A Subpopulation of Synovial Fibroblasts Leads to Osteochondrogenesis in a Mouse Model of Chronic Inflammatory Rheumatoid Arthritis

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ABSTRACT

Specific major histocompatibility complex (MHC) class II genes result in a high susceptibility to rheumatoid arthritis (RA), with co-stimulatory molecules working together with MHC class II during the progression of the disease. To elucidate the involvement of the B7.1 co-stimulatory molecule in RA, we analyzed the phenotype of B7.1 transgenic (named D1BC) mice and the sequential differentiation of synovial fibroblasts (SFs) by studying the expression of chondrogenic and osteogenic lineage markers together with lineage tracing experiment using B7.1 transgene in vivo. The B7.1 transgene was driven by a collagen type II (CII) promoter and enhancer in the D1BC mouse. A low-dose of bovine CII (bCII) was used to induce chronic articular inflammation with interstitial pneumonia. Joint damage was analyzed by histopathological examination and computed tomography. B7.1 was expressed in articular cartilage and SFs of D1BC mice. Chronic inflammatory arthritis in the bCII-D1BC mouse shared common features with those found in patients with RA, such as pannus formation, bone destruction, osteoporosis, and joint ankylosis. A subpopulation of SFs (Runx2+; Sox9+, Col10a1+; Osx+; and CX+) in the pannus was classified as osteochondrogenic lineage rather than mesenchymal stromal lineage. These cells underwent differentiation into osteogenic lineage via hypertrophic chondrocytes at the end of the chronic phase. The ectopic expression of B7.1 in chondrocytes and SFs leads to an increased susceptibility to chronic inflammatory arthritis and subsequent new bone formation, reminiscent of ankylosis. The regulation of cartilage remodeling in pannus tissue is an important consideration in the treatment of RA. © 2018 American Society for Bone and Mineral Research.

KEY WORDS: CHONDROCYTE; FIBROBLAST; INFLAMMATION; RHEUMATOID ARTHRITIS; SYNOVITIS

Introduction

Rheumatoid arthritis (RA) is a form of chronic inflammation, characterized by the onset of synovitis and progressive bone destruction in joints. In patients with RA, aggressive immune responses between T cells and antigen-presenting cells (APCs) result in robust inflammation, along with the production of cytokines and matrix metalloproteinases from synovial fibroblasts (SFs), augmenting bone destruction.1 Polymorphisms in major histocompatibility complex (MHC) class II genes are critical genetic risk factors for patient susceptibility to RA.2 The B7 family is a group of co-stimulatory molecules on APCs. The B7.1 molecule is thought to augment T cell proliferation, and leads to cytokine production.3 The expression of B7.1 may also be a risk factor in autoimmune disease, with B7 overexpression found in RA, systemic lupus erythematosus, Sjogren’s syndrome, and type I diabetes.4,5 Indeed, B7.1 is expressed in the synovial membranes of patients with RA.6 Thus, some studies have linked the pathogenesis of RA with a failure in self-MHC restriction together with co-stimulatory signaling. The interaction between B7 molecules and cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) on T cells leads to immune suppression, and the regulation of these two components is now regarded as a critical standard approach in clinical therapy.7

Synovial hyperplasia, frequently found in patients with progressive RA, is positive for S100A4 and vimentin, markers of mesenchymal stromal cells (MSCs), and podoplanin, a marker of SFs in the hyperplastic synovial lining layer.8,9,10 Also, extracellular matrix mineralization promotes podoplanin expression and drives osteocyte bone formation.11,12 S100A4 has been detected in SFs and immune cells (macrophages) of degraded cartilage in patients with RA.13 Other studies have cited osteirix (Osx, also osteoblast-specific transcription factor) and Meftin as distinct MSC markers associated with RA.14,15 Various in vitro studies have shown that SFs function as MSCs. Indeed, SFs from the synovium are capable of differentiating into chondrocytes, adipocytes, and...
osteonectin-17-19 as are primary fibroblast-like cells from amniotic membranes.20) These fibroblastic cells are functionally equivalent to MSCs, and SFs have been reported to express osteochondrogenic markers such as collagen type II (CII).21)

During osteogenic differentiation and in the repair of bone defects, extracellular matrix molecules like CII modulate epithelial–mesenchymal transition.22,23) Chondrocytes can differentiate into collagen type I (CI, a major component of the organic part of bone)–positive osteocytes (OCs) via hypertrophic chondrocytes (HCs) in the endochondral bone formation, which express Col10a1, collagen type X (CX), Osx, Runx2, and Sox9.24-27) Osx and Runx2 function as a master osteoblastogenic regulators during normal bone formation.23) Given the differentiation capacity of SFs, these cells may also be able to differentiate into HCs in RA. This may explain how ankylosis develops during RA disease progression.

Several in vitro and in vivo studies have suggested that SFs and/or fibroblastic MSCs can differentiate into cartilage; however, the fate of these cells remains unclear in an in vivo RA model.24,26,28,29) We thus investigated the properties of invasive SFs in pannus tissue using a mouse model of RA, and sought to determine how and whether these cells differentiate into cells of the osteochondrogenic lineage for chronic disease progression.

Materials and Methods

Animal care and use

The B7.1 transgenic mouse (named D1BC for DBA/1J, B7.1 gene; transcribed from the rat CII, rCII promoter and enhancer) was created using mouse full-length B7.1 cDNA; otherwise, the construct was the same as in the D1CC mouse described.30) The D1CC and DBA/1J mice were used as controls. The D1BC mouse was backcrossed with the DBA/1J mouse more than 15 times. All animal procedures were reviewed and approved by the Laboratory Animal Facility at the Nagoya City University.

Induction of inflammatory arthritis and clinical assessment

Inflammatory polyarthritis was induced as described.30) Briefly, D1BC, D1CC, and DBA/1J mice at 7 to 8 weeks of age were housed in a pathogen-free animal care facility in accordance with institutional guidelines. Mice were anesthetized with isoflurane before bovine CII (bCII) induction. Mice were immunized using one-tenth the normal concentration of bCII (low-dose; 0.02 mg/mouse), which was emulsified with an equal volume of Complete Freund’s Adjuvant (CFA) (day 0). This was followed by a booster injection with bCII with Incomplete Freund’s Adjuvant (IFA) on day 21. Mice were injected intradermally at the base of the tail, near the inguinal and axillary lymph nodes. For collagen-induced arthritis (CIA), the concentration of bCII was higher (high-dose; 0.2 mg/mouse). Mice were monitored each week from the onset of disease until the end of active inflammation.30) The clinical severity of arthritis was quantified for each limb according to a scoring system: 0 = no clinical symptoms; 1 = swelling and redness in one or two joints; 2 = moderate swelling and redness in more than three joints; 3 = severe swelling and redness of the entire paw; and 4 = deformity or ankylosis.

Histopathology and immunohistochemistry

Mice were euthanized, and limbs and lung were harvested, fixed in 4% paraformaldehyde diluted in PBS. Limbs were decalcified in 2.5% EDTA with 7% sucrose at 4°C for 1 month. Sections were stained by hematoxylin and eosin (H&E) and Masson’s trichrome stains. Paraffin sections (2 μm) were stained with the following primary antibodies: rabbit anti-S100A4 (Millipore, Billerica, MA, USA), rabbit anti-vimentin (Cell Signaling Technology, Beverly, MA, USA), mouse anti-CX (eBioscience, Santa Clara, CA, USA), rat anti-podoplanin (Medical & Biological Laboratories, Nagoya, Japan), rat anti-CD107b/Mac3 (Biosciences, South San Francisco, CA, USA), rabbit anti-SPP1/OSX (Abcam, Cambridge, MA, USA), rabbit anti-CI (Novus Biologicals, Littleton, CO, USA), and rat anti-Ki67 (DAKO, Carpinteria, CA, USA). The ABC method (Vector Laboratories, Burlingame, CA, USA) was used for B7.1 staining (biotin-conjugated hamster anti-CD80 [B7-1]; BD Biosciences, San Jose, CA, USA) of frozen sections. Histofine Simple Stain Mouse MAX-PO secondary antibodies were used, and visualized using the Histofine SAB-PO (M) kit (Nichirei Biosciences, Tokyo, Japan). For immunofluorescence staining, the Opal 4-color Fluorescent IHC kit (PerkinElmer, Waltham, MA, USA) was used according to the manufacturer’s protocol. Images were acquired by confocal (A1Rsi: Nikon, Tokyo, Japan) and fluorescence (BZ-X710; Keyence, Osaka, Japan) microscopy and analyzed by hybrid cell count (Keyence). We calculated intensity of podoplanin and Osx in S100A4-positive SFs, CI-positive HCs, or CI-positive OCs by using the data from three mice.

In situ hybridization

In situ hybridization for B7.1, Col2a1, Col10a1, Iislr (Meflin), Runx2, Sox9, and H2-Ab was performed using the RNAscope Multiplex Fluorescent Reagent Kit v2 (Advanced Cell Diagnostics, Newark, CA, USA), according to the manufacturer’s instructions. Briefly, 5-μm-thick tissue sections, cut from paraffin-embedded blocks, were deparaffinized and pretreated with heat and proteases before hybridization with the appropriate probe sets. Signals were detected using the Opal 4-color Fluorescent IHC kit. All sections were counterstained with Hoechst.

Bone mineral density and computed tomography

Bone mineral density and computed tomography (CT) were performed as described (LCT-1005, Hitachi-Aloka, Tokyo, Japan).30) To measure bone mineral density in the joint, measurements were taken from bone adjoining the articular cartilage (distal femur and the proximal tibia). Computed radiographs (CR) were obtained using the CR console (FCR5000plus; Fujifilm, Tokyo, Japan) with a high-resolution CR cassette.

Measurements of serum surfactant protein–D and the titer of anti-cyclic citrullinated peptide antibodies

Serum was collected from external jugular vein of each mouse. The quantitation of serum surfactant protein–D (SP-D) concentration was determined using ELISA according to the manufacturer’s instructions (Rat/Mouse SP-D kit; Yamasa, Chiba, Japan). For measurement of anti-cyclic citrullinated peptide (CCP) antibodies in serum, we performed ELISA.30) Serum samples were incubated at room temperature for 60 min in CCP-coated plates. After washing, trapped immunoglobulins were detected with goat anti-mouse IgG, peroxidase conjugate, and were quantified using QuantaRed Enhanced Chemiluminescent HRP substrate (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analyses

The results are shown the mean ± SE in the clinical score, titer of anti-CCP antibodies, bone mineral density, and immunohistochemical analyses. The significance of differences between DBA/1J and D1BC
Results

D1BC mice show severe inflammatory arthritis with low-dose bCII treatment

D1BC transgenic mice express the murine B7.1 transgene in chondrocytes because full-length B7.1 cDNA was fused with...
the rCII promoter, immediately upstream of the first exon of rCII, and the enhancer located between exons 1 and 2 (Supplemental Fig. 1A). We previously showed that chronic joint inflammation was induced in D1CC mice with a low-dose bCII injection, as compared with the conventional method of CIA. We examined whether joint arthritis could similarly be induced in D1BC mice. Joint inflammation commenced 3 weeks after the booster injection in both D1BC and D1CC mice, with maximum clinical scores of 8.7 ± 0.70 and 5.3 ± 0.58, respectively (Fig. 1A, B). Joint inflammation was

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**Fig. 2.** Joint space narrowing, erosion, and osteoporosis in bCII-D1BC mice. D1BC and DBA/1J mice were injected with a low-dose of bCII to induce joint inflammation. (A) Whole-mount bone and cartilage staining. All mouse limbs at 1 year after the first immunization were stained with Alizarin red S and Alcian blue, for bone (red) and cartilage (blue), respectively. Note the complete destruction of all joints (ankylosis) and shortening of the phalanges in the bCII-D1BC mouse joints. (B) X-ray panels at 6 weeks, 3 months, or 6 months after the first immunization in bCII-D1BC mice and at 6 weeks in bCII-DBA/1J mice. (C) CT scans in knee joints at 6 months after the first immunization in bCII-D1BC and bCII-DBA/1J mice. (D) CT scans revealed a decline in relative bone mineral density and joint space, with erosion in the forelimbs at 5 and 7 weeks and at 6 months after the first immunization. Data are presented as the relative percentage against non-CII induction. Error bars indicated SE (n = 10 per group).
also induced by the administration of high-dose and/or an additional booster of bCII (Supplemental Fig. 2). The incidence of disease between sexes was not statically different (data not shown). Chronic inflammatory arthritis was observed in bCII-D1BC mice but not in bCII-DBA/1J mice. Furthermore, we confirmed the expression of B7.1 in inflamed synovial cells (SCs) in bCII-D1BC mouse using immunohistochemistry (Fig. 1C). A clinical score of 4 indicates deformity or ankylosis.\(^{(30)}\) When a score of 4 was observed in 50% of bCII-treated mice, this was defined as the ICS50 for our study (ICS; incidence of clinical score 4, dotted line in Fig. 1D). In D1BC mice, the ICS\(^{(50)}\) was achieved at 11 weeks after the first immunization; however, D1CC mice were unable to reach the ICS\(^{(50)}\) by 13 weeks. This suggested that the period of inflammation in bCII-D1BC mice was shorter than that for bCII-D1CC mice.

Titers of serum anti-CCP antibodies in bCII-D1BC mice

We next examined titers of serum anti-CCP antibodies for inflammation and post-inflammation. In bCII-D1BC mice, antibody titers against CCP increased immediately after the first immunization, reaching a maximum at 7 weeks and persisting through to 18 weeks (Fig. 1E). Anti-CCP antibody titers were prone to be higher in bCII-D1BC mice than in bCII-DBA/1J mice.

Severe bone destruction with decreasing bone mineral density

Because severe joint inflammation causes destruction of the articular cartilage, bone loss, and a corresponding decrease in bone mineral density,\(^{(30)}\) we next confirmed changes in the joint space, measuring joint space narrowing and bone erosion by...
whole-mount bone and cartilage staining and radiographic examination in bCII-D1BC mice (Fig. 2A, B). Bone disruption with a loss of bone mineral density was further confirmed by CT (Fig. 2C). D1BC mice showed higher rates of bone loss as compared with bCII-DBA/1J mice (Fig. 2D), with 10% bone loss at 6 months after the first immunization. These findings suggest that the ectopic expression of B7.1 in the joints exacerbated bone loss together with ankylosis.

SFs share lineage markers with HC in pannus

We next used B7.1 mRNA as a tracing marker, because it was difficult to detect B7.1 expression in paraffin-embedded sections. B7.1 together with Col2a1 signals were detected simultaneously in the synovial membrane before arthritogenic stimuli (Fig. 3A) and in hyperplastic synoviocytes of bCII-D1BC mouse (Fig. 3B, upper panel). Only Col2a1 not B7.1 was observed in CIA, and this was used as a control (Fig. 3B, lower panel). H2-Ab (histocompatibility-2, MHC) was detected in only a limited number of hyperplastic synoviocytes (Fig. 3C). These hyperplastic synoviocytes also expressed MSC markers S100A4, vimentin, and Meflin (Fig. 3D, E). Podoplanin expressed in the synovial tissue in the pannus; however, its expression was not exactly restricted in the lining layer (Fig. 3D). We also observed infiltrated inflammatory lymphoid cells, such T cells and macrophages, into the region of synovial pannus hyperplasia. They were a relatively minor population, compared with S100A4-positive SFs (Fig. 3F, Supplemental

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**Fig. 4.** SFs differentiate into OCs via HCs in tissues with a clinical score of 4 or higher in bCII-D1BC mice. (A) Following chondrogenesis, HCs were localized adjacent to invasive SFs in pannus and preHCs (in the dotted frame, between pannus and HCs) in bCII-D1BC mouse joints (H&E staining). (B) B7.1 (green) and Col2a1 (red), Col10a1 (green), and Meflin (green) mRNA signals in differentiated HCs from SFs. Arrows indicate B7.1 (green), Col10a1 (green), Meflin (green), and Col2a1 (red) mRNA signals. (C) Immunohistochemical staining of CX (green), Osx (red), and CI (yellow), and CX (green), S100A4 (red) and vimentin (yellow) in chondrogenic joints. (D) Immunohistochemical staining of CI (yellow), Osx (red), and podoplanin (green), and S100A4 (red), vimentin (yellow), and podoplanin (green) in chondrogenic joints. (E, F) Fluorescence intensity for podoplanin (E) and Osx (F) in S100A4 (SFs in pannus), CX (HCs), or CI (OCs). Data are presented as the means ± SE. *p < 0.05, Tukey-Kramer test. (G) B7.1 (green) and Col2a1 (red), and Col10a1 (green) mRNA signals in OCs. Scale bars = 20 μm (yellow), 50 μm (black or white). Data are representative of more than three mice. ac = articular cartilage; b = bone; phc = preHCs; hc = hypertrophic chondrocyte; oc = osteocyte; p = pannus.
Fig. 3). Thus, these S100A4-positive hyperplastic synoviocytes were hereafter defined as SFs. SFs in the pannus also expressed Col2a1 and the HC lineage marker Col10a1. Its protein, CX, was not detected in SFs; it was detected in articular cartilage and HCs (Fig. 3E). The data suggested that SFs retain an ability to differentiate into HC.

SFs differentiate into HCs for cartilage remodeling

Meanwhile SFs may function as MSCs, these SFs may involve in subsequent direct hypertrophic cartilage remodeling in inflamed joints. We next examined whether the SFs detected in bCII-D1BC mice could also differentiate and produce regenerated HC tissue. We found relatively weak expression of Col2a1, Col10a1, Meelin, and B7.1 in morphologically identified preHCs and HCs adjacent to SFs (Fig. 4A, B). Next, we examined whether these HCs expressed CX besides S100A4 and vimentin. To confirm these expression patterns, we performed triple immunostaining using S100A4, vimentin, CX, and Osx. HCs expressed CX besides S100A4, vimentin, and Osx (Fig. 4C). We further investigated HC differentiation into osteogenic lineage. It was demonstrated that the expression of podoplanin was more predominant in OCs. In this model, podoplanin was expressed in SFs and osteochondrogenic cell lineage; however, its expression was transiently diminished in HCs (Fig. 4D, E). Spindle-shaped OC-like cells were localized around HCs expressed Col2a1 and B7.1 but not Col10a1 (Fig. 4G). These cells expressed podoplanin, Osx, and Ci, and thus were defined as osteochondrogenic lineage (Fig. 4C–F). As a control, CX and Ci were expressed in HCs, bone, respectively (Supplemental Fig. 4). Even though SFs retained the properties of MSCs, the SFs in the pannus was simultaneously rendered osteochondrogenic lineage just before terminally differentiating into HCs or OCs.

A subpopulation of SFs, Runx2, Sox9, and Col10a1 triple-positive cells toward osteochondrogenic lineage

To confirm SFs being in the osteochondrogenic lineage, we tested the expression of osteogenic lineage markers, Runx2 and Sox9 together with chondrocyte lineage marker Col10a1 or lineage tracing marker, B7.1. We found at least three different subpopulations, triple-positive (Runx2+, Sox9+, and Col10a1+), single-positive (Runx2+), and triple-negative (Runx2+, Sox9−, and Col10a1−) cells in B7.1-positive inflamed SCs (Fig. 5A). In HCs adjacent pannus, Runx2 and Col10a1 retained and Sox9 was diminished (Fig. 5B). Eventually a weak expression of Runx2 together with B7.1 was observed in OCs (Fig. 5C). Only triple-positive cells differentiated into in HCs and OCs because of the tracing of Runx2 and Sox9. On the other hands, single-positive cells in pannus, which was also observed in synovial membrane, and triple-negative cells spread in SFs (Fig. 5D).

Discussion

We demonstrated that B7.1 expression in chondrocytes and SFs led to a high susceptibility for chronic joint inflammation and interstitial pneumonitis (IP) mediated by arthritogenic stimuli. We followed the sequential differentiation of SFs into cells of the osteochondrogenic lineage in pannus, and hypothesized that the disorganized growth and differentiation in the pannus might be a consequence of a failure in the formation of proper articular structure during chondrogenesis and osteogenesis.

We previously showed that D1CC mice, which express human CIITA in chondrocytes, develop chronic joint inflammation in response to low-dose bCII injections. In this study, we established a similar transgenic mouse (D1BC) that expressed in chondrocytes a member of the B7 family of co-stimulatory factors, B7.1. B7.1 is thought to augment T cell proliferation.

Fig. 5. A subpopulation of SFs expresses Runx2, Sox9, and Col10a1 in pannus retain competence into OCs via HCs. Sox9 (red) and Runx2 (white), Col10a1 or B7.1 (green) mRNA signals in AC and SFs in pannus (A); HCs (B), and OCs (C) in bCII-D1BC inflamed joints and non-bCII D1BC joints (D). Scale bars = 20 μm. Data are representative of more than three independent experiments. ac = articular cartilage; hc = hypertrophic chondrocyte; oc = osteocyte; p = pannus; sc = synovial cell.
and play a role in autoimmune diseases, including RA.\(^{[4-8,32]}\) The D1BC mouse was phenotypically normal in the absence of an arthritogenic antigen, and we found that both low-dose and high-dose bCII could induce chronic inflammatory arthritis lasting 2 to 4 months (Fig. 1A; Supplemental Fig. 2). Approximately 90% of bCII-D1BC mice showed chronic joint inflammation with high titer of anti-CCP antibodies in our study (Fig. 1E). Our collective findings from the present study and previous work suggest that aberrant expression of B7.1 or CIITA in chondrocytes is associated with a high risk of developing joint inflammation. Yet, most B7.1-positive SFs did not convert to APCs, and only a small number of hyperplastic synoviocytes expressed H2-Ab (Fig. 3C).\(^{[21]}\) This aside, chronic disease with joint inflammation persists for longer than CIA, and this indicates that D1BC mouse is a useful animal model to study the progression of RA. Because many RA patients showed IP as a complication of RA, we found that bCII-D1BC mice also showed susceptibility for IP. Most of bCII-D1BC mice (~70%) showed higher serum surfactant protein-D (SP-D) levels, with evidence of rheumatoid lung fibrosis by histology (Supplemental Fig. 5A, B).

Synovial hyperplasia in RA is characterized by the proliferation and activation of SFs. SFs are regulated by chemokine-stimulated chondrocytes, and, during RA, this results in pannus formation. Both fibroblasts and immature chondrogenic cells can differentiate into cells of the chondrogenic and osteogenic lineages.\(^{[20,26,33]}\) Furthermore, SFs in the synovium are reported to participate in wound healing and tissue repair for bone erosion, which was triggered by immune response in the

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**Fig. 6.** Schematic representation of the stages of RA in D1BC mouse model. (1) SCs in synovial membranes express Runx2, Col2a1, and B7.1\(^*\) transgene (and lineage tracing marker) in neonatal D1BC. (2) The aggressive proliferation of SFs is induced by joint inflammation. Following pannus formation, bone erosion and destruction occurs in the inflamed joint. SFs share lineage markers with preHCs in bCII-D1BC mice. The most definitive feature of SFs in the pannus was the expression of Col10a1, but not its transcribed protein, CX. A subpopulation of SFs (B7.1, Col2a1, Runx2, Sox9, Col10a1, and Osx) differentiates into CX-negative pre-HCs (morphologically). (3) SFs differentiate into HCs (B7.1, Col2a1\(^{+/+}\), Runx2, Sox9\(^{+/+}\), Col10a1, CX, and Osx) by hypertrophic cartilage remodeling in the process of endochondral bone formation. (4) When HCs terminally differentiate into OCs, the expression of Cl is observed instead of CX. A failure in cartilage remodeling leads to new bone formation by ossified chondrocytes and resulting bony ankylosis.
In our study, both B7.1 and Col2a1 were detected simultaneously in the synovial membrane, but Col2a1 signals were weaker than those of B7.1; this was because 10 copies of the B7.1 transgene were integrated in the genome. These B7.1-positive and Col2a1-positive SFs expressed the Col10a1 lineage marker but showed no detectable levels of CX (Figs. 3E, 4C). As a control, synovial cells did not express both Col10a1 and CX in non-bCII-D1BC mouse (Supplemental Figs. 6, 7). Cells with a similar “intermediate phenotype” (between MSC and HC phenotypes) were also found in the deep zone of the growth plate, adjacent to HCs in the epiphyses (Supplemental Fig. 7).

Pannus hyperplasia is also characterized by differentiated chondrocytes and HCs. Even though CX was expressed in HCs and chondrocytes in the articular cartilage of adult mice, it was not detected in Col10a1-positive preHCS in bCII-D1BC mice (Supplemental Fig. 4; Fig. 4C). These preHCS and HCs expressed Mefrin, a marker of MSC and immature chondrocytes, but not in mature chondrocytes (Supplemental Fig. 8; Fig. 4B). Indeed, immature chondrocytes in 1-week juvenile mice expressed Mefrin (Supplemental Fig. 7B). Thus, most CX-positive HCs might be differentiated from invasive SFs via preHCS (Col10a1-<sup>CX</sup>-). We could not identify a specific lineage marker of this preHC, which were transient chondrocyte phenotype at the pre-hypertrophy stage, at this time.

Given that bCII-D1BC mice showed ankylosis at the end of disease progression, this de novo chondrogenesis might reflect a failure of hypertrophic cartilage remodeling in arthritic inflammation. Furthermore, we wanted to test whether these SFs regenerate joints through direct engagement into osteochondrogenesis. We identified Ocs adjacent to HCs (Col2a1, B7.1, Runx2, Sox9, and Col10a1), although these cells still expressed B7.1<sup>low</sup>, Runx2<sup>low</sup>, Oxs, podoplanin, and CI, but no detectable levels of Col10a1 or CX (Figs. 4D, 4G, 5C, 5D, 6I). In addition, the expression of S100A4, a negative regulator of mineralization in osteoblasts was decreased, suggesting increasing of mineralization in these cells (Fig. 4C, D).<sup>56,57</sup> This mineralization of extracellular matrix enhances the expression of podoplanin in osteocytes.<sup>13,14</sup> Thus, although the SFs might be associated with chondrogenesis, we see tissue dysfunction and a failure in joint remodeling in these tissues, with extra mineralization in parallel with osteoporosis. These de novo chondrogenic and osteogenic cells come from proliferative chondrogenic cells in the articular cartilage of adult mice, it still differentiate into OCs via HCs, resulting in endochondral bone formation. In addition, these HCs fail to reconstitute articular cartilage and bone in chronic disease progression, this inappropriate osteogenic differentiation leads to a malfunction in joint remodeling and the development of ankylosis. Taken together, the transcriptional regulation network via Runx2 in synovial fibroblasts will be a possible therapeutic approach to inhibit endochondral bone formation.

**Disclosures**

All authors state that they have no conflicts of interest.

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Authors’ roles: Study design: SK, MP, and GS. Study conduct: SK, YM, and SO. Data collection: YM and SO. Data analysis: SK, YM, and SO. Data interpretation: SK, YM, and MP. Drafting manuscript: SK and YM. SK takes responsibility for the integrity of the data analysis.

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