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# Site of invasion revisited: epigenetic drivers of joint destruction in RA

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## ABSTRACT

New analytical methods and the increasing availability of synovial biopsies have recently provided unprecedented insights into synovial activation in general and synovial fibroblast (SF) biology in particular. In the course of this development, SFs have become one of the most rapidly evolving and exciting fields of rheumatoid arthritis (RA) research. While their active role in the invasion of RA synovium into cartilage has long been studied, recent studies have brought new aspects of their heterogeneity and propagation in RA. This review integrates old and new evidence to give an overview picture of the processes active at the sites of invasive synovial tissue growth in RA.

Synovial invasion into adjacent joint structures is a pathognomic feature of RA. Although therapeutic options for patients with RA have improved significantly over the past decade, once structural damage to the joint has occurred, it is irreversible, and thus effective inhibition of joint destruction remains one of the most important goals of RA therapy. Destruction of cartilage and bone in RA is thought to be a result of pathological activation of osteoclasts (recently reviewed in Andreev *et al.*<sup>1</sup> chondrocytes and synovial fibroblasts (SF). Interestingly, subchondral bone marrow oedema is a strong predictor of bone erosions in RA<sup>2</sup> and cellular activation in the bone marrow adjacent to erosions could be demonstrated histologically.<sup>3</sup> However, how bone marrow cells are involved in joint destruction is not elucidated yet. Damaged cartilage facilitates adhesion and invasion of SF in mouse arthritis models<sup>4</sup> and fragments released from damaged cartilage might activate synovial cells via Toll-like receptors (TLR).<sup>5</sup> Recent studies could show sustained increased expression of matrix metalloproteinases (MMP-1 and MMP-13) and cytokines by cartilage explants from patients with RA compared with healthy controls over 14 days in culture,<sup>6</sup> supporting an active role of cartilage in promoting structural damage in RA. Nevertheless, compared with osteoarthritis (OA), very little is known about the activation of chondrocytes in RA, and on their role promoting the invasive capacities of the synovial tissue and the formation of what historically was called pannus (Latin for patch or cloth).

In the late 1970s/early 1980s, pannus was described as scar tissue that covers areas with cartilage damage and, by histological examination, was suggested to evolve from tumour-like, invasively growing SF that had undergone a mesenchymoid transformation.<sup>7,8</sup> Since then, SFs have been recognised as crucial effector cells in the

pathogenesis of RA and various factors have been described to promote and maintain this invasive aggressive RA SF phenotype. Novel single-cell-based analyses have more recently provided unprecedented insights into SF activation and delivered compelling evidence for fibroblast heterogeneity in the synovium.<sup>9–11</sup>

In this review, old and new insights into how SFs are involved in the development of joint damage are brought together to provide a new integrative picture of the processes active at sites of invasive synovial tissue growth in RA.

## THE SYNOVIAL ARCHITECTURE IS LINKED TO SPECIFIC CELLULAR FUNCTIONS

The synovial tissue is divided into the synovial lining (intima) and sublining layer (subintima). Expression of cadherin-11 by SF has been shown to be indispensable for the establishment of this organised synovial architecture.<sup>12</sup> In healthy synovium, the lining layer consists of a thin layer of tissue-resident macrophages (previously called type A synoviocytes) and SF (previously called type B synoviocytes). Lining SFs, which form the interface with the joint cavity, produce proteins, which are important in maintaining physiological lubrication and nutrition of the joint cartilage, such as hyaluronic acid and proteoglycan 4 (PRG4). The healthy sublining layer consists of loose connective tissue, blood vessels and a few scattered sublining SF. In inflammation, synovial cellularity changes dramatically. SF populations expand; the lining layer becomes hyperplastic and the sublining layer is populated by sublining SF and infiltrating immune cells.

Previously, histological and functional studies have indicated that lining and sublining SF may constitute distinct cell populations.<sup>13</sup> This could be confirmed more recently by single-cell analyses of the synovium, where CD90 expressing sublining SF clearly separated from the CD90<sup>−</sup> lining SF population.<sup>9–11</sup> There is, however, no defined structure such as a basement membrane or tight cell–cell interactions separating these two synovial compartments. Accordingly, synovial fluid flows unrestricted via the synovial capillaries through the synovium to the articular cavity and back again to be reabsorbed by the lymphatic vessels in the sublining layer. The various compartments of the synovium are thus more loosely organised and more permeable than other tissues with a similar structure such as the skin or the intestine.

To prove functional differences between lining SFs and sublining SFs, an elegant cell transfer approach was developed using an experimental arthritis model in mice.<sup>14</sup> This experiment showed



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that the severity of joint destruction, but not joint inflammation, increased when lining SFs were injected intra-articularly. On the other hand, when sublining SFs were injected, the inflammatory response increased, but joint destruction was unaffected. Although these experiments were performed in mice, it can be assumed that lining and sublining SF play a similar dichotomous role in human RA and that also in human disease synovial lining, but not sublining SF attach and invade adjacent joint structures. This is also supported by the strong expression of the lining cell marker cadherin-11 at sites of synovial invasion into cartilage and its ability to promote invasive growth of SF.<sup>15</sup>

### THE ORIGIN OF SF SUBPOPULATIONS

The origin and phenotypic stability of the different subpopulations of SF is not yet clearly understood. Lining and sublining SF might represent different phenotypic variations induced by local stimuli or different cell lineages. Trajectory analysis of single-cell RNA sequencing data suggests that the differences in gene expression between SF subpopulations are gradual, with CD90<sup>−</sup> lining SF on one side of the axis and CD90<sup>high</sup> SFs on the other.<sup>16</sup> This analysis, therefore, argues rather against the presence of distinct SF lineages in the synovium and for a gradual change in phenotype depending on the environment. Indeed, endothelial-derived NOTCH signals were found to induce and stabilise the CD90<sup>high</sup> SF subtype that is found around vessels in the synovium.<sup>16 17</sup> This could also mean that one SF subtype could transform into another depending on the stimulus and localisation within the synovium. Although distinct experiments addressing this ‘reprogramming’ are currently lacking, it is an attractive hypothesis for finding a therapeutic approach that can convert pathogenic SF into restorative, normal SF.

Lineage tracing studies showed that the majority of the synovium is populated by *Gdf5*-expressing joint interzone cells in mice.<sup>18</sup> Continuous, temporal-spatially regulated influx of *Gdf5*-lineage cells gives also rise to all other joint structures, such as cartilage, menisci and ligaments.<sup>19</sup> However, a fraction not derived from this lineage was found particularly in the sublining synovial compartment.<sup>20</sup> Interestingly, in antigen-induced arthritis in mice, the proportion of lining SF (CD90<sup>−</sup>) increased much more in the *Gdf5*-lineage SF than in SF that did not evolve from the *Gdf5*-lineage, where expansion of sublining SF (CD90<sup>+</sup>) dominated.<sup>21</sup> This could mean that in arthritic conditions the phenotypic differences between lining and sublining SF are reinforced by differential proliferative responses of different SF lineages. On the other hand, recruitment of *Gdf5*-lineage SF to perivascular areas was demonstrated after parapatellar arthrotomy in mice.<sup>18</sup> Intriguingly, perivascular CD90<sup>high</sup> human SF were suggested to expand in RA synovial tissues due to endothelial cell derived signals. Thus, a combination of local signals and lineage differences might shape the pathogenic architectural and cellular synovial changes seen in RA.<sup>16</sup>

However, to date, it is not clear whether and how these synovial changes differ between the various chronic arthritides. Although the clinical presentation and outcomes are very distinct, apart from its invasive growth in RA, the synovial response to inflammation seems rather uniform. Neither synovial lining hyperplasia, nor expansion of sublining SF populations is exclusive to RA and is similarly seen in psoriatic arthritis or reactive arthritis, and even in cases of OA.<sup>22</sup> Based on histomorphological investigations of the site of cartilage invasion in RA, it was speculated in the early 1990s that a mesenchymal cell of distinct origin arises from the perichondrial synovium in the early phases of RA and starts the invasive process.<sup>8 23</sup> Whether

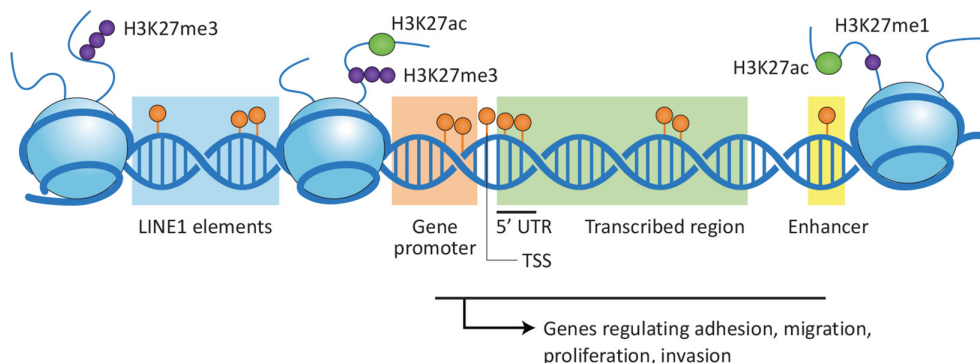
these RA-specific, invasive stromal cells in the lining layer, back then called pannocytes,<sup>23</sup> are transformed lining SF, stem from a distinct mesenchymal lineage or possibly are even chondrocyte-derived or bone marrow-derived cells has not yet been clarified. More detailed analyses focusing on the synovial lining and the site of invasion may reveal an RA-specific lining SF population. Until then, we can revisit studies analysing overall changes in RA SF to elucidate factors that are involved in the RA-specific invasive behaviour of lining SF.

### INVASIVE PROPERTIES OF RA LINING SF

Numerous studies analysed qualitative differences between RA and OA SF and many described molecules and pathways that are able to promote the invasive behaviour of SF, for example cadherin-11,<sup>15</sup> PI3Kδ,<sup>24</sup> hypoxia,<sup>25</sup> interleukin IL-17,<sup>26</sup> IL-21<sup>27</sup> just to name a few. High expression of MMPs in RA synovial tissues and prominent expression of MMPs by lining SF were already discovered in early studies and suggested as one of the major mediators of joint destruction in RA.<sup>28–30</sup> In particular, expression of MMP-1,<sup>31</sup> MMP-3,<sup>31</sup> MMP-13<sup>32</sup> and MMP-14 (MT-MMP1)<sup>33</sup> was connected to the cartilage damaging properties of RA SF. Their expression was found high in SF at sites of cartilage invasion and correlated with invasive behaviour of SF in vivo and in vitro. In addition, the cysteine proteinases cathepsin L and B<sup>34</sup> were found to be higher expressed in the lining layer of RA synovial tissues, as well as cathepsin K, which was directly connected to the degrading properties of SF in vitro.<sup>35</sup> Furthermore, upregulation of adhesion molecules<sup>36 37</sup> and local activation of SF via TLR2<sup>38</sup> was suggested to play a role in the pathological attachment and invasion of SF into cartilage. The production of RANKL, which is critical for osteoclast formation, has been described as an indirect mechanism for the promotion of bone erosion by RA SF.<sup>39</sup> However, as with the synovial inflammatory response, expression of these molecules and activation of these signalling pathways after stimulation is not specific to RA SF, and therefore, cannot fully explain why attachment of RA SF to cartilage occurs in RA but not in other joint diseases.

### EPIGENETIC CHANGES IN RA SF

In their seminal work in 1996, Müller-Ladner *et al* used a coimplantation model of cultured SF and human cartilage in immunodeficient severe combined immunodeficiency (SCID) mice and demonstrated that the invasive properties of RA SF are not dependent on an inflammatory environment and that they are maintained over several passages in cell culture.<sup>40</sup> This supported the notion of a ‘tumour-like’, stable transformation of RA SF, postulated by earlier studies.<sup>7</sup> Subsequently, differences in DNA methylation between cultured RA and OA SF could be shown and were suggested to underlie the phenotypic changes of RA SF. DNA methylation is an epigenetic mechanism that can mark specific genomic regions for silencing and thus regulates gene expression by influencing the accessibility of the transcriptional machinery to promoter regions. Furthermore, DNA methylation is an important mechanism to silence repetitive and retroviral sequences in the genome, such as retrotransposons. In 2013, several studies analysed DNA methylation in coding regions using microarray technology.<sup>41–43</sup> Similar to tumour cells, RA SF showed a loss of methylation in genomic regions containing retroviral LINE1 sequences<sup>44</sup> and focal hypermethylation/ and hypomethylation in coding regions.<sup>41–43</sup> First, these studies clearly showed that there a considerable changes in DNA methylation between cultured RA SF and OA/normal SF.



**Figure 1** Changes in epigenetic modifications in RA SF. DNA methylation (orange lollipops) in repetitive DNA sequences like line 1 (blue) is lost in RA SF, while in chromatin regions that are transcribed (green) or contain regulatory elements, such as enhancers (yellow) or promoters (red), DNA methylation can be lost or gained in RA SF. Differentially modified epigenetic regions (DMER) between RA and OA SF contained mainly the activating H3K27ac mark (acetylation of lysine 27 in histone 3) and H3K4me1, found in enhancer regions. H3K4me3 that marks promoter regions and H3K27me3, enriched in repressed chromatin, were found altered in the region encoding MMPs in RA SF. OA, osteoarthritis; RA, rheumatoid arthritis; SF, synovial fibroblast; TSS, transcription start site; 5'UTR, 5' untranslated region.

Second, further analysis of the affected regions indicated that the aberrant DNA methylation in RA SF is not random, because the implicated genes and pathways had a role in previously implicated inflammatory (e.g., TLR pathway<sup>43</sup>) and invasive pathways (e.g., extracellular matrix receptor interactions,<sup>42</sup> cell matrix adhesion<sup>41</sup>) in RA. In particular, pathways involved in cell adhesion, cell migration (actin cytoskeleton<sup>42</sup>, actin binding,<sup>43</sup> response to wounding<sup>41</sup>) and proliferation were recurrent themes that emerged in all three major studies and strongly pointed to regulation of invasive properties of lining SF by changes in DNA methylation (figure 1). Furthermore, treatment of RA SF with methyl donors and remethylation of their DNA inhibited their invasive behaviour in the SCID mouse model, further supporting a link between changes in DNA methylation and the 'tumour-like' behaviour of lining SF.<sup>45</sup>

However, there was relatively little overlap between the observed changes in DNA methylation and transcriptional changes in the associated genes. From 3470 differentially methylated sites being annotated to 1238 genes, only 17% (208) showed the expected inverse correlation of expression and changes in DNA methylation.<sup>41</sup> Mostly, hypermethylation or hypomethylation at transcription start sites (TSS) and 5' untranslated regions, but not in other genomic regions, associated with upregulated or downregulated expression of the respective genes in cultured RA SF. The lack of transcriptional effect of the differentially methylated sites could be due to other effects of DNA methylation (e.g., splicing effects), additional mechanisms regulating gene expression (e.g., histone modifications) or a lack of stimulus. In general, epigenetic modifications, including DNA methylation, do not initiate gene transcription themselves, but shape the chromatin landscape to promote or inhibit binding of relevant proteins, for example, transcription factors or the transcriptional machinery, in a specific environmental condition. Thus, repeated stimulation of macrophages changes the epigenetic landscape and induces tolerance or training.<sup>46</sup> Similar repeated TLR or tumour necrosis factor (TNF) stimulation have been shown to change epigenetic marks, in particular histone acetylation, in SF.<sup>47 48</sup> Specific environmental changes, for example, cytokines, damaged cartilage or the presence of autoantibodies might have supported transcriptional epigenetic reprogramming in RA SF that can only be recalled when the same stimulus is applied again. This hypothesis is corroborated by the fact that even though single-cell RNA sequencing analysis has shown that lining SF largely lose their transcriptional phenotype

in vitro,<sup>16</sup> cultured RA SFs still exhibit the invasive properties of in vivo lining SF in the SCID mouse model. Therefore, it can be assumed that epigenetic imprinting preserves the invasive RA lining phenotype in cell culture and that it can be reactivated under the right conditions. Direct contact with damaged cartilage and/or soluble factors secreted from activated chondrocytes might have a crucial role in this activation.

In addition to changes in DNA methylation, overall integration of epigenetic marks confirmed significant differences in the epigenetic makeup of RA and OA SF and defined several differentially modified epigenetic regions (DMER) (figure 1).<sup>49</sup> Pathway analysis of the genes associated with these DMER again pointed to epigenetic modulation of migration (eg, 'Signalling by Rho family GTPases'), and identified huntingtin-interacting protein-1 (HIP1) as additional modulator of invasive properties of RA SF.<sup>49</sup> Analysis of transcription factor binding sites within the identified DMER showed over-representation of AP-1 transcription factor binding sites in RA SF. AP-1 transcription factors are heterodimers/homodimers of JUN and FOS proteins. FOS was described to activate MMP production in RA SF already 30 years ago.<sup>50</sup> Enrichment of AP-1 transcription factor binding sites was also shown within chromatin regions that rearrange after TNF stimulation in SF.<sup>51</sup> Together, these studies indicate that AP-1 activation is an important step in reactivation of the epigenetically reprogrammed RA SF invasive phenotype. H3K4me3, the histone modification that is enriched in promoter elements, was found increased in MMP-1, MMP-3, MMP-9 and MMP-13 in RA SF compared with OA SF, while the repressive H3K27me3 mark was lost in RA SF in MMP-1 and MMP-9 promoters.<sup>52</sup> These changes could facilitate AP-1 binding to MMP promoters and thus promote high expression of these MMPs in RA SF.

However, based on single-cell analysis, it must be considered that SF cultures are not as homogeneous as previously thought. This raises the possibility that differences in the transcriptome and epigenome in RA SF do not represent qualitative differences, but reflect quantitative differences in the proportion of different SF subtypes in cultures. RA synovial tissues generally contain considerably more sublining SF than synovial tissues from OA patients or healthy controls. Thus, in RA cell cultures, lining SF might be overgrown by sublining SF more rapidly than in OA cultures. In this regard, it should be noted that 13 of the 20 major marker genes for lining SF, as defined by Zhang *et al*<sup>11</sup> by single-cell RNA sequencing, were differentially methylated, mostly hypermethylated in RA SF cultures, in at least one of the



three studies in which changes in DNA methylation were analysed.<sup>41–43</sup> These genes include for example PRG4, CD55, ITGB8 and CLIC5. It remains to be determined whether the differences of DNA methylation in these genes between RA and OA SF reflect epigenetic modulation of lining SF functions or are due to a higher proportion of sublining SF, in which these genes could be silenced by DNA methylation, in RA SF cultures.

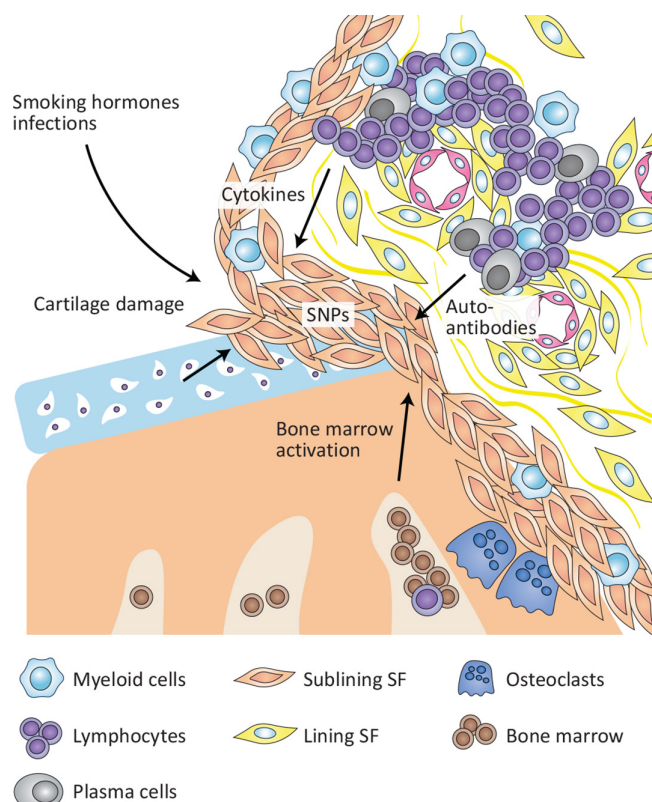
### WHEN AND HOW ARE RA SF IMPRINTED

Assuming that there is epigenetic imprinting of RA SF, the key question is how and when this imprinting occurs. Technically, epigenetic changes in RA SF can be triggered at any time during the lifetime, from embryonic development to disease flares, by a variety of environmental stimuli such as viral infections, hormones, smoking, etc. Research in this area is scarce and logistically difficult to implement. At least, studies comparing cultured SF from different stages of RA consistently showed that RA-specific changes in DNA methylation can be detected already in undifferentiated stages<sup>53</sup> and shortly after diagnosis,<sup>54</sup> but also that the DNA methylome is still changing over the course of the disease. For the first time, although with a small number of replicates, each of these studies provided evidence that the DNA methylome of RA SF is distinct from SF of other inflammatory arthritides (juvenile idiopathic arthritis,<sup>54</sup> undifferentiated resolving arthritis<sup>53</sup>). Most interestingly, pathway analysis of associated genes with sites that were already differentially methylated at undifferentiated disease stages once again highlighted migratory ('actin filaments') and cell adhesion ('cadherins') pathways.<sup>53</sup>

Furthermore, DNA methylation connected to genes involved in antigen presentation pathways was changed in SF at very early as well as late stages of RA.<sup>53</sup> Whether SF are true antigen-presenting cells is still under debate. Nevertheless, SF strongly upregulate MHC class II expression upon stimulation with interferon  $\gamma$ , IL-17 or coculture with neutrophil extracellular traps.<sup>55</sup> Of note, high expression of HLA-DR was also shown in a specific population of sublining SF that expanded in RA synovial tissue.<sup>11</sup> Therefore, again, it cannot completely be ruled out that differences in DNA methylation patterns are due to differential enrichment of different SF subpopulations in culture.

An intriguing possibility is that the presence of RA specific autoantibodies leads to the specific epigenetic imprinting of RA SF. A direct arthritogenic role of autoantibodies isolated from patients with RA has been suggested in several studies, showing that RA autoantibodies, in particular anticitrullinated peptide antibodies (ACPA) can promote osteoclastogenesis<sup>56,57</sup> and activate macrophages.<sup>58,59</sup> Importantly, SF incubated with polyclonal antibodies isolated from RA patients' blood or specific clones of monoclonal antibodies increased the migratory and adhesive properties of SF.<sup>60</sup> This activation was dependent on the presence of citrullination in SF, and thus, it might be concluded that this effect is indeed mediated via binding of ACPA. However, whether such effects of autoantibodies indeed depend on their reactivity and how they are mediated is as yet unclear. Furthermore, whether the presence of autoantibodies can induce changes in DNA methylation in SF has not been analysed.

Finally, epigenetic variability in SF could be mediated by variations in the DNA sequence. Some changes in DNA methylation levels are closely related to genetic variants (single-nucleotide polymorphisms, SNPs), so-called DNA methylation quantitative trait loci or meQTLs. To date, no study analysed meQTLs in SF, but analysis in peripheral blood cells demonstrated that genetic RA risk variants can influence DNA methylation in T-cells and



**Figure 2** Possible activators of lining SF transformation in RA. RA-independent factors (smoking, viral infections, hormones) as well as environmental changes connected to RA such as cytokines, autoantibodies, cartilage damage, bone marrow activation or genetic risk (single-nucleotide polymorphisms, SNPs) could have a role in the ongoing transformation of lining SF. RA, rheumatoid arthritis; SF, synovial fibroblast.

B-cells.<sup>61,62</sup> Several RA risk SNPs lie in open and active chromatin in SF<sup>51</sup> and thus might not only induce transcriptional changes in RA SF, but also changes in DNA methylation.

### CONCLUDING REMARKS

In summary, the data collected to date support the hypothesis that lining SF with an epigenetically imprinted phenotype, mainly affecting adhesive and migratory properties and probably activated via AP-1, promote invasive synovial growth in RA. This transformation occurs at early disease stages and might be induced by environmental and genetic factors (figure 2). Although many aspects of SF activation in RA have been clarified in recent years, some important questions remain. The origin of the invasive lining RA SF could be a distinct mesenchymal stem cell population within the joint ('pannocyte') or phenotypic changes of normal lining SF. For preventive measures, elucidation of the environmental factors which might induce the formation of invasive lining SF is crucial. While it is known that for instance smoking or viral infections can have a strong influence on DNA methylation, a direct link between an environmental factor and the RA SF pattern of DNA methylation changes has not been found yet. Given the typical pattern of joint involvement in RA, these triggering factors might be more present or act more strongly in certain joints than in others.<sup>63</sup> In this context, the role of cartilage and bone marrow cells in promoting invasive synovitis is completely unclear. Finally, yet importantly, a direct link between invasive growth and the observed epigenetic changes is still missing. Answering these questions will

substantially increase the knowledge on the invasive growth of RA SF and open up the way for direct therapeutic targeting of invasive RA lining FLS.

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