Chapter 7

Synoviolin is a Novel Pathogenic Factor of Arthropathy and Chronic Inflammation

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Abstract

Inflammation is classical pathogenic concept, but still very crucial for understanding many disorders even in twenty-first century. The purpose of inflammation is to eliminate the damaged tissues and to initiate tissue repair. By contrast, chronic inflammation leads to intractable diseases, including rheumatoid arthritis (RA), atherosclerosis, cancer, diabetes mellitus, and obesity. We recently cloned synoviolin, an E3 ubiquitin ligase, as an overexpressing molecule in rheumatoid synovium and has been verifying its critical roles in RA, inflammatory cytokine signaling, and fibrosis. Moreover, *synoviolin*-deficient mice exhibited severe anemia caused by defective nursing activity of erythrocytes in the fetal liver. This phenomenon resembles of RA that accelerates nursing activity. Our data indicate a close relationship between embryogenesis and RA. We successfully discovered synoviolin inhibitors, LS-101 and LS-102. These drugs have inhibitory effects to synoviolin in vitro and in vivo. We are now proceeding with the optimization of small compounds, and we hope our research will lead to the development of a new therapy for RA and fibrosis and other synoviolin-related diseases.

Keywords: synoviolin, synoviocyte, ubiquitin ligase, ERAD

1. Introduction

Rheumatoid arthritis (RA) has a tremendous negative impact on quality of life and affects nearly 1% of the adult population worldwide [1, 2].

Clinically, RA is characterized by multiple joint pain, stiffness, and swelling due to synovial inflammation and effusion [3–6]. The pathological features of RA result from multiple processes including chronic inflammation, overgrowth of synovial cells, bone and joint destruction, and as terminal phase tissue fibrosis.

2. Synoviolin is a causative factor for arthropathy

We cloned "synoviolin" by immunoscreening using anti-rheumatoid synovial cell antibody [7]. Synoviolin is a RING-type E3 ubiquitin ligase and is highly expressed in rheumatoid synovial cells [7]. Synoviolin is a mammalian homolog of Hrd1p/Der3p [8–10] and is involved in endoplasmic reticulum(ER)-associated degradation (ERAD) [7].

In eukaryotic cells, the balance of protein synthesis and degradation is strictly regulated, and the selective degradation of protein is carried out *via* the ubiquitin-proteasome system (UPS) [11, 12]. The proteins targeted for proteasomal degradation are ubiquitinated by three enzymes: ubiquitin-activating enzyme E1, ubiquitin-conjugated enzyme E2, and E3 ubiquitin ligases [11, 12]. Newly synthesized proteins are correctly folded in the ER and transported to the secretory pathway. When the amount of misfolded protein exceeds the protein folded capacity, result from ER stresses, it is eliminated by UPS-dependent degradation process of ERAD [13]. Synoviolin plays an important role in ERAD as an E3 ubiquitin ligase and involved in quality control of proteins.

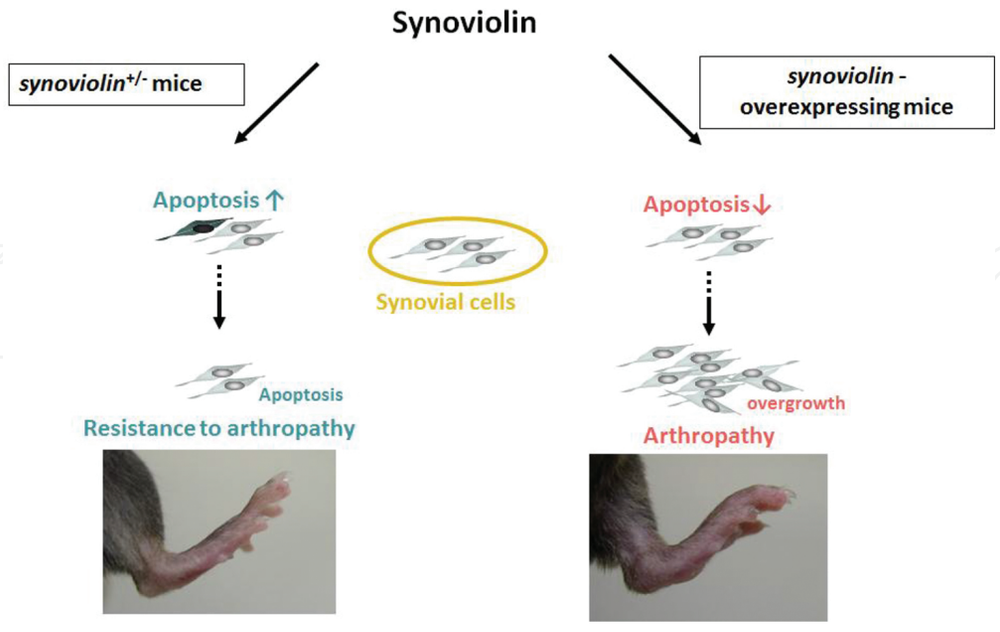

Figure 1. Synoviolin is a causative factor for arthropathy. "Gain-of-function" of synoviolin results in the development of spontaneous arthropathy through the anti-apoptotic effects of synoviolin. On the other hand, *syno*$^{+/-}$ mice are resistant to CIA model. Therefore, synoviolin is a novel causative factor for RA.

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Synoviolin is ubiquitously expressed in whole body, especially highly expressed in synoviocytes of patients with RA. Approximately 30% of overexpression of synoviolin in transgenic mice leads to advanced arthropathy caused by reduced apoptosis of synoviocytes (**Figure 1**) [7]. On the other hand, synoviolin-heterozygous mice demonstrate resistance to the development of collagen-induced arthritis (CIA) because of enhanced apoptosis of synovial cells (**Figure 1**) [7]. We postulate that the overexpression of synoviolin leads to hyperactivation of the ERAD and results in synovial hyperplasia. In addition, synoviolin negatively regulates the tumor suppressor p53 in the cytoplasm by ubiquitinating p53 [14]. Therefore, synoviolin regulates both apoptosis in response to ER stress and a p53-dependent apoptotic pathway. These studies indicate that synoviolin is a novel pathogenic factor of arthropathy through its anti-apoptotic effects [15].

3. The reason for death of *syno*$^{-/-}$ mice in utero

To gain insight into the function of synoviolin in vivo, we generated *synoviolin*-deficient (*syno*$^{-/-}$) mice by gene-targeted disruption. Strikingly, all fetuses lacking *synoviolin* died in utero by embryonic day 13.5 (E13.5) [16]. It is surprising that loss of synoviolin only can cause embryonic lethality, since Hrd1p/Del3p, a yeast homolog of synoviolin, is nonessential for survival [8]. Then, why is synoviolin deficiency associated with embryonic death? Morphologically, there was no remarkable difference between E13.5 wild-type and *syno*$^{-/-}$ embryos; however, the *syno*$^{-/-}$ fetal liver looked pale, suggesting an abnormal hematological status in *syno*$^{-/-}$ embryos, since the liver becomes the main hematopoietic organ by E12.5 [17–20]. Indeed, the number of peripheral blood cells was decreased in *syno*$^{-/-}$ embryos, and the level of β-major globin, which first appears in the fetal liver during definitive erythropoiesis, was markedly reduced in *syno*$^{-/-}$ embryos [16]. Subsequently, we examined erythropoiesis in *syno*$^{-/-}$ in vitro by colony-forming assay. Unexpectedly, we found that erythrocyte progenitors of *syno*$^{-/-}$ could differentiate in vitro to produce hemoglobin. Definitive erythropoiesis in embryos is controlled by cell autonomous and non-cell autonomous mechanisms. Therefore, it is expected that the abnormal cell morphology in *syno*$^{-/-}$ erythroid is secondary to changes in the local environment, that is, liver. Consequently, we analyzed the *syno*$^{-/-}$ fetal liver and found reduced number of hepatocytes and their augmented apoptotic cell death in *syno*$^{-/-}$ embryos compared to wild-type embryos [16]. Considered together, the above results indicate that the death in utero of *syno*$^{-/-}$ around E13.5 is caused by abnormal erythropoiesis in a non-cell autonomous manner, which depends on aberrant apoptosis in the liver.

4. Symmetric features of synoviolin in RA and embryogenesis

At a glance, embryogenesis and RA are non-related events. However, when considered through synoviolin, commonness becomes apparent. What is the common feature between these two processes? One answer could be the nurse-like cells. Nurse cells were first recog-

nized in a cell suspension from the thymus [21, 22]. Wekere and Ketelsen concluded that thymic nurse cells played an important role in the differentiation of thymocytes [23–25]. They referred to this phenomenon as pseudoemperipolesis. Pseudoemperipolesis has been observed also in the interaction between murine lymphocytes and murine bone marrow (BM) stromal cells [23–25]. Iwagami et al. [26] and Shimaoka et al. [27] reported cloning of nurse cells from synovial tissue of patients with RA. Moreover, BM stromal cells migrated from the BM into the affected joint cavity and contribute to synovial proliferation [28]. Clinically, BM stromal cells derived from donors show very little pseudoemperipoletic activity, and thus, nurse cell activity is considered a unique feature of BM stromal cells derived from RA [29]. That is, RA is a disease with accelerated nurse cell activity of BM stromal cells. In other words, increased nursing cell activity would enhance the cooperation between surrounding cells, BM stromal cells, and synovial cells.

When one compares RA with *syno*$^{-/-}$, these processes are symmetrical. In *syno*$^{-/-}$ fetal liver, there is a complete loss of nurse activity to erythrocytes. The excessive amount of synoviolin in RA and the lack of synoviolin in *syno*$^{-/-}$ are quite a symmetrical feature. These observations suggest the possible involvement of synoviolin in promoting the nursing activity in RA, a topic for future research. A more thorough analysis of *syno*$^{-/-}$ indicates that RA and embryogenesis are closely related processes (**Figure 2**).

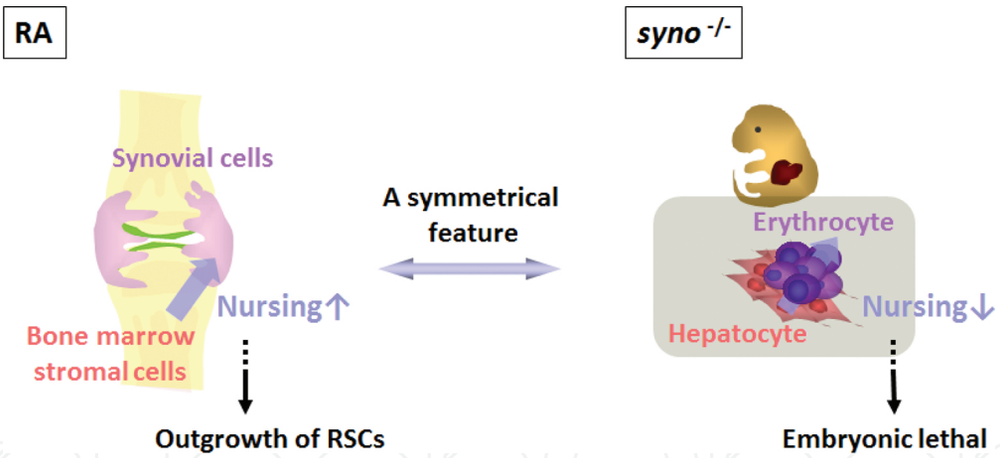

Figure 2. RA and embryogenesis have shared features. RA is a disease with accelerated nursing activity of bone marrow stromal cells. On the other hand, *syno*$^{-/-}$ fetal liver shows loss of nursing cell activity of erythrocytes. In this regard, RA and *syno*$^{-/-}$ demonstrate a symmetrical feature. The study of *syno*$^{-/-}$ has allowed the elucidation of new etiological factors of RA.

5. Impact of synoviolin on RA and embryogenesis

Embryogenesis, in which a single fertilized egg forms an individual consisting of millions of cells, is the most complicated process in higher eukaryote. That synoviolin shoulders this process highlights the importance of this protein. On the other hand, RA is a complex disease, in which all the details of its pathology are not yet understood. That this fundamental molecule

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is involved in the crisis of RA makes it conceivable that synoviolin is implicated in the intricacy of this disease. Furthermore, the joint cavity, representing the nidus of RA, is a complex space; it is formed of several types of cells, such as synovial cells, chondrocytes, osteoblasts, osteoclasts, and bone marrow cells. Just as there are many contacts with all sorts of cells in embryogenesis, the same is true for contacts between these numerous types of cells in the RA joint cavity. Except for the joint and eye, there is no space in our body formed by so many types of cells. In this regard, RA is a disorder of this complex space which needs to connect with the periphery, and the crisis of RA requires making sense of synoviolin function. Therefore, analysis of *syno*$^{-/-}$ has indicated that synoviolin is a molecule that connects embryogenesis and RA, and studies involving both processes would be the cutting edge in elucidating the pathogenesis of RA.

6. Synoviolin is participated in multiple processes of RA

RA consists of multiple processes including chronic inflammation, overgrowth of synovial cells, bone and joint destruction, and tissue fibrosis. Synoviolin plays an important role in over growth of synovial cells through hyperactivation of ERAD.

Inflammation is the most important process of RA. The synovial cells, macrophages, T cells, and B cells produce many kinds of cytokines, such as interleukin (IL)-1, IL-6, IL-10, tumor necrosis factor (TNF), and transforming growth factor β (TGF-β), and these cytokines stimulate the overgrowth of synovial cells [3–6]. Because it forms pannus, a mass of synovial tissue, inflammation leads to destruction of the bone and cartilage [3–6].

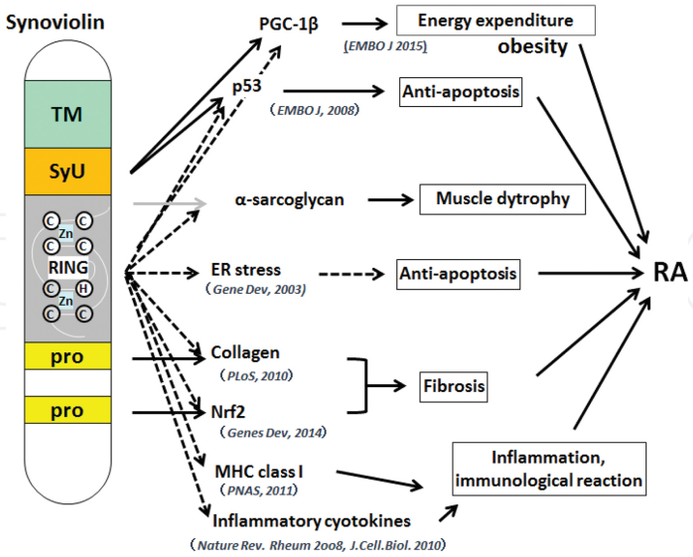

Figure 3. Synoviolin and its related disorders. The scientific summary of synoviolin function. Synoviolin is involved in a lot of disorders and would be a potential candidate for new drug of these disorders. Our compounds which inhibit the activity of synoviolin would be useful for these disorders. TM: transmembrane domain; SyU: SyU domain (amino acids, aa 236–270 of synoviolin); pro: prolin-rich region.

With regard to the relationship between synoviolin and inflammation, it was reported that IL-17 induction of synoviolin may contribute to RA chronicity [30], and synoviolin targets misfolded MHC class I heavy chains [31] and is a positive regulator of T-cell immunity [32]. Toh et al. demonstrated that synoviolin levels are elevated in circulating monocytes of RA patients [33]. It was reported that dual blockade of TNF and IL-17 decreased disease progression more effectively than when only one cytokine was blocked [34]. Therefore, it was suggested that synoviolin would be a potential candidate for new drug of chronic inflammation (**Figure 3**).

7. Synoviolin is also involved in fibrosis

Fibrosis is the terminal pathological feature of RA and results from excessive accumulation of the extracellular matrix (ECM) such as collagen and fibronectin [35]. Fibrosis is also major a pathological feature of chronic inflammatory disease. We previously demonstrated that synoviolin is upregulated in hepatic stellate cells (HSCs) of human cirrhosis, and synoviolin-heterozygous mice are resistant to CCl_4-induced hepatic injury [36]. Moreover, procollagen was abnormally accumulated in the ER of synoviolin-deficient mouse embryonic fibroblasts, suggesting the involvement of synoviolin in collagen secretion [36]. We also demonstrated that synoviolin expression and collagen secretion are enhanced in lung fibrosis using in vitro model, in which A549 human lung adenocarcinoma cells were transfected with exon-4 deleted surfactant protein C [37], which has been reported to induce ER stress [38, 39]. Li et al. demonstrated that synoviolin is involved in the renal fibrosis using the unilateral ureteral obstruction (UUO) model and plays an important role in the maturation of collagen [40]. These reports indicated that synoviolin plays an important role in fibrosis through the collagen expression and secretion (**Figure 3**).

8. Synoviolin as a therapeutic target for RA

During the past decade, biological agents have been approved for clinical use and dramatically have changed the treatment of RA. However, in some cases, patients fail to respond to the biologic treatment. It was reported that synoviolin overexpression of RA patients was associated with nonresponse to infliximab treatment (a monoclonal antibody against TNFα) [33]. Moreover, these agents are associated with high costs and discomfort arising from subcutaneous or intravenous administration. Thus, there is a clear need for the development of cheaper, orally administrated therapies with fewer side effects.

Since synoviolin is a pathogenic factor for chronic inflammation including RA and fibrosis (**Figure 3**), inhibition of synoviolin activity may be a useful therapeutic approach for the treatment of RA. Then, synoviolin is a drug-able molecule because: (1) synoviolin is an enzyme; (2) synoviolin localizes in cytoplasm; (3) the structure of synoviolin has been determined

(Nakajima T, unpublished data); and (4) specific substrates of synoviolin have been identified such as p53 [14]. Moreover, synoviolin may be a disease-modifying molecule because synoviolin may be involved in RA and fibrosis and may be implicated in some severe diseases such as interstitial pneumonia and systemic sclerosis. In making synoviolin a therapeutic target, downregulation of synoviolin and/or inhibition of its activity might be useful.

In order to reduce the amount of synoviolin, it is important to elucidate the transcriptional regulation of synoviolin. Establishing the mechanisms of transcriptional regulation of synoviolin should allow suppression of synoviolin transcription. We identified Ets binding site in the *synoviolin* proximal promoter as for crucial site of synoviolin expression. Moreover, the GA-binding protein (GABP) α/β complex is essential for its transcriptional regulation [41].

9. Development of synoviolin inhibitors

Next, in order to block the enzymatic activity of synoviolin, we performed high-throughput screening that inhibits the auto-ubiquitination activity of synoviolin. Over four million compounds from Pharmacopeia's compound collection were screened, and we found two unique compounds, termed LS-101 and LS-102 [42]. LS-101 and LS-102 demonstrated an inhibition of synoviolin auto-ubiquitination with IC_{50} of ~15 and 20 μM, respectively. LS-101 demonstrates stronger efficacy than LS-102, but less selectivity to synoviolin among other RING-type E3 ubiquitin ligases. Administration of either LS-101 or LS-102 also suppressed the clinical severity scores in mice collagen-induced arthritis (CIA) model. There was no difference in the protective effect between high dose of LS-101 and LS-102.

Moreover, it was also reported that LS-102 was able to suppress CCl_4-induced elevation of alanine aminotransferase (ALT) and restored normal liver morphology in CCl_4-induced liver cirrhosis mice model [43]. We also demonstrated that collagen secretion is suppressed by LS-102 in lung fibrosis using in vitro model [37]. Therefore, LS-102 is a novel potential drug for synoviolin inhibition. Thus, we proceed toward the optimization of LS-101 and LS-102 and get the derivative compounds from these compounds named LS-302 (Nakajima T, unpublished data). The arthritis scores of mice injected with LS-302 were also decreased. We hope our research will lead to the development of a new therapy for synoviolin-related diseases and serve as an example for the therapeutic benefit from E3 ligase inhibitors.

According to the UPS, a proteasome inhibitor has been developed. Bortezomib (BTZ) is the first proteasome inhibitor to gain the U.S. Food and Drug Administration (FDA) approval [44, 45]. BTZ induces apoptosis of a wide variety of cancer cells, and however, there are some patients who do not respond to therapy [44, 46]. There are second-generation proteasome inhibitors: carfilzomib [44, 47–49], ixazomib [44, 47, 48, 50], delanzomib [44, 47, 48, 51], oprozomib [44, 47, 48, 52], and marizomib [44, 48, 53]. These drugs are global inhibitors of the proteasome, and therefore, the associated toxicities prevent their use for the treatment of chronic disease such as RA. It is important to develop inhibitors of the UPS enzymatic cascade, and E3 ubiquitin ligase is suitable target given their large number and substrate specificity [54].

There is HDM2, the E3 ubiquitin ligase that regulates the degradation of p53 [55, 56], inhibitor currently in clinical trials [57, 58]. Then, synoviolin inhibitor would be a drug that follows a HDM.

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