC, LPS and TNF</td>
<td>• NOD-2 is expressed by RASFs predominantly at sites of cartilage invasion and can be induced.</td>
<td>68</td>
</tr>
<tr>
<td>TLR2, TLR4 and TLR6</td>
<td>Lipoproteins, glycolipids; e.g. fibrinogen, heparin sulphate and hyaluronic acid fragments; multiple diacyl lipopeptides</td>
<td>• Synergistic effects of TLR activation on the induction of TNF and IL-1 in RASFs isolated from an IL-1Ra-deficient mouse model.</td>
<td>69</td>
</tr>
<tr>
<td>(b) Inflammatory factor</td>
<td>Source</td>
<td>Finding</td>
<td>Ref.</td>
</tr>
<tr>
<td>TNF</td>
<td>Inflammatory cells</td>
<td>• Induction of RIG-I in RASFs, a DExH box protein with immunomodulatory potential, which in turn might induce chemokines that recruit lymphocytes and monocytes into synovial tissue.</td>
<td>70</td>
</tr>
<tr>
<td>TNF, IL-17</td>
<td>Inflammatory cells; mainly Th17 cells</td>
<td>• IFNβ abolishes TNF-induced RIG-I expression in RASFs. After an initial priming step by IL-17, making RASFs more responsive to TNF, the synergistic increase of IL-23 p19 production is observed in RASFs.</td>
<td>71</td>
</tr>
<tr>
<td>IL-6</td>
<td>Inflammatory cells, fibroblasts</td>
<td>• Stimulation with IL-6 and soluble IL-6 receptor but not IL-6 alone induces VEGF in RASFs.</td>
<td>42</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>Osteoblasts, placenta, chondrocytes, macrophages, ECs</td>
<td>• RASFs expressing a 75 kDa form of osteopontin produce more IL-6 when cocultured with B lymphocytes.</td>
<td>28</td>
</tr>
<tr>
<td>Galectin 3</td>
<td>Inflammatory cells, chondrocytes, fibroblasts</td>
<td>• Induction of chemokines and cytokines in RASFs.</td>
<td>72</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Inflammatory cells, platelets</td>
<td>• When cultured on laminin, TGF-β1 induces the production of IL-16.</td>
<td>73</td>
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<tr>
<td>Oncostatin M</td>
<td>Inflammatory cells (including activated neutrophils, monocytes and T cells)</td>
<td>• Strong induction of CCL13 in RASFs.</td>
<td>74</td>
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<td>CCR7 ligands</td>
<td>CCL19 and CCL21</td>
<td>• RASFs migrate towards the CCR7 ligands.</td>
<td>75</td>
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<tr>
<td>Adiponectin</td>
<td>Synovial cells inducing RASFs and adipocytes</td>
<td>• Induction of IL-6.</td>
<td>76</td>
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<tr>
<td>(c) Cell-cell contact</td>
<td>Molecule(s)</td>
<td>Finding</td>
<td>Ref.</td>
</tr>
<tr>
<td>RASF-lymphocytes</td>
<td>VCAM-1</td>
<td>• Analysis of intracellular mechanisms of TNF-induced VCAM-1 upregulation confirmed the increase of VCAM-1 by the adhesion of lymphocytes to RASFs.</td>
<td>78</td>
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<td></td>
<td>LFA-1 and ICAM-2</td>
<td>• Coclure of lymphocytes with RASF contributes to the activation of both cell types and involves interactions between LFA-1 and ICAM-2.</td>
<td>79</td>
</tr>
<tr>
<td>RASF–T cells</td>
<td>Surface IL-15</td>
<td>• Coclure using T cell subsets and RASFs shows that RASF-derived factors including surface IL-15 on RASFs promote a shift in T cell differentiation towards a proinflammatory state.</td>
<td>80</td>
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Abbreviations: dsRNA; double-stranded RNA; IFN interferon; NOD-2 nucleotide-binding oligomerization domain 2; RIG-1 retinoic acid-inducible gene-1.
Interestingly, PGE2 synthesis by RASFs is also increased in tissue [2,34], actively contributing to the COX-1/COX-2 destructive properties of RASFs [36]. The inflammatory cascade affecting the proinflammatory and matrix-degrading enzymes in RASFs, contributing to the central features of RA: matrix degradation, inflammation and cellular activation. However, although inflammation and disease progression can be efficiently stopped or slowed by anti-TNF treatment in many patients, some patients do not respond [88]. Furthermore, joint erosion is progressive and affects additional joints over time. Thus, the destructive potential of RASFs is reduced under biologics treatment owing to the reduction of further activating cytokines, but permanent RASF activation is not completely suppressed. Similar to anti-TNF strategies, the inhibition of IL-6 effectively reduces inflammation and disease progression in many patients with RA [89]. Additional approaches to inhibit the proinflammatory cascade in RA are currently under investigation.

Box 2. Central cytokines of synovitis in RA

TNFα, IL-1β and IL-6 are key cytokines in the inflammatory cascade of RA and have been intensely studied for therapeutic interventions [for reviews see [1–4,87]]. Multiple cell types release these cytokines in the RA synovial tissue, including macrophages and SFs. In turn, proinflammatory cytokines enhance the transcription of matrix-degrading enzymes in chondrocytes and RASFs. The inhibition of IL-1, IL-6 and TNF is now part of RA treatment. TNFα induces a variety of proinflammatory cytokines and matrix-degrading enzymes in RASFs, contributing to the central features of RA: matrix degradation, inflammation and cellular activation. However, although inflammation and disease progression can be efficiently stopped or slowed by anti-TNF treatment in many patients, some patients do not respond [88]. Furthermore, joint erosion is progressive and affects additional joints over time. Thus, the destructive potential of RASFs is reduced under biologics treatment owing to the reduction of further activating cytokines, but permanent RASF activation is not completely suppressed. Similar to anti-TNF strategies, the inhibition of IL-6 effectively reduces inflammation and disease progression in many patients with RA [89]. Additional approaches to inhibit the proinflammatory cascade in RA are currently under investigation.

IL-8 release in RASFs via the long isoform of the leptin receptor (OBRI) and related signaling pathways [33].

Another important proinflammatory factor potentially contributing to RASF activation in RA is prostaglandin E2 (PGE2) [2]. Proinflammatory factors and epidermal growth factor (EGF) induce PGE2 synthesis in RASFs [2,34]. Interestingly, PGE2 synthesis by RASFs is also increased by the adipokine adiponectin [35]. Furthermore, RASFs are the main source of cyclooxygenase (COX)-2 in synovial tissue [2,34], actively contributing to the COX-1/COX-2 system in RA.

Interestingly, neuroendocrine-immune interactions including neurotransmitters are also important modulators of inflammation affecting the proinflammatory and matrix-destructive properties of RASFs [36]. The α7 subunit of the nicotinic acetylcholine receptor is expressed by RASFs and the activation of the receptor promotes the production of proinflammatory cytokines (IL-6 and IL-8) [37]. Sympathetic nerve fibers are lost at sites of inflammation. Interestingly, the density of neuropilin-2-positive sympathetic nerve fibers is reduced in RA compared with OA, whereas the expression of the sympathetic nerve repellent, semaphorin 3F, is comparable [38]. Therefore, the nervous and immune systems regulate each other in RA.

RASF activation via cell–cell contact

Cell–cell interactions are required for the long-term hyperplastic growth of synovium in RA. In addition to the cellular interactions previously mentioned, additional interactions are important for RASF function [2]. ICAM-1 is upregulated in response to inflammatory cytokines, facilitating the interaction of RASFs with T cells. Additional adhesion molecules including vascular cell adhesion molecule (VCAM)-1 and its ligand very late activation antigen (VLA)-4 mediate the RASF-dependent mechanisms of activation. VCAM-1 is expressed in RASFs invading the cartilage and endothelial cells (ECs) in the synovial microvasculature [2], whereas VLA-4 is expressed in lymphocytes. These molecules contribute to cell–cell interactions between RASFs and lymphocytes. Furthermore, RASFs release chemotactic molecules after stimulation via the CD40 ligand/CD40 system, for instance by cell–cell contact with T cells, contributing to the complex network of inflammatory interactions during synovitis. Of note, inflammatory cytokines induce VCAM-1 expression in RASFs and can contribute to transmigration in and out of vessels and potentially migration to distant cartilage. McGettrick and colleagues established an in vitro flow system to study cellular RASF interactions with other cell types [39]. Endothelial cells cultured with fibroblasts on porous filters were placed into a flow chamber and perfused with lymphocytes. The adhesion of lymphocytes to the ECs on the filters was recorded. In this assay, ECs cocultured with RASFs bound perfused lymphocytes better than dermal fibroblasts or trauma fibroblasts from the same patients [39].

Additional findings are summarized in Table 1C. Interestingly, microparticles can induce the release of MMPs and proinflammatory factors in RASFs. Cellular interactions including direct cell–cell contact between RASFs and between RASFs and other cell types seem to be an important feature for RASF function. This is consistent with the observation that RASFs in culture require specific cell–cell contacts for proliferation and migration and are contact-inhibited at high densities in culture [1–4,40].

In summary, RASFs not only mediate cartilage destruction and attract additional RASFs to sites of cartilage and bone erosion, they also actively contribute to chronic inflammatory processes in joints affected by RA. In turn, RASF activation is enhanced by inflammatory cytokines and factors produced by inflammatory cells within the joint. The innate immune system contributes to the vicious inflammatory cycle of RA and directly affects RASF via factors of the complement system. TLR activation, the release of proinflammatory cytokines and through direct cellular interactions. RASFs produce proinflammatory factors; owing to the enhanced migratory potential of RASFs, these cells potentially carry factors promoting inflammation to sites of cartilage and bone destruction and to unaffected sites within the joint.

RASFs in joint remodeling and disease progression

Activated RASFs actively degrade and invade cartilage and mediate cartilage and bone erosion. The factors involved and mechanisms promoting RASF-mediated joint destruction have been well described. However, the contribution of RASFs to RA progression and the mechanisms involved are still under investigation. These studies could lead to therapies specifically targeting the joint destructive processes mediated by RASFs in RA as opposed to the most current therapies that target inflammation.

RASFs actively invade the matrix leading to cartilage and bone destruction

The destruction of articular cartilage and bone is mediated by the release of matrix-degrading enzymes (Box 1). RASFs are the major source of MMPs and cathepsins in the synovium and actively drive joint destruction via these enzymes.

The inhibition of MMPs can significantly reduce RASF cartilage invasiveness [41]. Tissue inhibitors of metalloproteinases (TIMPs) are antagonists of MMPs. However,
the normal levels of TIMPs found in joints affected by RA are not sufficient to counteract the matrix-degrading effects of MMPs and cathepsins. Of note, the overexpression of TIMPs such as TIMP-1 or TIMP-3 reduces RASF-mediated cartilage destruction in the SCID mouse model [2].

RASFs also influence bone erosion via cathepsin K and the modulation of osteoclastogenesis. RANK, a member of the TNF receptor family, and its ligand RANKL induce osteoclastogenesis. In RA, RANKL is strongly expressed at sites of bone erosion [1,2]. RANKL is induced in RASFs by several factors, such as proinflammatory IL-6 trans-signaling [42] and via TLRs [43].

RASF–matrix contact enhances the activation and migration of RASFs and is mediated via adhesion molecules. Small matrix components and matrix-associated components are liberated from the matrix during joint destruction. These fragments further activate RASFs, leading to the increased production of matrix-degrading enzymes and inflammatory factors [1–3]. RASFs migrate towards matrix fragments [6]. Factors produced by activated RASFs lead to the degradation of the ECM via the increased release of matrix-degrading enzymes, which exacerbates matrix invasion and destruction. In RA, SFs showing matrix-destructive properties seem to be shifted from the normal fibroblast function of tissue repair and wound healing towards constant tissue degradation.

**Altered apoptosis and proliferation rates of RASFs contribute to synovial hyperplasia**

The dysregulation of apoptosis and proliferation could cause synovial hyperplasia. Proliferating RASFs have a reduced susceptibility to apoptosis [44]. The increased survival of RASFs owing to reduced apoptosis susceptibility and increased proliferation could contribute to synovial hyperplasia in RA, which is generally characterized by increased numbers of RASFs in the synovial lining and especially at the invasion zone. However, the mechanisms underlying resistance to apoptosis and increased proliferation are largely unknown, and their contribution to RASF activation and RASF-mediated disease progression are under investigation.

Multiple mechanisms might contribute to reduced apoptosis in RASFs. For example, RASFs express increased amounts of the small ubiquitin-like modifier (SUMO)-1. The covalent attachment of SUMO-1 to cellular proteins modifies their function. This process, called SUMOylation, is a post-translational modification that can affect several cellular processes [45]. Increased SUMO-1 expression and the increased SUMOylation of nuclear proteins in RASFs results in resistance to Fas-induced apoptosis [46]. Additionally, the nuclear SUMO-protease SENP1, which can revert the apoptosis-inhibiting effects of SUMO-1, is reduced in RASFs. SENP1 overexpression in RASFs decreases MMP-1 secretion and RASF invasiveness and, therefore, represents a potential new target [47].

Survivin, also expressed by RASFs, belongs to the inhibitor of apoptosis family and represents another factor contributing to decreased RASF apoptosis [48]. Silencing survivin reduces urokinase production by RASFs [49]; urokinase signaling pathways are involved in RA inflammation and matrix degradation. Interestingly, the knockdown of Hsp70 in mice protects RASFs from nitric oxide-induced apoptosis, demonstrating the proapoptotic effect of Hsp70 in RASFs [50]. The susceptibility of RASFs to apoptosis is cell cycle-dependent [51]. Proliferating RASFs are less sensitive to the Fas ligand and TRAIL-induced apoptosis than RASFs with a decreased proliferation rate (e.g. during contact-dependent growth inhibition).

In addition to reduced apoptosis, increased proliferation could contribute to the accumulation of RASFs in the hyperplastic synovium and at sites of invasion. Factors such as IL-17 induce the proliferation of RASFs [51]. Furthermore, the contact-mediated growth inhibition of RASFs is reduced under hypoxia owing to the reduced expression of the adhesion molecule N-cadherin compared with cells cultured at low density under normoxia [53]. In vivo, these mechanisms could lead to the local enhancement of RASF proliferation at sites of local synovial hypoxia (e.g. at the invasion zone and the lining layer).

Both reduced apoptosis and increased proliferation could contribute to the accumulation of RASFs in the synovial lining, the sublining at sites of inflammation and especially at sites of cartilage invasion. However, the mechanisms responsible for the observed apoptosis resistance and increased proliferation are still under investigation and further analysis of the respective pathways will determine whether understanding these mechanisms might lead to new treatments.

**Hypoxia activates RASFs and induces the release of angiogenic factors**

A region deprived of an adequate oxygen supply is called hypoxic, and neoangiogenesis is induced in these areas to overcome a pathological lack of oxygen. Synovial hyperplasia in RA shows the characteristic increase in neoangiogenesis of newly formed tissue [2,3]. In joints affected by RA, hypoxic areas are present at sites of cartilage invasion and in the synovial lining. Neoangiogenesis in RA increases the number of synovial capillaries with activated endothelium.

Local hypoxia stimulates proangiogenic and chemotactic factors, matrix-degrading enzymes and osteoclastogenic factors [1–3]. Activated RASFs induce neoangiogenesis and are activated by proangiogenic factors, for example angiogenin [54] and angiopoietin-1, which is inducible by TNF [55]. Both factors are also produced by RASFs. Growth factors such as FGF-2 [56] and placenta growth factor (PIGF) [57] produced by and affecting RASFs also contribute to vessel formation within the synovium. PIGF is induced by TNFα and IL-6 in RASFs; the binding of PIGF to its ligand on monocytes induces proinflammatory cytokines in these cells [57]. Microparticles from the synovial fluid can also induce angiogenesis by inducing proangiogenic chemokines in RASFs [58,59].

One hypoxia-induced factor in RASFs, the inhibitor of DNA binding-2 (ID-2), is induced especially at sites of bone invasion [60]. In cocultures, ID-2-overexpressing RASFs induce the increased osteoclastogenesis of bone marrow precursors, potentially contributing to the bone erosion mediated by RASFs. Furthermore, hypoxia increases migration and the proangiogenic potential of RASFs in vitro.
Box 3. Cell migration

Cell migration is a central process for maintaining tissue homeostasis under physiological conditions. Wound healing, immune defense and matrix remodeling require the migration of several cell types including lymphocytes, macrophages, fibroblasts and ECs [85]. Cells required for matrix remodeling and homeostasis also have central functions in wound healing and tissue repair. Therefore, many cells including macrophages and fibroblasts migrate locally within a tissue to sites of damage. The ability of fibroblasts to migrate locally to sites of matrix destruction is well known [85]. Fibroblast migration to damaged tissue and subsequent tissue repair includes a temporary change from a “resting” to an "activated" phenotype [86].

Under pathophysiological conditions, local cells can acquire the potential for long distance migration (i.e. in tumor metastasis). During (chronic) inflammation the increased migration of inflammatory cells to the site of inflammation is observed [1–4]. Cells from the vascular system and local, resident cells are recruited to the site of inflammation in response to the activation of ECs, leading to the expression of more adhesion molecules and chemokines within the inflamed tissue. Many processes visible during inflammation are also active during wound healing.

[61]. Hypoxia-inducible transcription factor (HIF)-1α and the hypoxia-induced expression of VEGF by RASFs also contribute to neoangiogenesis [59]. Owing to the central role of angiogenesis at sites of inflammation and joint destruction, therapeutic approaches have been discussed to target proangiogenic factors in RA and the inhibition of HIF-1 is a candidate for potential therapeutic interventions [62].

Synovial hyperplasia is accompanied by increased tissue formation, subsequent local hypoxia followed by neoangiogenesis. Increased vessel formation and activation contribute to the influx of inflammatory cells from circulation. However, the significance of neoangiogenesis in early arthritis might be limited and, therefore, might not contribute to early RASF activation.

Increased RASF migration contributes to matrix invasion and the spreading of RA
Cell migration is an important part of many physiological processes and many cells can migrate after activation (Box 3). The potential of transformed-appearing RASFs to emigrate from affected joints has been shown in the SCID mouse model of RA [6]. RASFs migrate from an affected site (simulated by an RASF-containing cartilage complex) into a distant healthy site (simulated by a RASF-free cartilage implant), resulting in the subsequent cartilage invasion. The ability of RASFs to migrate to a distant implanted cartilage exceeds short-range intra-organ movement and seems to be unique. In addition, migration to the cartilage is independent of the site of application (subcutaneous, intravenous, intraperitoneal [6] or under the renal capsule [63]).

One central clinical characteristic of RA is the continuous spreading of disease. It has been speculated that the involvement of unaffected joints is caused by systemic humoral or cellular factors. However, the long-distance migration of RASFs is independent of an inflammatory environment. Wound healing and established vasculization seem to promote RASF migration [6]. RASF migration takes place through the blood, and human RASFs can be detected in the blood of the animals. This finding was surprising because fibroblasts, in contrast to fibrocytes, were not known to transmigrate into and out of blood vessels. RASFs can adhere to ECs [63] and transmigrate through EC layers in vitro [6]. RASFs adhere better to the ECM in comparison to other fibroblasts, and small (soluble) cartilage components attract RASFs [63]. The exposed cartilage matrix seems to be necessary because RASFs do not migrate into healthy joints but into joints with damaged cartilage and bone surfaces, for example after the injection of collagenase into the knees of the SCID mice. Therefore, the RASF invasion of unaffected joints most likely requires microlesions to initiate RASF attachment to the matrix. This hypothesis is in accordance with the observation of early joint erosion in individuals with RA that are similar to that visible after collagenase injection and RASF application in mice.

The increased migratory and adhesive potential of RASFs might contribute to the attachment of RASFs to the cartilage and bone surfaces, forming the hyperplastic tissue growing over the cartilage surface and potentially initiating hyperplasia. In addition, long distance RASF migration could spread RASF-mediated erosion and inflammation to unaffected regions within a joint and potentially between joints because RASFs mediate matrix degradation and are strong inducers of inflammation. The attachment of RASFs to cartilage seems to require exposed, damaged cartilage surfaces, and could in part explain the symmetry of joint involvement in RA. However, the findings are new and further analyses are required to understand the significance of RASFs in spreading RA between joints in humans.

RASFs are central cells of matrix degradation, leading to cartilage invasion and destruction, and actively contribute to bone erosion. Proinflammatory factors and degradation products contribute to RASF activation, resulting in an increased release of matrix-degrading enzymes. Furthermore, reduced apoptosis and the increased proliferation of RASFs might increase the numbers of activated RASFs contributing to hyperplastic tissue formation. RASFs contribute to the increased angiogenesis visible in RA by releasing proangiogenic factors and are further activated by hypoxia-induced factors within the hyperplastic tissue. Furthermore, the increased migratory potential of RASFs might spread inflammation and matrix erosion to hitherto unaffected areas within a joint and potentially between joints.

Concluding remarks
RASF activation requires the combination of several factors including inflammatory stimuli. Most likely, a collection of different factors at early stages of synovitis or even before the adapted immune system is involved is required to permanently activate SFs in RA. In turn, RASF activation leads to massive matrix destruction within the joint and contributes to spreading RA to unaffected joints. RASFs, a central cell type of matrix destruction, are in turn activated by factors released from the degrading matrix, such as matrix-associated factors and small matrix components. These factors have chemoattractant properties and recruit additional RASFs to the site of cartilage.
and bone erosion. Intracellular alterations within RASFs (epigenetic modifications as well as the altered regulation of oncogenes and tumor-suppressor genes) might contribute to RASF activation, even in the absence of inflammation, and might occur at early stages of RA. The inhibition of these pathways might inhibit RASF-mediated tissue destruction within the joints (Table 2). Owing to the multifaceted roles of RASFs in RA pathophysiology, the inhibition of the prodestructive and proinflammatory properties of activated RASFs is of central interest. Indeed, new therapeutic approaches targeting these properties are currently under investigation. However, to identify RASF-specific mechanisms causing or contributing to RASF activation and to specifically target these mechanisms without interfering with the more general mechanisms active in fibroblasts, which are important for wound healing and are present throughout the body, is a remaining challenge for the development of new treatments. Alternatively, new methods for site-specific application or tissue-specific drug delivery including tissue-specific carrier vehicles (e.g. microparticles) represent promising strategies to target RASFs.

Table 2. Therapeutic strategies in development.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mechanism of action</th>
<th>Current status</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF receptor tyrosine kinase inhibitors (i.e. imatinib)</td>
<td>Inhibition of activated RASF proliferation</td>
<td>Preclinical investigation</td>
<td>12</td>
</tr>
<tr>
<td>OPG (osteoprotegerin) (i.e. denosumab)</td>
<td>Inhibition of RANKL binding to its receptor RANK</td>
<td>Preclinical investigation</td>
<td>42, 43</td>
</tr>
<tr>
<td>miRNAs (i.e. miRNA-124a)</td>
<td>Downregulation of the release of MMPs and proinflammatory cytokines by RASFs</td>
<td>Preclinical investigation</td>
<td>19, 23, 81</td>
</tr>
<tr>
<td>PTEN agonists/overexpression of PTEN</td>
<td>Blocking PI3K (phosphatidylinositol 3-kinase)/Akt pathway activity</td>
<td>Preclinical investigation</td>
<td>18</td>
</tr>
<tr>
<td>SSRIs (i.e. fluoxetine and citalopram)</td>
<td>Inhibition of TLR3 activation with potential anti-inflammatory effects</td>
<td>Preclinical investigation</td>
<td>27</td>
</tr>
<tr>
<td>SENP-1</td>
<td>Reduced MMP-1 secretion and RASF invasiveness, possibly rendering RASF susceptible to apoptosis</td>
<td>Preclinical investigation</td>
<td>19, 46</td>
</tr>
<tr>
<td>Inhibitors of the TSP-1/TGF-β/CTGF axis</td>
<td>Antiangiogenic</td>
<td>Preclinical investigation</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Preclinical investigation</td>
<td>10</td>
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