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Original Article

A protocol for humanized synovitis mice model

Junlong Dang[1,2], Shangling Zhu[3], Julie Wang[2,4]

[1]*Department of Clinical Immunology, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China;* [2]*Division of Rheumatology, Department of Medicine, Penn State University College of Medicine, Hershey 17033, USA;* [3]*Department of Rheumatology, The Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou, China;* [4]*Division of Immunology and Rheumatology, Department of Internal Medicine, Ohio State University College of Medicine, Columbus 43210, USA*

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Abstract: Rheumatoid arthritis (RA) is a debilitating autoimmune disease that causes progressive chronic inflammation of the joints and destruction of articular cartilage and bone erosion. Cartilage destruction is a key characteristic in patients with RA. RA fibroblast-like synoviocytes (FLS) mainly contributes to local production of cytokines, inflammatory mediators and MMPs, and to migrate and destruct joint cartilage. Here, we summarized a detailed protocol for developing a humanized synovitis animal model. A cartilage-sponge complex without RA FLS was implanted under the left flank skin of a SCID mouse primarily, two weeks later, cartilage-sponge complex containing RA FLS was inserted under the right skin of the contralateral flank. The H&E staining clearly helps to identify the cartilage damage on the day 45 after second implantation. This model is highly significant to investigate the role and mechanisms of agents or cells in targeting RA FLS *in vivo*.

Keywords: Rheumatoid arthritis (RA), RA FLS, cartilage, humanized synovitis animal model

Introduction

Rheumatoid arthritis (RA) is a debilitating autoimmune disease that causes progressive chronic inflammation of the joints and destruction of articular cartilage and bone erosion [1-3]. It has been documented that interleukin 17 (IL-17) secreting CD4+ T helper (Th17) cells plays a key role in the pathogenesis and severity of RA [4-6]. Th17 cells contribute to bone destruction by increasing expression of receptor activator of nuclear factor-κB ligand (RANKL) and activating osteoclast [7]. Moreover, IL-17 exacerbates synovitis through promoting the production of pro-inflammatory cytokines including IL-1β, IL-6, tumor necrosis factor-α (TNF-α), and matrix metalloproteinases (MMPs) [7-9]. Therefore, targeting Th17 cells may relieve or delay the degree or severity of RA. Recently, we and others have documented that lots of candidates showed therapeutic effects on collagen induced arthritis (CIA) mice model, such as 1, 25-dihydroxyvitimin D3 [10], Apremilast (a phosphodiesterase 4 inhibitor) [11], regulatory T cells [12, 13], and human gingiva-derived mesenchymal stem cells (GMSCs) [14-16]. Other T helper or T effector cells, such as Th1, Th9, Th22 and T follicular helper cells may also directly or indirectly involve in the pathogenesis of autoimmune arthritis [17-20]. Conversely Treg cells have an opposite role in controlling Th17 cells and other pathogenic cells and eventually prevent and treat autoimmune arthritis and other diseases [21-23].

Bone loss is a hallmark of RA in 3 different forms: (1) joint margins and subchondral bone erosion; (2) periarticular bone loss adjacent to the inflamed joints; (3) systemic bone loss (osteoporosis) [24]. Normal bone remodeling is a balance of bone resorption by osteoclast and bone matrix synthesis by osteoblast [25]. However, osteoclast triggered bone remodeling balance losing appears early in the course of RA, which causes joint damage and functional capacity impairment [26]. Therefore, the correction of bone imbalance may alleviate bone erosion in RA. Indeed, we and others have reported that induced regulatory T cells [27] and GMSCs [28] can suppress osteoclast in CIA

Table 1. Reagents and material involved in this protocol

Items	Cat. No	Manufacturer
Absorbable Gelatin Sponge	1972	ETHICON
Surgical Gloves Powder-free	ZD72N70X	Cardinal Health
Drapes	GEM1154S	Medline
Disposable Skin Stapler	DS-15	Precise
Skin Staple Remover	DYNJ04058	Medline
Antiseptic Non-sterile solution (10% Povidone-iodine)	-	-
Ketamine (100 mg/ml)	NDC 50989-161-06	Keta Ved
Xylazine (20 mg/ml)	NADA#139-236	Ana Sed
Meloxicam	-	-

mice and thus protect joints from bone destruction.

In addition, cartilage destruction is a key feature in patients with RA. It is clear that synovial lining fibroblasts (also refer to fibroblast-like synoviocytes, FLS) in RA patients mainly contribute to local production of cytokines, inflammatory mediators and MMPs [29]. Importantly, RA FLS transform from quiescence cells to hyperplastic cells teeming with immunocompetent, and thus, are the primary effects of cartilage destruction due to their migration and invasion capacities and prodigious production of proteinases [29]. A lot of factors may contribute to the aberrant behaviors and properties. For instance, long non-coding RNA GAPLINC (LncRNA GAPLINC) may promote RA FLS proliferation, migration, invasion and pro-inflammatory cytokines production in a miR-382-5p dependent and miR-575 dependent manner [30]. Additionally, Sonic Hedgehog (SHH) signaling pathway is involved in proliferation and migration capacities of RA FLS *via* mitogen-activated protein kinases/extracellular-regulated kinases (MAPK/ERK) signaling pathway [31]. Smoothened (Smo), a key component of SHH, is of vital in RA FLS migration and inhibition of Smo decreases RA FLS proliferation [32, 33]. Urokinase-type plasminogen activator receptor (uPAR) also promotes tumor-like behaviors through PI3K/Akt signaling pathway [34]. Therefore, the regulation of RA FLS may have an alleviating effect on protecting joint cartilage from destruction.

Joint inflammation in RA starts in the first joint but can spread to all joints during the course of RA [35]. RA FLS have the abilities to product cytokines, inflammatory mediators and MMPs, and to migrate and destruct joint cartilage [29, 36]. Therefore, it is of vital to study how RA FLS migrates and destructs the joint cartilage. Fortunately, Lefevre et al [35] have reported a humanized synovitis animal model by implanting healthy human cartilage together with RA FLS subcutaneously into one flank of a severe combined immunodeficient (SCID) mouse and healthy human cartilage only into contralateral flank of the same SCID mouse. Their model showed that RA FLS is able not only to invade and degrade cartilage without additional stimuli from the mouse or human immune system but also to maintain and transfer its properties to a distant and hitherto unaffected joint [35].

Interestingly, there is a significant difference between implant a cartilage containing RA FLS primary and cartilage without RA FLS two weeks later and implant the two items conversely [35]. As for the importance of this animal model, we here summarized a detailed protocol for developing the humanized synovitis animal model. The protocol primarily derived from the original description by Lefevre et al [35]. In order to study the migrative and invasive abilities of RA FLS *in vivo* more significantly, we implanted a cartilage-sponge complex without RA FLS, under the left flank skin of a SCID mouse primarily. Two weeks later, we inserted cartilage-sponge complex containing RA FLS under the right skin of the contralateral flank. With the H&E staining on day 45, we noted that RA FLS migrates and degrades the healthy cartilage clearly in SCID mice.

Reagents

All the reagents related to this protocol are listed in **Table 1**.

Statistical analysis

For statistical comparison of the treatment groups, one-way ANOVA was employed. Data were presented as Means ± S.E.M. All statistical analyses were performed using GraphPad Prism Software (version 7.00). $P<0.05$* was considered as statistically significant.

Procedur

First operation

1. A piece of fresh normal human cartilage will be cut into 0.7*0.4*0.2 (cm^3), and a piece of sterilized synthetic gelatin sponge will be cut into 0.7*0.6*0.6 (cm^3). All the processes will be performed under sterile condition. 2. The cut cartilage will be inserted into the gelatin sponge like a sandwich. 3. 5-wks-old CrlscidBR SCID mice will be employed in this protocol. Mice will be anesthetized intraperitoneally with 10 mg/kg of Xylazine and 90 mg/kg of Ketamine in an isotonic solution. 4. After fur shaving, the anesthetized mice will be put on a piece of sterile drape and incision site will be disinfected as follows: a. Using a surgical grade detergent first, wiping from the site of the incision outward; b. Rinse with alcohol (alcohol is not a sterilant, but it rinses soap and debris well); c. Swab with antiseptic solution from the incision site outward. 5. A 1-cm incision will be made on the left flank of skin on a SCID-mouse, and a small space will be opened using a sterile ophthalmic scissors by closing and opening the scissors. 6. One sponge including normal human cartilage will be inserted subcutaneously into the left side of SCID-mouse (contralateral). 7. The incision will be closed using Disposable Skin Stapler and wore with 10% Povidone-iodine. 8. Thereafter, post-surgical pain will be controlled with Meloxicam (5 mg/kg) subcutaneously injected prior to surgery and a second dose for the following day. 9. The skin stapler will be removed with a stapler remover at day 5.

Second operation

10. At day 14, a piece of fresh normal human cartilage will be cut into 0.2*0.2*0.2 (cm^3), and a piece of sterilized synthetic gelatin sponge will be cut into 0.7*0.6*0.6 (cm^3). 11. Repeat the steps 2-5. 12. One sponge including normal human cartilage will be inserted subcutaneously into the right side of SCID-mouse (primary). After being inserted, RA FLS (5×10^5 cells) will be injected into the sponge. 13. Repeat the steps 7-9.

Endpoint of the experiment

14. At day 60, mice will be euthanatized, and the implants removed, blood will be collected, implants and organs will be snap-frozen. Tissue preparation include fixation in 4% buffered formalin and paraffin embedding according to standard procedures. For cytokines detection, H&E staining, immunohistochemical staining and Immunofluorescence staining are used. 15. Using a standard hematoxylin and eosin staining, each specimen will be evaluated in a blinded manner by 4 independent examiners for the degree of destruction of the implanted cartilage as follows:

Criterion for clinical score

Invasion: No or minimal invasion, 0; visible invasion (two cell depths), 1; invasion (five cell depths), 2; deep invasion (more than ten cell depths), 3.

Perichondrocytic cartilage degradation: No degradation (sharp, intact halo of the chondrons compared with the cartilage stored before implantation), 0; visible degradation (one diameter of the chondron), 1; degradation (between one and two diameters of a chondron), 2; intensive degradation (more than two diameters of a chondron), 3.

All single implants from all mice were scored by five different trained researchers.

Anticipated results

1. The animal model schedule was showed in **Figure 1A**. 2. H&E staining of cartilages from different flanks is shown in **Figure 1B**. In this protocol, we implanted a cartilage-sponge complex without RA FLS, under the left flank skin of a SCID mouse primarily. Two weeks later, we inserted cartilage-sponge complex containing RA FLS under the right skin of the contralateral flank. By comparing the invasion and degradation scores of the different flanks of implants, the scores of primary implants inserted before the contralateral cartilage were significantly higher than contralateral implants inserted

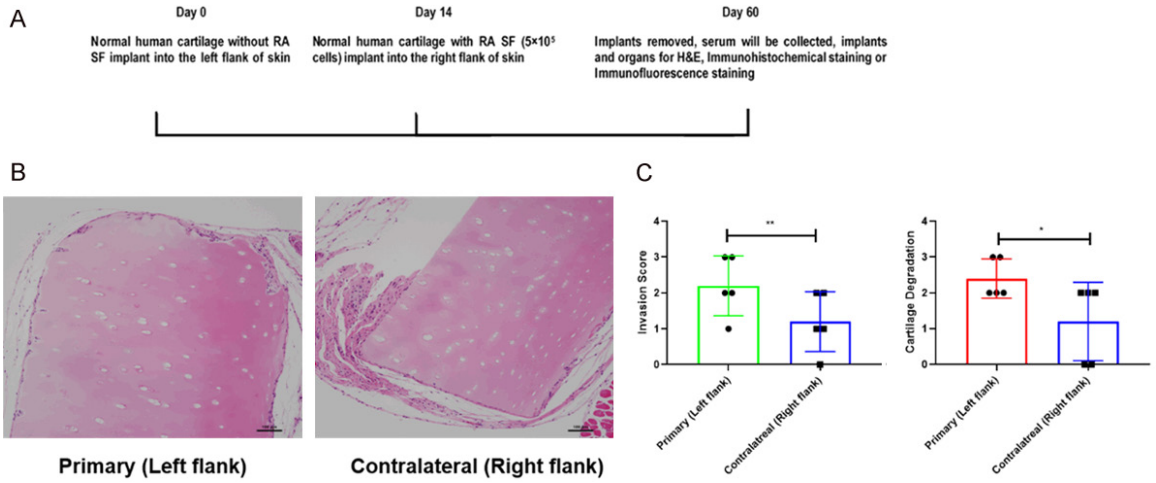

Figure 1. RA FLS migrates and degrades the unaffected cartilage. (A) Schedule of humanized synovitis model in SCID mice. (B) H&E staining of cartilages from different flanks of SCID mice. (C) Summary data from (B).

Table 2. Scoring results of invasion and perichondrocytic degradation of SCID mouse implants [35]

	Implant	Invasion (mean ± s.d.)	Perichondrocytic degradation (mean ± s.d.)
Implantation of contralateral cartilage at day 0, subsequent implantation of primary cartilage after approximately 14 d (n=13)	Primary (with RASFs)	1.8±0.7	2.3±0.7
	Contralateral (without RASFs)	2.8±0.5	2.5±0.7
Implantation of the primary cartilage at day 0, subsequent implantation of contralateral implant after approximately 14 d (n=10)	Primary (with RASFs)	2.7±0.4	2.5±0.7
	Contralateral (without RASFs)	1.5±0.6	1.7±0.8

after the primary cartilage, which is consistent with the results from Lefevre et al (**Table 2**) [35].

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Julie Wang, Division of Immunology and Rheumatology, Department of Internal Medicine, Ohio State University College of Medicine, 480 Medical Center, Columbus, OH 43210, USA. Tel: 614-293-6415; E-mail: Julie.Wang@osumc.edu

References

[1] Zou Y, Xu S, Xiao Y, Qiu Q, Shi M, Wang J, Liang L, Zhan Z, Yang X, Olsen N, Zheng SG and Xu H. Long noncoding RNA LERFS negatively regulates rheumatoid synovial aggression and proliferation. J Clin Invest 2018; 128: 4510-4524.

[2] Chen D, Liu D, Liu D, He M, Peng A, Xu J, Lin L, Luo F, Chen L, Huang X, Zhuang J and Xu J. Rheumatoid arthritis fibroblast-like synoviocyte suppression mediated by PTEN involves survivin gene silencing. Sci Rep 2017; 7: 367.

[3] Wangyang Y, Yi L, Wang T, Feng Y, Liu G, Li D and Zheng X. MiR-199a-3p inhibits proliferation and induces apoptosis in rheumatoid arthritis fibroblast-like synoviocytes via suppressing retinoblastoma 1. Biosci Rep 2018; 38.

[4] McInnes IB and Schett G. The pathogenesis of rheumatoid arthritis. N Engl J Med 2011; 365: 2205-2219.

[5] Li N, Wang JC, Liang TH, Zhu MH, Wang JY, Fu XL, Zhou JR, Zheng SG, Chan P and Han J. Pathologic finding of increased expression of interleukin-17 in the synovial tissue of rheumatoid arthritis patients. Int J Clin Exp Pathol 2013; 6: 1375-1379.

[6] Sandal I, Karydis A, Luo J, Prislovsky A, Whittington KB, Rosloniec EF, Dong C, Novack DV, Mydel P, Zheng SG, Radic MZ and Brand DD. Bone loss and aggravated autoimmune arthritis in HLA-DRbeta1-bearing humanized mice following oral challenge with Porphyromonas gingivalis. Arthritis Res Ther 2016; 18: 249.

[7] Jung SM, Kim Y, Kim J, Jung H, Yi H, Rim YA, Park N, Kwok SK, Park SH and Ju JH. Sodium chloride aggravates arthritis via Th17 polarization. Yonsei Med J 2019; 60: 88-97.
[8] Luo Y and Zheng SG. Hall of fame among pro-inflammatory cytokines: interleukin-6 gene and its transcriptional regulation mechanisms. Front Immunol 2016; 7: 604.
[9] Pan HF, Leng RX, Feng CC, Li XP, Chen GM, Li BZ, Xu WD, Zheng SG and Ye DQ. Expression profiles of Th17 pathway related genes in human systemic lupus erythematosus. Mol Biol Rep 2013; 40: 391-399.
[10] Zhou L, Wang J, Li J, Li T, Chen Y, June RR and Zheng SG. 1,25-dihydroxyvitamin D3 ameliorates collagen-induced arthritis via suppression of Th17 cells through miR-124 mediated inhibition of IL-6 signaling. Front Immunol 2019; 10: 178.
[11] Chen W, Wang J, Xu Z, Huang F, Qian W, Ma J, Wee HB, Lewis GS, June RR, Schafer PH, Lin J and Zheng SG. Apremilast ameliorates experimental arthritis via suppression of Th1 and Th17 cells and enhancement of CD4(+) Foxp3(+) regulatory T cells differentiation. Front Immunol 2018; 9: 1662.
[12] Kong N, Lan Q, Chen M, Wang J, Shi W, Horwitz DA, Quesniaux V, Ryffel B, Liu Z, Brand D, Zou H and Zheng SG. Antigen-specific transforming growth factor beta-induced Treg cells, but not natural Treg cells, ameliorate autoimmune arthritis in mice by shifting the Th17/Treg cell balance from Th17 predominance to Treg cell predominance. Arthritis Rheum 2012; 64: 2548-2558.
[13] Zhou X, Kong N, Wang J, Fan H, Zou H, Horwitz D, Brand D, Liu Z and Zheng SG. Cutting edge: all-trans retinoic acid sustains the stability and function of natural regulatory T cells in an inflammatory milieu. J Immunol 2010; 185: 2675-2679.
[14] Chen M, Su W, Lin X, Guo Z, Wang J, Zhang Q, Brand D, Ryffel B, Huang J, Liu Z, He X, Le AD and Zheng SG. Adoptive transfer of human gingiva-derived mesenchymal stem cells ameliorates collagen-induced arthritis via suppression of Th1 and Th17 cells and enhancement of regulatory T cell differentiation. Arthritis Rheum 2013; 65: 1181-1193.
[15] Zhao J, Wang J, Dang J, Zhu W, Chen Y, Zhang X, Xie J, Hu B, Huang F, Sun B, Bellanti JA and Zheng SG. A preclinical study-systemic evaluation of safety on mesenchymal stem cells derived from human gingiva tissue. Stem Cell Res Ther 2019; 10: 165.
[16] Huang F, Chen M, Chen W, Gu J, Yuan J, Xue Y, Dang J, Su W, Wang J, Zadeh HH, He X, Rong L, Olsen N and Zheng SG. Human gingiva-derived mesenchymal stem cells inhibit xeno-graft-versus-host disease via CD39-CD73-Adenosine and IDO Signals. Front Immunol 2017; 8: 68.
[17] Garcia-Arellano S, Hernandez-Palma LA, Bucala R, Hernandez-Bello J, De la Cruz-Mosso U, Garcia-Iglesias T, Cerpa-Cruz S, Padilla-Gutierrez JR, Valle Y, Sonanez-Organis JG, Parra-Rojas I, Pereira-Suarez AL and Munoz-Valle JF. Th1/Th17 cytokine profile is induced by macrophage migration inhibitory factor in peripheral blood mononuclear cells from rheumatoid arthritis patients. Curr Mol Med 2018; 18: 679-688.
[18] Pan HF, Leng RX, Li XP, Zheng SG and Ye DQ. Targeting T-helper 9 cells and interleukin-9 in autoimmune diseases. Cytokine Growth Factor Rev 2013; 24: 515-522.
[19] Mao YM, Zhao CN, Leng J, Leng RX, Ye DQ, Zheng SG and Pan HF. Interleukin-13: a promising therapeutic target for autoimmune disease. Cytokine Growth Factor Rev 2019; 45: 9-23.
[20] Lin S, Yang X, Liang D and Zheng SG. Treg cells: a potential regulator for IL-22 expression? Int J Clin Exp Pathol 2014; 7: 474-480.
[21] Horwitz DA, Gray JD and Zheng SG. The potential of human regulatory T cells generated ex vivo as a treatment for lupus and other chronic inflammatory diseases. Arthritis Res 2002; 4: 241-246.
[22] Yang M, Liu Y, Mo B, Xue Y, Ye C, Jiang Y, Bi X, Liu M, Wu Y, Wang J, Olsen N, Pan Y and Zheng SG. Helios but not CD226, TIGIT and Foxp3 is a Potential Marker for CD4(+) treg cells in patients with rheumatoid arthritis. Cell Physiol Biochem 2019; 52: 1178-1192.
[23] Deng YN, Bellanti JA and Zheng SG. Essential kinases and transcriptional regulators and their roles in autoimmunity. Biomolecules 2019; 9.
[24] Panagopoulos PK and Lambrou GI. Bone erosions in rheumatoid arthritis: recent developments in pathogenesis and therapeutic implications. J Musculoskelet Neuronal Interact 2018; 18: 304-319.
[25] Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J and Boyle WJ. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 1998; 93: 165-176.
[26] Welsing PM, van Gestel AM, Swinkels HL, Kiemeney LA and van Riel PL. The relationship between disease activity, joint destruction, and functional capacity over the course of rheumatoid arthritis. Arthritis Rheum 2001; 44: 2009-2017.
[27] Kong N, Lan Q, Chen M, Zheng T, Su W, Wang J, Yang Z, Park R, Dagliyan G, Conti PS, Brand

D, Liu Z, Zou H, Stohl W and Zheng SG. Induced T regulatory cells suppress osteoclastogenesis and bone erosion in collagen-induced arthritis better than natural T regulatory cells. Ann Rheum Dis 2012; 71: 1567-1572.

[28] Luo Y, Wu W, Gu J, Zhang X, Dang J, Wang J, Zheng Y, Huang F, Yuan J, Xue Y, Fu Q, Kandalam U, Colello J and Zheng SG. Human gingival tissue-derived MSC suppress osteoclastogenesis and bone erosion via CD39-adenosine signal pathway in autoimmune arthritis. EBioMedicine 2019; 43: 620-631.

[29] Bartok B and Firestein GS. Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. Immunol Rev 2010; 233: 233-255.

[30] Mo BY, Guo XH, Yang MR, Liu F, Bi X, Liu Y, Fang LK, Luo XQ, Wang J, Bellanti JA, Pan YF and Zheng SG. Long Non-Coding RNA GAPLINC promotes tumor-like biologic behaviors of fibroblast-like synoviocytes as MicroRNA sponging in rheumatoid arthritis patients. Front Immunol 2018; 9: 702.

[31] Liu F, Feng XX, Zhu SL, Huang HY, Chen YD, Pan YF, June RR, Zheng SG and Huang JL. Sonic hedgehog signaling pathway mediates proliferation and migration of fibroblast-like synoviocytes in rheumatoid arthritis via MAPK/ERK signaling pathway. Front Immunol 2018; 9: 2847.

[32] Peng WX, Zhu SL, Zhang BY, Shi YM, Feng XX, Liu F, Huang JL and Zheng SG. Smoothened regulates migration of fibroblast-like synoviocytes in rheumatoid arthritis via activation of Rho GTPase signaling. Front Immunol 2017; 8: 159.

[33] Zhu SL, Huang JL, Peng WX, Wu DC, Luo MQ, Li QX, Li ZX, Feng XX, Liu F, Wang MX, Chen WQ, Olsen N and Zheng SG. Inhibition of smoothened decreases proliferation of synoviocytes in rheumatoid arthritis. Cell Mol Immunol 2017; 14: 214-222.

[34] Liu Y, Pan YF, Xue YQ, Fang LK, Guo XH, Guo X, Liu M, Mo BY, Yang MR, Liu F, Wu YT, Olsen N and Zheng SG. uPAR promotes tumor-like biologic behaviors of fibroblast-like synoviocytes through PI3K/Akt signaling pathway in patients with rheumatoid arthritis. Cell Mol Immunol 2018; 15: 171-181.

[35] Lefevre S, Knedla A, Tennie C, Kampmann A, Wunrau C, Dinser R, Korb A, Schnaker EM, Tarner IH, Robbins PD, Evans CH, Sturz H, Steinmeyer J, Gay S, Scholmerich J, Pap T, Muller-Ladner U and Neumann E. Synovial fibroblasts spread rheumatoid arthritis to unaffected joints. Nat Med 2009; 15: 1414-1420.

[36] Muller-Ladner U, Kriegsmann J, Franklin BN, Matsumoto S, Geiler T, Gay RE and Gay S. Synovial fibroblasts of patients with rheumatoid arthritis attach to and invade normal human cartilage when engrafted into SCID mice. Am J Pathol 1996; 149: 1607-1615.