[Frontiers in Bioscience 5, d594-601, June 1, 2000]

# CELL CYCLE IMPLICATIONS IN THE PATHOGENESIS OF RHEUMATOID ARTHRITIS

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## 1. ABSTRACT

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by hyperplasia of the synovial lining cells, angiogenesis, and infiltration of mononuclear cells resulting in pannus formation, cartilage erosion and ultimately joint destruction. Synovial tissue (ST) fibroblast hyperplasia is reminiscent of tumor-like proliferation and is a major cause of cartilage destruction in the RA joint. The RA joint is replete with cytokines and growth factors which exert a synergistic mitogenic effect on ST fibroblasts. As a result, RA ST fibroblasts exhibit elevated gene expression of proto- oncogenes, such as *c-Myc, c-Ras, and c-Jun* and apoptosis inhibitors such as Bcl-2. At the same time, RA ST fibroblasts contain mutations in tumor suppressor genes such as p53. The altered rates of proliferation and apoptosis of RA synovial cells result in the hyperplasia of synovial tissue and in concert with the chronic inflammatory environment ultimately lead to the destruction of the RA joint.

## 2. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic disease characterized by polyarticular inflammation in which hyperplasia of the synovial lining cells and pannus formation are observed, along with angiogenesis and infiltration of inflammatory mononuclear cells. Erosion of articular cartilage occurs predominantly in areas adjacent to proliferating cells, suggesting that these cells release proteolytic enzymes capable of degrading the collagens and proteoglycans of cartilage and bone (1). Synovial hyperplasia has been characterized as a tumor-like proliferation and is thought to be a major cause of destruction of the RA joint.

In RA and animal models of arthritis, transformed-appearing cells with large pale nuclei, prominent nucleoli and abundant cytoplasm typically are found adjacent to the affected cartilage and bone of the joint (2). RA synovial tissue (ST) fibroblasts in culture, have a tendency to grow in disorganized monolayers, proliferate in an anchorage-independent manner, lack contact inhibition, and form microfoci (3-6). Many RA ST fibroblasts are multinucleated and have the ability to form colonies in soft agar (6). RA ST fibroblasts co-implanted with cartilage into SCID mice autonomously invade the cartilage matrix, while ST fibroblasts from patients with osteoarthritis (OA) or from non-arthritic patients do not (7). ST from patients with RA can form short-lived tumor-like nodules when implanted into nude, athymic mice (6,8). Finally, in RA ST, but not OA ST, the inactivation pattern of X chromosome-linked phosphoglycerate kinase-1 gene indicates a monoclonal or oligoclonal cell expansion (9). All these studies demonstrate that RA ST fibroblasts are actively in the cell cycle and have properties of transformed cells.

The RA synovial environment is replete with cytokine and inflammatory mediators which have been described as exerting a synergistic mitogenic effect on ST fibroblasts (10). In RA ST fibroblasts, abnormal gene programs for nuclear proteins including protooncogenes, are activated, resulting in altered rates of proliferation and invasion of cells into the periarticular cartilage and bone. This chronic inflammatory milieu also induces DNA damage within synoviocytes, increasing the expression of DNA repair genes such as p53 and ultimately dominant negative mutations of p53, resulting in increased mutant cell proliferation and greater joint destruction (11). Thus, the RA synovial environment may be responsible for the changes in ST fibroblasts resulting in cells entering the cell cycle and releasing destructive molecules into the joint.

**Table 1. Expression of cyclin inhibitors by ST fibroblasts**

| Cyclin Inhibitors | Expression in ST fibroblasts |
|---|---|
| **Ink4** | |
| • p15 | No |
| • p16 | No* |
| • p18 | ND |
| • p19 | ND |
| **Cip/Kip** | |
| • p21(Cip1) | No** |
| • p27(Kip1) | Yes |
| • p57(Kip2) | ND |

* Inducible in RA, OA, or non-arthritic ST fibroblasts upon growth inhibition. ** Inducible only in RA ST fibroblasts upon growth inhibition. ND, Study not performed

## 3. RA AND THE CELL CYCLE

The mammalian cell cycle is made up of four stages, a growth phase (G0/G1), a DNA synthesis phase (S), a second growth phase (G2), and finally mitosis (M). Cell progression through the cell cycle is regulated by cyclin-dependent kinases (Cdks) and their regulatory subunits, the cyclins, which catalyze transitions into S phase and mitosis (12,13). Cyclins and Cdks have preferential binding patterns to each other and the resulting Cdk/cyclin complexes are active at various stages throughout the cell cycle. In G0/G1 the hypophosphorylated tumor suppressor protein, retinoblastoma protein (Rb), is bound to the transcription factor, E2F, resulting in the suppression of its transcriptional activity. In mid to late G1 phase, cyclin D/Cdk4 and/or cyclin D/Cdk6 are activated and hyperphosphorlylate Rb resulting in the release of the bound transcription factor, E2F (14). E2F then initiates transcription of cyclin E and other genes needed to initiate S phase. Cyclin E binds Cdk2 and the resulting active kinase, cyclin E/Cdk2, phosphorylates Rb, Cdk inhibitor p27 (Kip 1), and additional substrates required for DNA replication (15). During the S and G2 phases, cyclin E is degraded and cells accumulate cyclin A/Cdk2 and cyclin B/Cdk2 maintaining Rb in it hyperphosphorlyated state and triggering entry into M phase and the completion of the cell cycle.

The activation of cyclin/Cdks is carefully regulated as DNA replication and mitosis are irreversible processes. Cyclins are synthesized only at specific times within the cell cycle and Cdks must be phosphorlyated on certain residues and inactivating phosphates must be removed on other residues in order to activate the cyclin/Cdk complex (16). In addition two families of cyclin dependent kinase inhibitors have been identified, inhibitors of Cdk4 (INK4) and Cdk2-interacting proteins (Cip/Kip). The INK4 family consists of p15, p16, p18, and p19 and is specific in the inhibition of Cdk4 and Cdk6, while the Cip/Kip family consists of p21 (Cip1), p27 (Kip1), and p57 (Kip2) and inhibits a broad range of Cdks (16). In the presence of INK4 proteins, cyclin D/Cdk4 and cyclin D/Cdk6 are inhibited, freeing Cip/Kip family members to bind and inhibit cyclin E/Cdk2, resulting in cell cycle arrest in the G1 phase (17). In response to a mitogenic signal, cyclin D/Cdk4 and cyclin D/Cdk6 bind to Cip/Kip family members preventing them from binding and inactivating cyclin E/Cdk2 (18). Removal of mitogens stops cyclin D synthesis, increasing the amount of Cip/Kip family proteins, resulting in the inhibition of cyclin E/Cdk2 and G1 arrest.

In RA ST very little is known about the role cyclin/Cdk complexes play in regulating the cell cycle (Table 1). One study showed that ST from RA, OA, and non-arthritic patients expressed p27 but not p21, p16, or p15 (13). This study also showed that growth inhibited RA, OA and non-arthritic ST fibroblasts could all be induced to express the Cip/Kip family member, p21, but only RA ST fibroblasts could be induced to express the INK4 family member, p16. Subsequent experiments by the same group, demonstrated p16 inducibility in growth inhibited RA ST fibroblasts was at least partially mediated through the Cip/Kip family member p21 (19). Since the expression of p16 was specifically induced in RA ST fibroblasts upon growth inhibition, the authors next showed that an adenoviral vector containing p16 could be used *in vitro* on RA ST fibroblasts to inhibit their growth. In an adjuvant-induced arthritis rat model of RA, therapeutic injection of adenovirus encoding p16 into the afflicted joint efficiently inhibited arthritis. Future studies should address the expression of the remaining INK 4 and Cip/Kip family members, p18, p19, and p57 in RA ST and ST fibroblasts.

During each cell cycle, specifically during chromosomal replication telomeric DNA is lost at the chromosomal ends. This process of shortening telomeric DNA is believed to be a timed mechanism resulting in cellular senescence (20). Telomerase is a ribonucleoprotein polymerase that maintains chromosomal length by synthesizing telomeric DNA at the ends of chromosomes. Cells with telomerase activity acquire the capacity for indefinite growth (21). Greater than half of RA STs have telomerase activity while OA STs have no telomerase activity (22,23). This telomerase activity in RA STs was specific to mononuclear infiltrating cells and not to RA ST fibroblasts. Another study confirmed that RA ST lymphocytes had high telomerase activity and that RA ST fibroblasts had no telomerase activity (24). This study however also found slight telomerase activity in OA ST lymphocytes. Thus, telomerase activity is elevated in RA ST infiltrating monocytes and lymphocytes but not in RA ST fibroblasts. This is contrary to what one would expect for RA ST fibroblasts given their transformed phenotype.

## 4. THE P53 TUMOR SUPPRESSOR GENE

The p53 tumor suppressor gene is a nuclear phosphoprotein that serves as a critical regulator of cell survival and proliferation. p53 is a sequence specific DNA binding protein which initiates and facilitates DNA repair and apoptosis. p53 functions as a transcriptional activator of genes that block progression from G1 to S phase in mammalian cells (25). p53 gene expression is induced by DNA damage resulting in cell cycle arrest, allowing time for DNA to be repaired or in cases of extensive DNA

damage to apoptose (26). P53 expression levels are extremely low in normal cells and it has a very short half life (<20 minutes) (27-29). Proto-oncogenes including *c-myc* can control p53 transcription (30). The loss of p53 is often associated with neoplasia *in vivo* and cell transformation *in vitro* (31,32).

In RA ST lining, expression of both wild type and mutant p53 are increased relative to ST lining of OA or non-arthritic patients (33). RA ST fibroblasts also express more p53 protein than do OA ST fibroblasts or dermal fibroblasts (34-38). P53 expression in RA is believed to be induced by DNA strand breaks and *c-myc* expression (34,39). Although the RA synovial environment is replete with cytokines [ie. tumor necrosis factor-alpha (TNF-alpha)] and oxygen free radicals, which are potentially responsible for a synovial lining DNA fragmentation rate of almost 50% of cells *in situ*, only a rare cell completes apoptosis (34,36-38). This suggests ineffective apoptosis possibly due to abnormal p53 (26). Somatic mutations similar to mutations previously identified in human tumors, occur in the p53 gene in 40% of RA ST and in cultured ST fibroblasts (33,40). This significant rate of mutation of the p53 gene in RA supports the hypothesis that there is a dysfunctional cell cycle in RA ST fibroblasts. Recently, two RA ST mutant p53 genes were shown to be dominant negative mutations (11). Another group inhibited RA ST fibroblast p53 by virally administering E6 protein and showed that infected ST fibroblasts had increased growth, increased invasiveness into cartilage extracts, and were more resistant to oxidant-induced apoptosis *in vitro* (26). Recently, this same group showed increased cartilage invasion of E6 virally transduced ST fibroblasts when co-implanted with non-arthritic human cartilage into severe combined immunodeficient mice (41). In contrast to the previous reports, Kullmann and coworkers found no specific p53 mutations in the genome of RA ST fibroblasts from German patients (42). While the exact percentage of mutant p53 which is present in RA ST has not been precisely determined due to the lack of antibodies specific for only mutant p53, the presence of mutant p53 identifies it as a critical regulator of RA ST fibroblasts proliferation, apoptosis, and invasiveness. Future experiments characterizing the function of specific p53 mutations found in RA ST fibroblasts and identifying the percentage of RA patients with these mutations will better define the role of mutant p53 in the pathogenesis of RA.

## 5. PROTO-ONCOGENES AND EARLY RESPONSE GENES

Proto-oncogenes encode proteins that are involved in cellular growth and differentiation. Normal expression of cellular proto-oncogenes takes place during predetermined steps of mitosis and cellular differentiation (2). Proto-oncogene products have a variety of functions including acting as cell to cell and cell to matrix ligands, growth factors, growth factor receptors, triphosphate-binding proteins, cytoplasmic regulators, intracellular protein kinases, and nuclear transcription factors (43).

c-*myc* family members encode DNA-binding nuclear phosphoproteins that function as transcription factors and are critical signals that initiate cell proliferation (2). *c-myc* induction is observed in quiescent cells upon mitogen induction and is associated with the transition from G0 to G1 phase in these cells (2). In RA, approximately 30% of ST fibroblasts are positive for Myc protein (6,39,44). *c-myc* gene product expression was demonstrated in an abundant number of proliferating synovial lining cells at the site of cartilage and bone destruction (6). In a rat model of RA, streptococcal cell wall- induced arthritis, *c-myc* was expressed by ST fibroblasts in culture and in the synovial lining layer *in vivo* (45). Hashiramoto and coworkers, using a *c-myc* antisense oligodeoxynucleotides, inhibited RA ST fibroblast proliferation and induced apoptosis *in vitro* (10).

The *c-ras* family encodes second messenger molecules which act as nucleotide-binding regulatory proteins similar to G proteins (43). Ras is a central mediator of growth factor-induced cell proliferation and differentiation and is required throughout G1 phase and is essential for S phase progression of fibroblasts (46). Specifically, *c-ras* can increase cyclin D expression and activate another oncogene, *raf*, a serine/threonine kinase (2). In RA, Ras protein was detected by immunohistochemistry, at similar expression levels as Myc, in the synovial lining layer at the site of invasive growth and in endothelial cells of synovial terminal vessels, while it was absent in OA ST (47).

The c-*jun* family of proto-oncogenes are classic immediate early response genes based on their rapid, large, and transient increase in transcription following stimulation of cells with mitogens (48,49). Both c-*jun* and *jun-b* encourage the growth of fibroblasts, promoting cells into S and $G_2$/M phases, while overexpression of *jun-d* results in slower fibroblast growth and an increase in the percentage of cells in $G_0/G_1$ (50). Jun along with Fos form the heterodimeric activation protein-1 (AP-1) which regulates the production of tissue degrading molecules, especially matrix metalloproteinases (51,52). In RA, *c-jun* expression is induced in about 50% of STs from RA patients and *c-jun* is constitutively expressed in ST fibroblasts *in vitro* (53). ST fibroblasts from RA patients have greater expression of *jun-b* compared to ST fibroblasts from patients with OA or joint trauma (54). Transfection with a plasmid containing *jun-d* inhibited the proliferation of and proinflammatory cytokine and matrix metalloproteinase production by RA ST fibroblasts mainly by inhibiting their transcription via down modulation of the AP-1 transcription factor (55). In RA, increased AP-1 activity has been reported in ST fibroblasts, macrophages, and T-cells, presumably triggered by cytokines such as TNF-alpha and interleukin (IL)-1, as the constitutive expression of *c-fos* and *c-jun* in RA ST most probably results from a continuing inflammatory stimulus (53,56). Overexpression of both proliferative, *c-jun* and *jun-b*, and downregulation of the inhibitory, *jun-d*, in RA synovial cells may contribute to synovial growth.

The Fos family of proteins can form dimers with Jun proteins to form AP-1, an early response gene and a transcription activator, which triggers phosphorylation of proteins in the nucleus (57). Growth factors including epidermal growth factor, fibroblast growth factor, platelet derived growth factor (PDGF), TNF and IL-1 mediate the cellular production of AP-1 (58). One relevant function of the translated Fos proteins in RA is their control of the activation of tissue degrading molecules such as collagenase and stromelysin (59,52). RA ST fibroblasts constitutively express *c-fos* resulting in greater levels of *c-fos* than expressed by OA ST fibroblasts (2,53). Fos proteins are present in 12% of RA synovial cells in tissue (44). RA ST fibroblast growth in culture was suppressed with antisense *c-fos* mRNA (60). Destructive arthritis spontaneously occurred in *c-fos* transgenic mice (61). Finally, AP-1 activity was upregulated in synovium of RA vs. OA and correlated with the expression of *c-fos* RNA *in situ* (62). Thus, RA ST fibroblasts express elevated levels of Fos and Jun leading to an increase in the activity of AP-1 which results in more degrading molecules and greater tissue damage.

*erg-1* is another family of early response genes which regulate the transcription of other proto-oncogenes such as *sis* and *ras* (2). In RA ST fibroblasts *erg-1* is significantly upregulated (1). *in situ* hybridization revealed *erg-1* mRNA expression in numerous cells of the proliferating RA synovium (2). As Egr-1 regulates the expression of other proto-oncogenes (like *sis* and *ras*), which are also overexpressed in RA synovium, it is thought to possibly be one of the initial steps in the pathogenesis of RA (2). Presently, in RA only the transcription of *egr-1* has bee studied, future research will need to focus on the expression of the Egr-1 protein to fully assess its role in the pathogenesis of RA.

## 6. APOPTOSIS

Apoptosis is a cell death pathway in which an apoptotic stimulus induces a series of events resulting in membrane blebbing, mitochondrial dysfunction, cellular shrinkage, chromosomal condensation, and DNA fragmentation into characteristic 180 basepair nucleosomal segments. Proliferation and apoptosis are coupled events in that cells proliferating uncontrollably undergo elimination via apoptosis (63). In RA, synoviocytes undergo very little apoptosis. The low level of synovial apoptosis has been shown by several groups using several techniques, including *in situ* end labeling of DNA to detect DNA fragmentation, and electron microscopy to identify cells with morphological features of apoptosis (36,37,64). In contrast, apoptotic cells were frequently seen in RA cartilage relative to non-arthritic cartilage (65).

The *B-cell lymphoma-2* (*Bcl-2*) family is a group of integral membrane proteins localized to the outer mitochondrial membrane (66). *Bcl-2* has been shown to inhibit apoptosis in several different type of cells and to be involved in cell cycle progression (67-71). In RA, the level of *Bcl-2* expression is controversial. Matsumoto and co workers showed that Bcl-2 protein is expressed in 1% of RA ST fibroblasts (64). In contrast, Liang and coworkers found 70% of RA ST lining cells express *Bcl-2* mRNA and Perlman and coworkers showed by immunohistochemistry high expression of Bcl-2 in RA ST fibroblasts (72,73). These differences in Bcl-2 expression may be due to different anti-Bcl-2 antibodies used in these studies. Bcl-2 temporal expression in rat adjuvant-induced arthritis was correlated with the absence of apoptosis (74). Rapamycin treatment of RA ST fibroblasts induced a decrease in Bcl-2 and rendered the cells more susceptible to Fas-mediated apoptosis (66). Fibroblast mitogens PDGF and transforming growth factor-beta1 (TGF-beta1) increased RA ST fibroblast proliferation and upregulated *bcl-2* (75). In contrast to ST fibroblasts, Bcl-2 expression is significantly lower in RA cartilage relative to non-arthritic cartilage (65).

Growth arrest-specific gene 6 (Gas6) is a multidomain protein containing both epidermal growth factor-like repeats and a sex hormone-binding globulin-like domain (76). Gas6 is produced by cells in the quiescent (G0) phase of the cell cycle and binds to the receptor tyrosine kinase Ax1 (77,78). O'Donnell and coworkers identified a novel function for the Gas6/Ax1 interaction in human umbilical vein endothelial cells as a protector from apoptosis (79). In the same report, both Gas6 and Ax1 were detected in RA and OA ST and Gas6 was also detected in RA SF. In RA ST Ax1 was expressed by endothelial cells, smooth muscle cells, and some synovial lining cells. This report concluded that the Gas6/Ax1 interaction may promote survival of endothelial cells and synovial cells in RA ST. Further studies are needed to better characterize the expression of Gas6 in RA ST and the effect of the Gas6/Ax1 interaction on the survival of other cells, including ST fibroblasts.

## 7. APOPTOSIS INDUCERS

Fas (CD95) is a type 1 membrane protein in the TNF-alpha receptor family that can promote apoptosis and is involved in clonal deletion (80-82). In RA ST Fas mRNA was present in endothelial cells and sublining scattered cells, while Fas protein was detected in 3% of ST fibroblasts (2,64). Whether RA ST fibroblasts undergo Fas mediated apoptosis is a matter of contention. Several groups have shown RA ST fibroblasts to be resistant to Fas mediated apoptosis and have hypothesized that additional proteins are required to induce Fas mediated apoptosis in ST fibroblasts (83,84). In contrast, other groups have shown Fas-mediated apoptotic cell death in RA ST fibroblasts (36,37). Mitogens and cytokines present in RA tissue such as PDGF, TGF-beta1, and IL-1beta inhibited Fas mediated apoptosis and increased proliferation of ST fibroblasts while downregulating Fas (75,85). There is also evidence that some Fas-mediated apoptosis is observed in ST fibroblasts of RA patients, but not OA or non-arthritic ST fibroblasts, despite equal expression of the Fas antigen on their cell surface (37). This study concluded that downstream signaling of the Fas molecule is responsible for the regulation of Fas-mediated apoptosis in RA ST fibroblasts. This signaling may involve multiple pathways as Fas ligation with an anti-Fas antibody induced a rapid phosphorylation of JNK (c-Jun amino-terminal kinase) and formation of AP-1, coinciding with apoptosis of RA but not

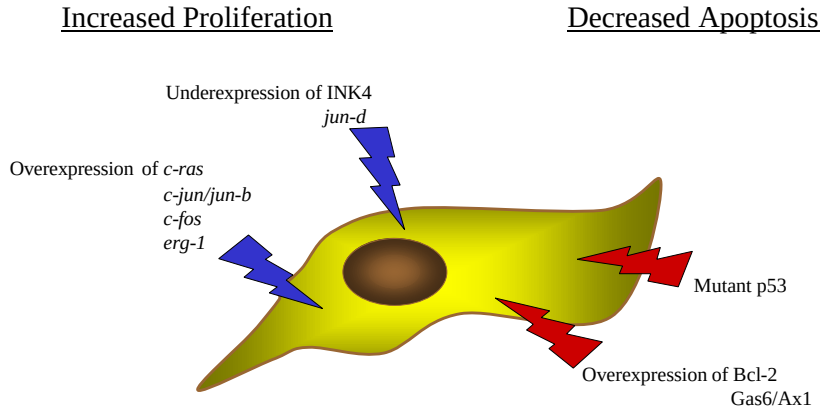


**Figure 1.** RA ST fibroblasts gene expression is in part responsible for their reduced proliferation (blue bolts) and decreased apoptosis (red bolts).

OA ST fibroblasts (86). Fas ligation also induced activation of Caspase-8, which is upstream of Caspase-3 activation, resulting in subsequent cleavage of poly(ADP-ribose) polymerase, reported to be involved in DNA repair (87-89). Alternatively, the source of the fas ligand may be the differentiating factor between RA and OA ST fibroblast undergoing apoptosis. Future experiments addressing the mechanism by which Fas mediates apoptosis in fibroblasts are need to clear up whether RA ST fibroblasts are capable of Fas mediated apoptosis.

## 8. THERAPIES

Future therapies for RA may include strategies to decrease ST fibroblast proliferation and increase ST fibroblast apoptosis. *In vitro*, antisense *c-fos* mRNA suppressed RA ST fibroblast growth (40). Adenoviral vectors containing the INK4 family member p16, when introduced into RA ST fibroblasts, inhibited their proliferation. The same p16 containing adenovirus efficiently inhibited arthritis in the adjuvant-induced arthritis rat model *in vivo* (13). Recently, ablation of Bcl-2 in ST fibroblasts by adenoviral-mediated hammerhead ribozyme induced apoptosis of these cells *in vitro* (90). *In vitro*, RA ST fibroblasts, which express Fas antigen, were induced into apoptosis by incubation with anti-Fas antigen antibodies (37,75,91-93). In animal models of RA, both anti-Fas antibody and an adenovirus expressing Fas ligand ameliorated experimental arthritis (92,94-96). Thus, RA future therapies may include viral vector delivery into the joint of apoptosis inducers and humanized antibodies against proto-oncogenes.

## 9. SUMMARY

In RA, ST fibroblasts take on a transformed phenotype, in which they are multinucleated and proliferate in an anchorage-independent manner. These cells clonally expand, aggressively invade cartilage matrix, and form tumor-like nodules. RA ST fibroblasts express several genes which are involved in the entry and progression into the cell cycle including c-myc, c-ras, c-jun, jun-b, c-fos, and erg-1 (Figure 1). While reports have shown some fas-mediated apoptosis in RA ST fibroblasts, generally these cells have an inability to undergo apoptosis. RA ST fibroblasts have increased Bcl-2 expression and mutated p53 expression, both of which may be responsible for the decreased apoptosis. The hyperproliferative, anti-apoptotic state of RA ST fibroblasts leads to potential therapeutic treatments involving inhibition of the cell cycle and the induction of apoptosis.

## 10. ACKNOWLEDGMENTS

We thank Dr. Harris Perlman for his insightful discussion. Support for this work was provided by NIH grants AI40987 and HL58695, the Veterans' Administration Research Service, and the Gallagher Professorship for Arthritis Research.

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**Key Words:** Rheumatoid arthritis, apoptosis, proliferation, synovial fibroblast, cell cycle, Review

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