Review

# Relevance of the stroma and epithelial-mesenchymal transition (EMT) for the rheumatic diseases

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## Abstract

Epithelial-mesenchymal transition (EMT) is a term applied to the process whereby cells undergo a switch from an epithelial phenotype with tight junctions, lateral, apical, and basal membranes, and lack of mobility into mesenchymal cells that have loose interactions with other cells, are non-polarized, motile and produce an extracellular matrix. The importance of this process was initially recognized from a very early step in embryology, but more recently as a potential mechanism for the progression and spread of epithelial cancers. As the sequence of morphological changes has become understood in molecular terms, diseases characterized by alterations in stromal elements and fibrosis are being considered as examples of EMT. This review will focus on the pathogenetic features of immune-mediated renal disease, systemic sclerosis and rheumatoid arthritis that could be explained by EMT.

## The relevance of the stroma and epithelial-mesenchymal transition for rheumatic diseases

Epithelial-mesenchymal transition (EMT) describes a process wherein static epithelial cells lose cell-cell contacts, acquire mesenchymal features and manifest a migratory phenotype. Multiple alternative terms, including epithelial-mesenchymal interactions, transformation, transdifferentation, and transition, have been used interchangeably to describe this process. I've chosen 'transition' for the reasons elaborated by Kalluri and Neilson [1], whose excellent publication is recommended to any reader interested in the entire subject. EMT, which was first appreciated by developmental biologists in the 1980s, is now attracting the attention of investigators interested in metastatic cancers and diseases characterized by fibrosis [1,2]. This review will explain these observations briefly and consider how they might be relevant to certain rheumatic diseases.

In the embryo the first and only tissue formed is epithelium [3]. Sheets of epithelial cells are held together tightly at strong adherens junctions containing E-cadherin in complexes with catenins linked to the actin cytoskeleton. The epithelial cells are firmly attached through integrins to an underlying extracellular matrix (ECM) containing type IV collagen and laminin; the basement membrane. Around day 15 the epiblast cells of the developing human embryo migrate into a structure called the primitive streak [4]. Once in place they assume the features of embryonic mesoderm and endoderm in a process known as gastrulation. From the mesoderm arise the visceral and limb bud mesenchyme. The latter is the source of bone, cartilage, fibroblasts, fat, skeletal muscle and the bone marrow stroma.

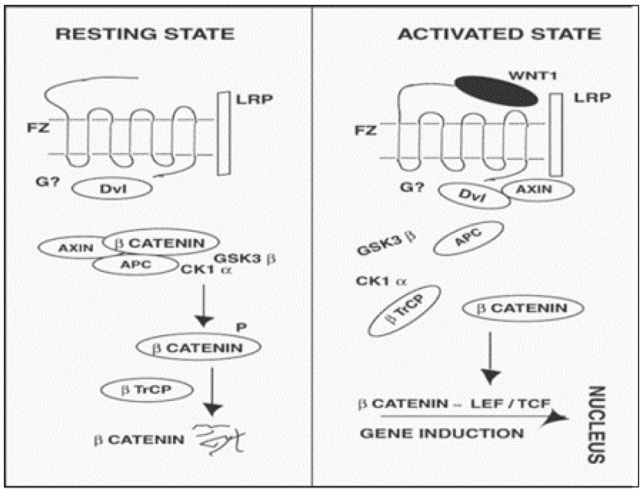
Although mesenchymal cells are secretory and produce collagens, fibronectin, vimentin, and alpha smooth muscle actin (αSMA), no one of these is unique to this cell type. The attribute that sets mesenchymal cells apart is their ability to invade and move through the three-dimensional structure of the ECM. Accordingly, mesenchymal cells are defined by morphology and behavior: front end to back end polarity; elongated morphology; filopodia; and invasive motility [3].

## Signaling pathways used in development

The wnt and transforming growth factor (TGF)-β signaling families are essential for development of the primitive streak and the induction of EMT [5,6]. Each acts through the transcription factor LEF-1/TCF, a member of the family of HMG-box DNA binding proteins, which has binding sites for both Smads and catenin signaling molecules [7]. The primacy of LEF-1/TCF can be demonstrated by transfecting epithelial cells with LEF-1/TCF DNA and observing that they lose their epithelial features and acquire a motile mesencyhmal

αSMA = alpha smooth muscle actin; BMP = bone morphogenic protein; CAF = cancer associated fibroblast; ECM = extracellular matrix; EMT = epithelial-mesenchymal transition; FLS = fibroblast-like synoviocyte; FSP-1 = fibroblast specific protein 1; MMP = matrix metalloproteinase; MPC = mesenchymal progenitor cell; MSC = mesenchymal stem cell; RA = rheumatoid arthritis; RTE = renal tubular epithelium; SDF = stromal derived factor; SSc = systemic sclerosis; TGF = transforming growth factor; TNF = tumor necrosis factor.

**Figure 1**



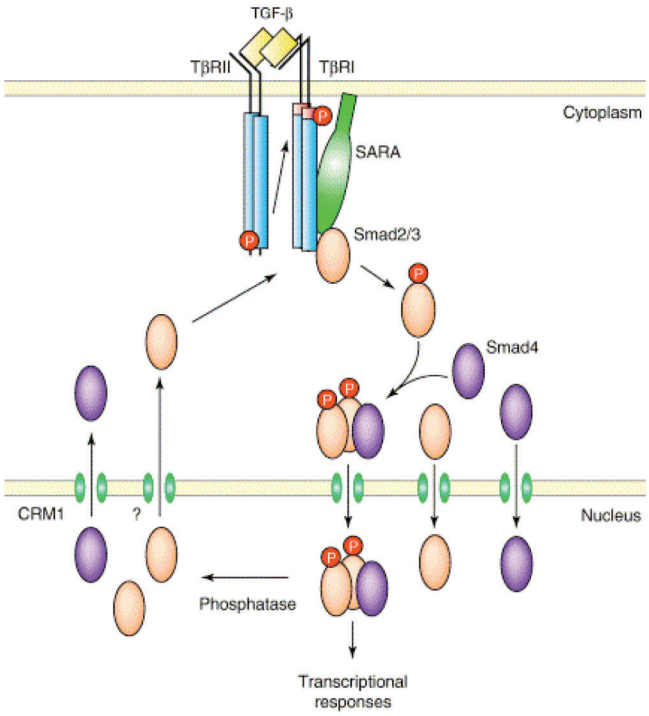

Wnt/β-catenin signaling pathway. In resting cells, glycogen synthase kinase 3 (GSK3β) is in a complex with CK1, β-catenin, axin and adenomatous polyposis coli protein. In this state, β-catenin is primed for phosphorylation by GSK3β. The phosphorylated β-catenin is degraded by ubiquitination. In the activated state (upon Wnt binding to Fz), Wnt-Fz and LDL receptor-related protein 5/6 (LRP) coordinate Dvl (disheveled, an adaptor protein) activation, which results in recruitment of axin to the plasma membrane. This leads to dissociation and inactivation of GSK3β, which can no longer phosphorylate β-catenin. Free β-catenin translocates to the nucleus and induces gene expression in a complex with LEF-1/T cell factor (TCF) family transcription factors, down regulating E-cadherin genes and initiating epithelial mesenchymal transition. (Adapted from [8].)

phenotype. Conversely, mesenchymal cell lines become epithelial when transformed by E-cadherin genes [6].

The wnt signaling pathway regulates the amounts of β-catenin protein available within the cell for binding to the cytoplasmic tail domain of cadherins, which mediates cell-cell adhesion, and to cytoskeletal (F actin) elements [8]. In the resting state, β-catenin is in the cytoplasm associated with adenomatous polyposis coli protein and axin, which results in its ubiquination and subsequent degradation by the proteosome (Figure 1). Normally, a balance is maintained between a relatively stable pool of β-catenin associated with adherens junctions and a small, rapidly degraded cytosolic pool [9]. Engagement of wnt glycoprotein by cell surface frizzled receptors results in an excess of free cytosolic, non-phosphorylated β-catenin, which can enter the nucleus and engage LEF-1/TCF DNA binding proteins, transforming them into transcriptional activators of the genes central to EMT, including the down-regulation of E-cadherin genes.

Binding of TGF-β ligands to their tetrameric type I and II receptors causes sequential activation of MKK-4/JNK and the complex of Smad 2/3 and Smad 4 proteins (Figure 2). This complex can enter the nucleus and engage LEF-1/TCF at a site separate from the β-catenin binding site [7], but with

**Figure 2**




The canonical transforming growth factor (TGF)-β/Smad signaling pathway. Members of the TGF-β family of growth factors (TGF-βs, activins, nodals) interact sequentially with two membrane receptors. TGF binds first to the constitutively active type II receptor (R) and then the ligand-recepor complex associates with type I TGF-R. TGF-IIR (TβIIR) phosphorylates TGF-IR (TβIR) on a cluster of serine threonine residues. Activated TGF-RI propogates the signal downstream by directly phosphorylating Smad2 and Smad3. These form heterodimeric or trimeric complexes with Smad 4 and translocate into the nucleus where, in combination with LEF-1/T cell factor (TCF) family transcription factors, they down-regulate E-cadherin genes and initiate epithelial-mesenchymal transition. Complexes of Smad7 and Smurf1 or Smurf2 promote ubiquination and degradation of activated receptors limiting the intensity and duration of signaling. P, phosphorylation sites; SARA, small anchor for receptor activity. (Adapted from [61].)

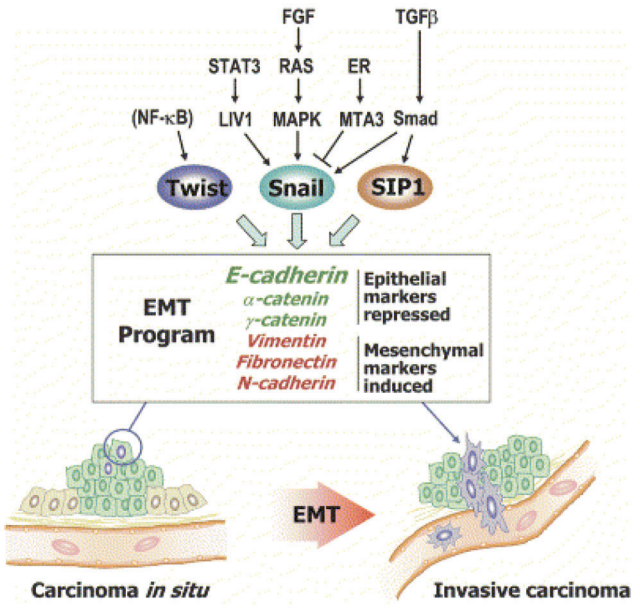
similar results; namely, induction of EMT genes, E-cadherin down-regulation, and acquisition of mesenchymal features [10,11].

In addition to LEF-1/TCF, a family of transcription factors that can cause EMT and down-regulate E-cadherin expression has recently been identified (Figure 3). These repressors, bearing fanciful names like Snail, Slug, Sip-1, and Twist, exert their effects by binding to different E-boxes in the E-cadherin promoter [12]. Wnt and TGF-β can also up-regulate these E-cadherin repressors.

## EMT and malignancies of epithelial cells (carcinomas)

Often genes important in embryogenesis have an oncogenic potential (i.e., the ability to initiate tumors), but the

**Figure 3**




Drivers and mediators of epithelial-mesenchymal transition (EMT). Early stage tumor cells maintain epithelial properties similar to the neighboring normal epithelium. The accidental overexpression of master regulators of EMT, such as the transcription factors Twist, Snail, and SIP1, in cancer cells leads to dramatic changes in gene expression profiles and cellular behavior. Twist, Snail, and SIP1 repress the expression of E-cadherin via E boxes in its promoter and trigger expression of an entire EMT transcriptional program through as yet unknown mechanisms. Several pathways are known to regulate Twist, Snail, and SIP1 expression in tumor cells while others (shown in parentheses) do so at least in developmental contexts. (Adapted from [21].)

propagation and spread of these tumors depends on several different processes [13]. Many separate steps are involved in metastasis of neoplastic epithelial cells, namely expansion into local tissues, penetration of blood and lymphatic vessels, entrance into the systemic circulation (intravasation), subsequent extravasation through the vascular endothelium at distant locations, and the establishment of new tumors. Each of these steps has been analyzed by gene-expression microarrays in both experimental animals and man [14-17]. The conversion from a sessile tumor to an invasive carcinoma results from the loss of constraints imposed by cell-cell adhesion, that is, EMT. The level of E-cadherin expression is often inversely correlated with tumor grade and stage and inactivating mutations of E-cadherin are present in 50% of lobular breast carcinomas [18,19].

Equally important are E-cadherin repressors. In a very influential paper, Yang and colleagues [20] found elevated levels of Twist expression in mouse mammary gland tumors at every stage of metastasis. Reduction of the expression level of Twist substantially reduced tumor cell intravasation, but had no effect on the histology or growth rate of the primary tumor. Kang and Massague [21] recently reviewed the contribution of additional pathways and E-cadherin repressors to metastatic disease (Figure 3). They also pointed out that the number of carcinoma cells that have undergone EMT and appear as stromal elements is likely to be underestimated. This is an important consideration given the interest in the influence of the stromal environment on neoplasia.

## The role of the ECM and stroma in cancer

Stroma is the tissue that forms the ground substance, framework or matrix of an organ. New studies suggest that the cancer cell microenvironment not only facilitates tumor progression, but also may occasionally initiate the oncogenic conversion of epithelial cells [22,23]. An example of the former is the study of Orimo and colleagues [24], who isolated cancer associated fibroblasts (CAFs) from six human breast cancers and compared them to fibroblasts isolated from a nearby non-cancerous region of the same breast (counterpart fibroblasts). CAFs were more competent in supporting *in vivo* growth of tumor cells and enhanced tumor angiogenesis and the recruitment and mobilization of endothelial progenitor cells. Cancer associated fibroblasts express traits of activated fibroblasts (myofibroblasts with increased αSMA staining) when compared to counterpart fibroblasts or normal fibroblasts. CAFs expressed high levels of stromal derived factor (SDF)-1, which is responsible for the chemotaxis of endothelial progenitor cells and contributes to angiogenesis and tumor growth by acting in a paracrine manner on the CXCR4 receptors of tumor cells. A comprehensive gene expression profile of breast carcinomas noted significant overexpression of the chemokines CXCL14 and CXCL12 in tumor myoepithelial cells and myofibroblasts [25]. These authors proposed that locally produced chemokines bind to receptors on epithelial cells, enhancing their proliferation, migration, and invasion.

Rat mammary adenocarcinomas develop when just the stroma is treated with a carcinogen (N-nitrosomethyl-urea) regardless of the exposure of epithelial cells [26]. In a related study, TGFβ-1 and the extracellular matrix protein laminin-5 induced EMT and hepatocellular carcinoma cell invasion by upregulating Snail and Slug, down regulating E-cadherin, translocating β-catenin into nuclei, and inducing dramatic spreading and morphological changes in the cancer cells [27]. Similar changes were not observed with the peritumoral tissues from the same hepatocellular carcinoma patients. EMT was blocked by antibody to alpha 3, but not alpha 6 integrins, supporting the critical role of laminin 5 in these processes [27]. In a related study, tissue derived fibroblasts modulated the integrin-dependent interactions (alpha-5, alpha-6, beta 1) between the gastric cell line HGT-1 and fibronectin [28]. Hepatocyte growth factor produced by autologous stromal fibroblasts augments the growth of human small cell lung cancer in nude mice [29]. Exposure to CAFs transformed non-tumorogenic prostate epithelial cells into neoplasms [30,31] and fibroblasts from tumor stroma

induced malignant transformation with dysregulation of several chromosones in the non-tumorogenic SV40 immortalized, prostate line BPH-1 [32].

Conversely, in some experimental models, the stroma can normalize carcinomatous epithelial cells. For instance, mammary gland stroma from mature and multiparous rats interferes with the development of neoplastic breast tissue and encourages normal ductal growth of grafted epithelial cancer cells, whereas 6 months after inoculation tumors developed in 75%, 100% and 50% of 24-, 52-, and 80-day old virgin rats [33]. These observations, although unexplained, have obvious clinical implications.

## Fibrotic disorders

### Kidney disease

Wound healing results from a sequential process of inflammation, leukocyte infiltration, cytokine and growth factor release, and formation of a scaffold composed of collagens and other matrix molecules into which fibroblasts enter and proliferate. Healing and fibrotic scaring are advantageous in wounds, but they can be pathological in the kidneys, the lungs and the liver [1]. Extensive investigations in mice, rats, and man of acute and chronic renal fibrinogenesis implicate EMT as the cause for cells of the renal tubular epithelium (RTE) becoming interstitial fibroblasts [34,35].

For instance, *in vitro* exposure of isolated RTE to graded doses of cyclosporine A results in cellular elongation, detachment and cytoskeletal reorganization. αSMA expression occurred in the treated cells with a concomitant dose-dependent production of TGF-β [36].

A model of unilateral hydronephrosis provides a comparison of events in the obstructed and normal kidneys [37]. Unlike the lung, the kidney interstitium normally has few fibroblasts. Ureteral obstruction causes rapid (within days) interstitial fibrosis, while the control kidney remains normal. The sequence of events was traced with an antibody to a 'fibroblast specific protein 1' (FSP-1), which disclosed staining of RTE trapped in damaged nephrons [38]. The FSP-1 positive epithelial cells traversed the damaged tubular basement membrane and increasing numbers of FSP-1 positive fibroblasts appeared in the interstitium. The cells had lost their epithelial markers and gained a fibroblast phenotype [38]. Unfortunately, anti-FSP-1 staining is not exclusive for fibroblasts [39]. The cascade of EMT, interstitial infiltration, and eventual renal fibrosis resulting from an interplay of hypoxia, inflammatory mediators, growth factors and matrix metalloproteinases (MMPs) produced by invading inflammatory cells, resident fibroblasts and RTE is illustrated in Figure 4 [40]. But TGF-β is central to the fibrotic process and strategies that reduce TGF-β levels restore the loss of E-cadherin and inhibit EMT. In cell culture systems, bone morphogenic protein (BMP)-7, an intracellular competitor of TGF-β signaling, reverses interstitial fibrosis and impaired renal function in several murine models of kidney failure, including lupus nephritis [34,41-43].

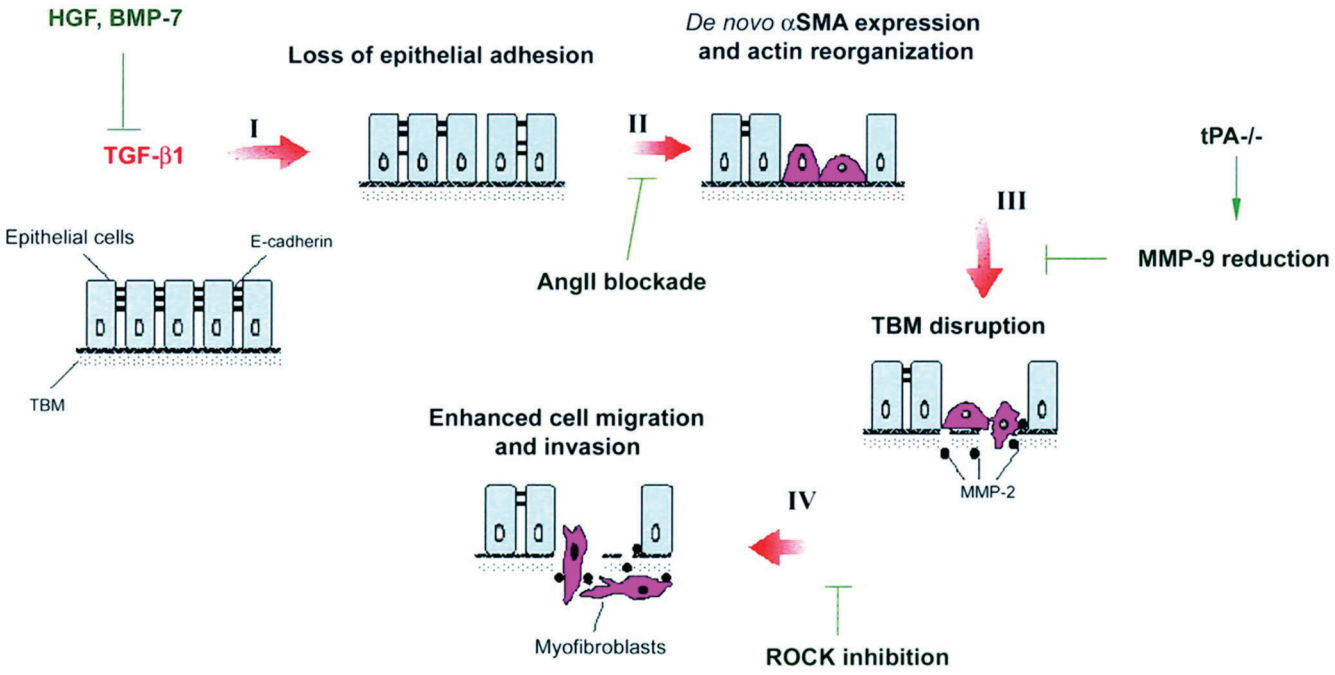
The myofibroblast was initially described as a reversibly activated fibroblast found in healing wounds that exhibits contractility needed for scar retraction and strong expression of αSMA. Subsequently, this same myofilament protein (αSMA) was demonstrated in most fibrotic processes in multiple tissues from various species [44,45]. Faulkner and colleagues [46] questioned if prior renal injury accelerates the progression of glomerulo-sclerosis and interstitial fibrosis caused by sustained renal vascular injury. Glomerular injury was induced in rats by Habu venom; immediately thereafter they were exposed to continuous infusions of angiotensin II. End-stage renal disease and severe fibrosis developed in 14 days and the combination treatment was more damaging than either one alone. Within 24 to 48 hours, αSMA(+) myofibroblasts appeared in the peritubular interstitial spaces, while αSMA(–), Na+,K+-ATPase(+), Texas red-dextran labeled RTE was excluded. Over the next two weeks the tubular cell loss was seen to result from encroachment by interstitial myofibroblasts; not by EMT [46].

The origins of myofibroblasts have not been established, but it's unlikely that they originate from a single source. An earlier study with bone marrow chimeras and transgenic reporter mice showed that 36% of the new fibroblasts responsible for renal fibrogenesis came from local EMT, 14% to 15% came from the bone marrow and the rest from local proliferation [1]. Thus, a failure to identify a 'final common pathway' probably reflects differences in the kinds of insults used to create the individual renal injury.

## Fibroblasts, fibrosis and systemic sclerosis

Until recently, scleroderma research focused mainly on the unique nature of the systemic sclerosis (SSc) fibroblast, its ability to produce ECM molecules, especially collagens, and the responsible growth factors, especially TGF-β [47-49]. Lately, the emphasis has shifted, prompted by recognition of the heterogeneity in the origins and phenotype of fibroblasts [50]. But, as with renal fibrosis, opinions about the SSc fibroblast vary. Postlewaite and colleagues [51], in an admirable review, elaborated the prevailing theories and, based on studies from their own laboratory, suggested that conventional, circulating CD14(+) monocytes transdifferentiate into SSc fibroblasts. Another cell, the fibrocyte, initially described in the context of wound repair, can participate in granuloma formation, antigen presentation and is a source of contractile myofibroblasts found in a variety of fibrosing lesions [52] (discussed below). British workers favor a link between vascular damage (an essential requirement in any scheme of SSc pathogenesis) and the formation of myofibroblasts from pericytes [53]. The latter are derived primarily from mesenchymal cell precursors. Under the influence of various growth factors they become either endothelial cells (vascular endothelial growth factor) or pericyte/smooth muscle

**Figure 4**




Schematic illustration of the key events of epithelial-mesenchymal transition (EMT) involving the renal tubular basement membrane (TBM) and possible therapeutic interventions. The diagram illustrates four key events essential for the completion of EMT: loss of epithelial adhesion properties; *de novo* alpha smooth muscle actin (αSMA) expression and actin reorganization; disruption of TBM; and enhanced cell migration and invasion capacity. Transforming growth factor (TGF)-1 alone is capable of inducing tubular epithelial cells to undergo all four steps. Strategies to block any steps during EMT would have a major impact on EMT and, thereby, on renal fibrosis. For instance, hepatocyte growth factor (HGF) and bone morphogenic protein (BMP)-7 could antagonize TGF-1 and consequently inhibit the initiation of EMT (step 1). Blockade of angiotensin (Ang)II by losartan abolishes its activity as an EMT promoter and attenuates renal fibrosis (step 2). Preservation of TBM integrity in tPA–/– mice selectively blocked EMT in obstructive nephropathy (step 3). Finally, pharmacological inhibition of ROCK kinase impairs cell migration and reduces renal fibrosis (step 4). MMP, matrix metalloproteinase. (Adapted from [35].)

cells (platelet-derived growth factor-BB) [54]. A monoclonal antibody, STRO-1, identifies a subpopulation of bone marrow stromal cells that give rise to fibroblasts (colony forming units [CFUs]) [55]. Yet the same antibody applied to rheumatoid arthritis (RA) synovium only stains periadventitial vascular cells (pericytes) (Figure 5). Pericytes provide structure to blood vessel walls, synthesize basement membrane proteins, and regulate blood flow and vascular permeability. In their capacity as primitive mesenchymal cells, pericytes can be a source of several tissues, including cartilage and bone [56,57]. Thus, both tissue fibrosis and ectopic calcification (features of SSc) could be attributed to pericytes.

Human myofibroblasts reside in a fraction of fibroblasts that react with Thy-1 antibody [58]. Myofibroblasts are the hallmark of idiopathic pulmonary fibrosis [58,59]. Rat alveolar epithelial cells exposed *in vitro* to TGF-β for 6 days develop a fibroblast morphology and molecular markers associated with EMT. This effect is enhanced by tumor necrosis factor (TNF)-α [59]. Cells co-expressing epithelial markers and αSMA are abundant in lung tissues from idiopathic pulmonary fibrosis patients. Mice with a targeted deletion of Smad3, a critical molecule in the TGF-β signaling pathway, fail to develop EMT and tissue fibrosis in experimental models of pulmonary, renal, liver, ocular and radiation induced skin injury [60].

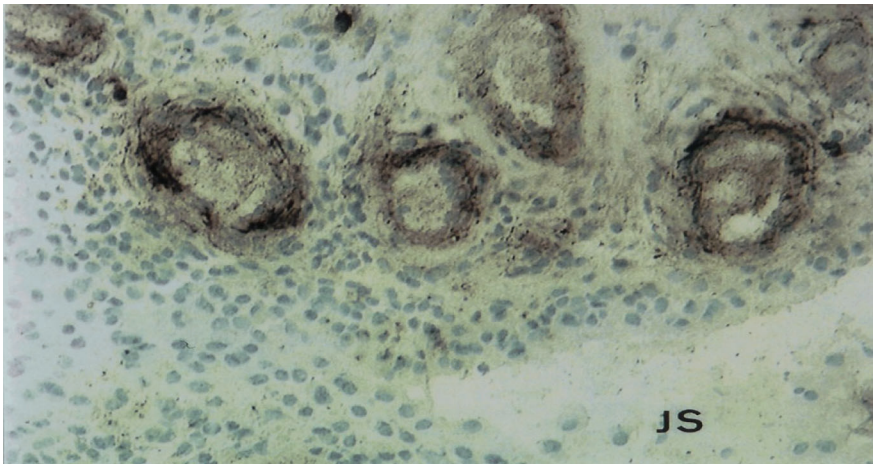
Overexpression of the inhibitory Smad7 protein or treatment with a small molecule inhibitor of Smad 3 reduces the fibrotic response in all of these animal models (including murine systemic lupus erythematosus) and holds out a promise for treatment of pathological fibrotic human diseases [60,61].

EMT cannot explain all fibrotic conditions, however. Bleomycin treatment is complicated by pulmonary fibrosis, akin to SSc. Repeated local injections of bleomycin induces a murine model of scleroderma [62]. Yet *in vitro* studies of alveolar epithelial cell lines and immunohistochemical analysis of pulmonary fibrosis from bleomycin-treated mice and rats show no features of EMT [63].

## Rheumatoid arthritis as a disease of stroma?

The tissue invasion and destruction of cartilage and bone by stromal elements (known as pannus) as seen in RA joints is often compared to cancer. HG Fassbender, a student of RA

**Figure 5**




Microscopic sections of synovial tissue from a patient with rheumatoid arthritis stained with the STRO-1 antibody. In the bone marrow STRO-1 reacts with stromal elements that are progenitors of fibroblasts (CFU-F) [55], but staining in the synovium is limited to perivascular mesenchymal cells (pericytes). JS, joint space.

pathology, remarked on the changes in the synovial stroma: "Normally this consists of loosely arranged collagen fibers with a small number of spindle shaped fibrocytes. In association with exudation of fibrin the local connective tissue cells proliferate. These cells may resemble the cells of the surface layer to such an extent that recognition of separate layers becomes impossible. In particularly gross examples, these large cells may lie so close together that any interstitial substance becomes unrecognizable" – he called this appearance "mesenchymoid transformation" (figures 124 to 126 in [64]). More recent research on RA pathogenesis has concentrated on the immuno-hematological and angiogenic elements found in the synovium. Mast cells, important in modifying ECM by elaborating proteases and tryptic enzymes, are reviewed elsewhere [65]. Only in the past 10 to 15 years has the import of synovial fibroblasts, lining cells and other mesenchymal elements been reconsidered [66-69].

How might such cells contribute to the pathogenesis of joint inflammation and bone and cartilage destruction? First, by their sheer bulk and metabolic needs. Most standard texts report that the number of intimal cells (fibroblast like synoviocytes (FLSs)) increase with inflammation from a few cells to 8 to 10 lining cells. But this is only what can be seen in thin (5 to 6 micron) histological sections. In reality, however, even in a large joint like the knee, the normal synovial membrane is a thin, filmy structure weighing just a few milligrams, whereas the inflamed, redundant synovium that is removed at surgery can weigh kilograms, a million-fold increase over normal. Much of the increased weight results from tissue edema, hypervascularity and the ingress of numerous blood cells, but tissue fibroblasts and FLSs also make a significant contribution

Second, fibroblasts are not inert cells. They both make and degrade matrix elements, especially collagen and fibronectin, into numerous bioactive peptides. Fibroblasts operate through both cytokine independent and dependent pathways; they recruit and stimulate T cells and monocytes by the production of chemokines, especially IL-6 and SDF-1 (CXCL12) and they can attract and retain B lymphocytes by B cell activation factor of the TNF family (Blys) production. Fibroblasts are antigen presenting cells and elaborate numerous pro-inflammatory cytokines, including TNF-α and IL-1 (detailed in [68]).

What accounts for the massive increase in fibroblasts? Knowledge of their origins, or the origin of any RA stromal element, is incomplete. Local proliferation of resident fibroblasts responding to the inflammatory milieu of the RA synovium is certainly a possibility [66]. This explanation was initially invoked, then rejected, and later reconsidered, but proliferation alone cannot account for all of the increase. Subsequently, a prolonged life span of FLSs was recognized (reviewed in [68,69]), although even a combination of enhanced proliferation of the normally slow growing FLSs plus defective apoptosis seems an insufficient explanation.

What about EMT? Several factors that can modulate fibroblast formation are found in the RA synovium, either as genes or proteins; for example, large amounts of both latent and activated TGF-β I and II are present in RA synovium and synovial fluids [70-72]. Rheumatoid articular tissues have mesenchymal appearing cells that stain with an antibody to phosphorylated Smad 2/3, suggesting engagement of TGF-β receptors and activation of ECM through the TGF-β signaling pathway [73] (Figure 2). Myofibroblasts and/or cells that react with an antibody to αSMA are absent from normal or

osteoarthritis synovium, but are detected in a proportion of synovial fibroblasts [74,75]. Common constituents of the ECM, such as MMPs and hyaluronan, can stimulate fibroblast formation through EMT. For example, ectopic expression of MMP3 (also known as stromelysin-1) in normal epithelial cells induces a fibroblast-like phenotype by mediating transcriptional upregulation of Rac-1b and enhanced production of reactive oxygen species. This results in genomic instability and increased expression of the Snail transcription factor. Snail down modulates E-cadherin and initiates the EMT cascade [76] (Figure 3). Hyaluronan (a major glycosaminoglycan of the ECM) is critical for EMT in the embryo [3]. It can induce a fibroblast morphology, anchorage independent growth, loss of adhesion molecules at cell junctions, upregulate vimentin expression in epithelial cells and supports tumor growth and invasion *in vivo* [77,78]. However, there are some important reservations about the role of EMT in the synovium because: very few cells have epithelial features; classical E-cadherins are scant; and the synovial lining lacks a basement membrane [79]. Normal FLSs probably stick together through homotypic adhesion mediated by a newly described molecule, cadherin 11 [80], whose regulation and role in the RA synovium is currently under investigation [81].

Since neither increased proliferation, inadequate apoptosis, nor EMT is responsible for the accumulation of fibroblasts in the joint, how do we explain abnormalities, quantitative or qualitative, of the articular stroma? The ingress of mesenchymal elements or their progenitors must be considered. There is certainly a precedent, because most inflammatory, immunological, and angiogenic cells in the synovium come from the blood. Are there such mesenchymal cells? One candidate is the fibrocyte, a marrow derived cell of hematopoetic lineage, thus CD34+, that circulates in the blood and responds to inflammatory cues [52]. Fibrocytes participate in wound healing [82], are thought to be responsible for the thick, hard skin seen in some dialysis patients with renal insufficiency (nephrogenic fibrosing dermopathy) [83], and could have a role in other fibrotic disorders [51]. However, fibrocytes have not been reported in synovial tissues and their numbers in the blood of RA patients are not different from normal individuals (NJZ, personal observation).

A second candidate, a mesenchymal stem cell (MSC) or mesenchymal progenitor cell (MPC), resides in the bone marrow [84], circulates in the blood [85], and has been found in a variety of normal tissues, including periarticular marrow, periosteum and synovium [86-89]. MSCs/MPCs are CD34(–) and lack a single, defining antigen, but can be phenotyped by a combination of cell surface markers, including thy-1 (CD90), endoglin (CD105), ALCAM (CD166) [84], and receptors for low affinity nerve growth factor (LNGFR1) and BMP (BMPR1A and II) [89]. Cells with these features are present in joints. Marinova and colleagues [90] recognized a small population of large, adherent, stromal-appearing cells in primary cultures of inflammatory joint effusions. These stained with antibodies to mesenchymal elements (collagen I, vimentin, αSMA and BMP receptors), and maintained this phenotype through multiple passages in tissue culture [89]. An anti-BMPR II antibody reacted with 11.6% of the FLSs from RA synovial fluids (passages 3 to 6), but only 2% from non-inflammatory osteoarthritis fluids. BMPR IA and II expressing cells were identified in RA synovial tissues – approximately 25% of intimal lining cells and 7% in the sublining tissues. Strong staining was seen at the advancing front of pannus and sites of bone erosions [90].

Jones and colleagues [91] used a fibroblast CFU assay to quantify MPCs in synovial effusions from various kinds of arthritis (53 RA, 20 osteoarthritis, 27 miscellaneous). Unlike the earlier study [90], the numbers of MSCs per ml of synovial fluid was higher in osteoarthritis than in RA effusions. Fibroblasts from synovial fluids had trilineage potential and under appropriate conditions could be induced to become either fat, cartilage, or bone cells. The synovial fluid fibroblasts stained with standard mesenchymal cell antibodies. Rare cells expressed the low affinity nerve growth factor receptor. Whether they are the same as the BMPR(+) cells remains to be determined. The authors interpreted their findings as evidence that the MSCs were derived from "injured joint structures" (i.e., cartilage) [91]. Synovial tissues were not examined in this study.

Patients with a diagnosis of RA differ from each other in many ways: clinical features, disease course, response to treatment, serologies and synovial immunohistology can all be cited. Of late, cDNA microarray technology has identified distinctive profiles among articular tissues from RA subjects and the relationship of particular genes to specific disease features is being examined [75,92-95]. Given the complex cellular makeup of RA synovitis, the finding of different gene patterns in intact synovial tissues is not surprising. Less anticipated have been the differences found in presumably homogeneous FLS 'lines' [75,92,94,95].

But how 'homogenous' are FLSs from intact synovial tissues? Several potentially confusing methodological problems must be recognized. Typically, synovium obtained either by arthroscopic biopsy or at joint surgery is enzymatically digested, disrupted, and maintained as single cells in tissue culture. The cells that adhere and grow are designated as FLSs, but no markers exist to indicate whether they originated as lining cells or came from subintimal stroma. Death and attrition eliminate blood cells in the cultures. Leukocytes and non-adherent lymphocytes go first, but monocyte/macrophages remain through several passages, during which time the slow growing fibroblasts are exposed to their cytokines and growth factors. To minimize contamination with other cells, FLS analysis is usually performed around the fourth passage or later. But the question arises: are changes observed at that time inherent to all the fibroblasts or did they develop during culture?

What is the impact of inflammatory cells present at the initiation of the culture on subsequent features of the FLSs? For instance, certain genes are found in FLSs from inflamed RA synovial tissues, but they differ from genes in the FLSs from non-inflammatory RA lesions. Were these genes induced *in vivo* or could products from the inflammatory cells in the primary culture (*in vitro*) have influenced them? Zimmermann and colleagues [96] used negative selection with anti-CD14 magnetic beads to obtain a relatively clean population of RA FLSs (passage 1). These differed considerably from conventional fourth passage FLSs in phenotype and proliferation rates. Thus, depending on the isolation procedures, gene arrays might also be different.

Do culture conditions modify FLSs? The growth of FLSs maintained at low density is faster than in high density cultures because proliferation is impeded by contact inhibition. For instance, Masuda and colleagues [97] compared the molecular profile of the same RA FLSs cultured at low density (proliferating) and high density (quiescent). Certain genes were only identified in the low density-proliferating cells. For some this was not a tissue culture artifact, because the genes were present in intact RA synovium, as confirmed by *in situ* hybridization The authors concluded, however, that the expression of many other genes likely depends on the stage of FLS proliferation in the culture. If FLSs are heterogeneous, then might certain culture conditions favor the expression of one subpopulation over another? For instance, low cell density, selected growth media, and low oxygen tensions are known to favor rapidly growing MSCs [98].

Might a small number of 'activated' or 'aggressive' FLSs present in a primary culture (passage 1) overgrow other elements and appear as a major population in later (passage 4) cultures? Is either normal or osteoarthritis synovium an appropriate control for RA synovitis or should RA synovium only be compared to other forms of chronic inflammatory synovitis? And might the influence on gene profiles depend on the stage and duration of the disease or prior treatment? Finally, the RA pannus invading cartilage and bone needs to be analyzed for unique mesenchymal elements, perhaps analogous to the CAFs found in the tumor stroma. For instance, there is evidence that cells isolated from RA tissues eroding cartilage have a distinctive morphology and features of both FLSs and chondrocytes (pannocytes) [99,100]. They also are oligoclonal, whereas non-erosion FLSs are polyclonal [101]. Might pannocytes have a different profile of chemokines and tumor suppressor genes?

With these caveats in mind, several recent studies should be considered. Evidence for genetic heterogeneity of FLSs obtained from individual RA patients was described by Kasperkovitz and colleagues [75]. Employing gene arrays they identified two distinctive patterns in multipassaged RA synovial fibroblasts. The FLSs from highly inflamed RA synovium had significant up-regulation of genes associated with immune activity and high expression signatures of several genes in the TGF-β signaling pathway, as seen in myofibroblasts. The molecular features that identified myofibroblasts were confirmed by immuno-histochemistry of cultured FLSs and in companion synovial tissues, which makes it less likely that the findings were artifactual. Material from a second group of RA patients with little inflammatory synovitis had a gene profile consistent with low immune activity and increase in the insulin-like growth factor/insulin-like growth factor binding protein pathway. The idea of two separate pathogenic mechanisms in RA synovitis – one T cell mediated and the other a T cell independent (stromal?) pathway – has been proposed before [69]. But linking the immune (T cell) activated pathway to TGF-β (which is associated with myofibroblast formation and stromal activation) is counter-intuitive, given that TGF-β is known to suppress a number of T lymphocyte functions [72]. Perhaps differences in stroma are dictating the type of cells found in the joint?

Evidence in support of differences in the stromal elements in some RA patients comes from an analysis of synovial tissue samples from 17 early RA patients, obtained prior to disease-modifying anti-rheumatic drug (DMARD) therapy. These were examined by immunohistochemistry and microarrays [102]. In both whole tissues and FLS cultures, two clearly separate groups were identified. Samples from 10 patients had very high co-expression of genes encoding MMP1 and MMP3 and a collection of nuclear factor κB genes. Increased expression of these genes was not identified in tissues from the other seven patients. Other MMPs, cytokine, chemokine, and T and B cell related genes were similar in the two sets of patients and no other clinical, serological, or histological features distinguished them. Long-term follow-up will be needed to see if the two groups have a different outcome.

The idea that cells behave in a context-dependent manner and that stromal elements can modify the behavior of carcinomas (described above) is provocative. Can this be translated to RA synovium?

As noted by Fassbender, there is considerable histological evidence of stromal abnormalities [64]. Significant differences in cell cycle related gene products were found in synovial stroma and lining cells in tissues from RA patients with active compared to quiescent disease [103]. RA synovial tissues obtained by arthroscopic biopsy before and 10 months after adalimumab treatment were analyzed by western blot and histochemistry with antibodies to phosphorylated Smad1-5-8.9 [73]. A variety of p-Smad positive mesenchymal appearing cells were identified in synovial sections located around blood vessels (pericytes?) and in the stroma. The mononuclear cells in the pretreatment biopsies were reduced after anti-TNF therapy, but Smad staining was unchanged. Joint inflammation usually recurs soon after stopping anti-TNF

agents. Is that because even after anti-inflammatory treatment a unique stromal environment remains, which attracts and retains inflammatory and immunological cells; a view championed by Buckley and Salmon [104]? If this were the case, then therapies that modify the mesenchymal elements of the synovium will be needed.

## Conclusion

This review is meant to introduce the rheumatological community to a rapidly emerging area of great biological and medical interest. References were not selected for the *cognoscenti* and are not comprehensive. Rather they were chosen to stimulate the reader unfamiliar with this area of inquiry. Thus, many are recent reviews or commentaries. Only time will tell how these concepts of the stroma and EMT will influence future thinking about the pathogenesis and treatment of rheumatic diseases. But new viewpoints are always worth considering, for as John Maynard Keynes famously said, "the difficulty lies not so much in developing new ideas, as in escaping from the old ones."

## Competing interests

The author declares that they have no competing interests.

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