CHAPTER IV

DISCUSSION AND CONCLUSION

4.1 Discussion for conditional knockout mice of versican model

In this study, we have generated the conditional versican-null mice, in which the versican gene *Vcan* is conditionally inactivated in limb buds and a subset of mesenchyme tissues starting at very early embryonic limb development. These mice, termed *Prx1*-Cre/*Vcan*$^{flox/flox}$, grow normally and are fertile, although they grossly show distorted digits. Histologically, their digits display hypertrophic chondrocyte nodules, tilting and clefting of the joint surface, and a slight delay of cartilage development. The joint interzone at E15.5 of these mice exhibited a decrease in TGF-β in the extracellular matrix, concomitant with a substantially decreased number of nuclei positive for phospho-Smad2/3. Our micromass culture system of limb bud mesenchymal cells demonstrated diffuse TGF-β localization in the mutant micromass, contrasting to its restrictive localization to regions surrounding developing cartilaginous nodules in *Prx1*-Cre/*Vcan*$^{+/+}$. These results strongly suggest that versican localizes TGF-β in the extracellular matrix and regulates its signaling. Our study uncovers, for the first time, the *in vivo* role of versican in skeletogenesis.

4.1.1 Versican is required for TGF-β signaling during joint morphogenesis

Joint formation begins with the condensations of uninterrupted mesenchyme differentiating to chondrocytes. In the sites of joint formation, the mesenchymal cells

remaining undifferentiated or conversely dedifferentiated from chondrocytes become densely packed to form a region termed the joint interzone, followed by joint cavity formation (136, 146). In this study, we have shown that whereas the metatarsophalangeal joint interzone at E15.5 of *Prx1*-Cre/*Vcan*$^{+/+}$ digits gives rise to a well-defined space representing early joint cavities, that of *Prx1*-Cre/*Vcan*$^{flox/flox}$ forms a tilted joint interzone filled with closely packed mesenchymal cells. Interestingly, the deposition of TGF-β in the ECM and the number of nuclei positive for phosphoSmad2/3 were markedly diminished. Recently, TGF-β type II receptor gene (*Tgfbr2*) conditional knockout mice under the control of *Prx1* promoter have demonstrated a failure of interphalangeal joint interzone development, resulting from an aberrant persistence of differentiated chondrocytes and a failure of Jagged-1 expression, thereby indicating an essential role of TGF-β signaling in joint formation (167). The absence of versican in the joint interzone of *Prx1*-Cre/*Vcan*$^{flox/flox}$ digits may disturb levels and spatially ordered distribution of TGF-β signaling, leading to altered localization of mesenchymal cells in the joint interzone. Despite marked inhibition of TGF-β signaling in the joint interzone of *Prx1*-Cre/*Vcan*$^{flox/flox}$ digits, accumulation of mesenchymal cells was observed in the joint interzone. Therefore, TGF-β signaling necessary for maintaining a mesenchymal phenotype appears viable. It has also been proposed that mesenchymal cells in the interzone are derived from de-differentiation of chondrocytes. Since chondrocytes were absent in the interzone of *Prx1*-Cre/*Vcan*$^{flox/flox}$ as evaluated by immunostaining for aggrecan, the process of their de-differentiation to mesenchymal cells is likely intact, even with considerable reduction of TGF-β signaling.

HA and its receptor CD44 have been proposed to play key roles in joint cavitation (138, 188, 189). It is also known that HA binding to CD44 can regulate specific ERK signaling events (245, 246), and the MEK-ERK pathway is regulated by mechanotransduction pathway (197, 198). We have shown that unincorporated HA in the joint interzone of both *Prx1*-Cre/*Vcan*$^{+/+}$ and *Prx1*-Cre/*Vcan*$^{flox/flox}$ is accumulated at similar levels in the joint interzone, and the expression of CD44 in the joint was not detected until E18.5. In addition, the immunostaining pattern for pERK1/2 was similar between *Prx1*-Cre/*Vcan*$^{+/+}$ and *Prx1*-Cre/*Vcan*$^{flox/flox}$. These observations may exclude direct involvement of HA and CD44-mediated signaling and the mechanotransduction through MEK-ERK pathway in the joint abnormalities of *Prx1*-Cre/*Vcan*$^{flox/flox}$ digits.

Wnt/β-catenin canonical signaling pathway has been reported to be necessary and sufficient for the induction of synovial joints in the limb (148). By immunostaining of the joint interzone at E15.5, β-catenin was expressed in closely associated mesenchymal cells at similar levels between *Prx1*-Cre/*Vcan*$^{+/+}$ and *Prx1*-Cre/*Vcan*$^{flox/flox}$, although more mesenchymal cells were observed in the interzone. Together with the fact that the joints themselves are formed in *Prx1*-Cre/*Vcan*$^{flox/flox}$, versican is unlikely to have direct effects on Wnt signaling.

It is intriguing that the abnormalities of *Prx1*-Cre/*Vcan*$^{flox/flox}$ appeared only in autopods, although versican is expressed in both proximal and distal joint interzone, articular cartilage, and synovia of limbs (148). Our immunostaining confirmed the absence of versican in joints where no abnormalities were observed, suggesting that the role of versican in joint formation is confined to digits. It is of note that TβRII conditional knockout mice exhibit impaired joint formation only in digits, although

TβRII is also expressed in proximal joints. These observations support the notion that versican contributes to joint formation by regulating TGF-β signaling, and that versican does not play a major role in formation of the joints where TGF-β is unnecessary.

4.1.2 Versican accumulates TGF-β to perinodular regions in mesenchymal condensation

Since versican was identified in mesenchymal condensation areas of chick limb bud, this proteoglycan has been assumed to play an important role in cartilage development (6). Our previous study using N1511 chondrogenic cells clearly demonstrated that inhibition of versican expression at an early stage substantially decelerates following chondrocyte differentiation (17). High-density micromass culture systems of limb bud obtained from versican-null *hdf* homozygote embryos demonstrated that versican is necessary for mesenchymal cell aggregation but that these mesenchymal cells undergo chondrocyte differentiation in three-dimensional type I collagen gel culture (16). These results suggest that versican interacts with other ECM molecules and cell-membrane molecules and mechanically ties up individual cells to form aggregates. In contrast to these results, the fact that *Prx1*-Cre/*Vcan*$^{flox/flox}$ mice undergo almost normal cartilage development suggests that versican does not have a profound effect on mesenchymal cell aggregation *in vivo*, although a slight delay of cartilage development occurs.

It has not been understood why mesenchymal condensation is required for proper chondrocyte differentiation. It may facilitate signal transduction by increasing local concentrations of growth factors and cytokines secreted by the cells, and by gathering

target cells close with each other. We have shown that both versican and TGF-β are restricted to the area surrounding the cartilaginous nodules in *Prx1*-Cre/*Vcan*$^{+/+}$, whereas TGF-β is widely distributed in the *Prx1*-Cre/*Vcan*$^{flox/flox}$ micromass. This data suggests that versican accumulates TGF-β in the ECM and elicits a strong TGF-β-mediated signal to the target cells. Indeed, phosphoSmad2/3 was strongly immunostained in the nuclei of cells in the perinodular regions in *Prx1*-Cre/*Vcan*$^{+/+}$ micromass, whereas it was immunostained weakly in *Prx1*-Cre/*Vcan*$^{flox/flox}$ micromass.

4.1.3 Functional domain of versican

The functional domain of versican that participates in mesenchymal condensation has not yet been determined. By chondroitinase ABC treatment of *Prx1*-Cre/*Vcan*$^{+/+}$ micromass during 48 h before fixation, both versican and TGF-β retained essentially the same localization patterns as the untreated micromass, eliminating a direct function of CS chains in accumulating TGF-β in the ECM.. In addition, CS chains attached to versican in cartilage are undersulfated compared with those of aggrecan (247). Furthermore, gene-trapped mice of chondroitin-4-sulfotransferase 1 (*C4st1*) *C4st1*$^{gt/gt}$, with a substantial decrease in CS amounts, show no abnormalities at E11.5 and E13.5 when mesenchymal condensation occurs in limb bud (248). Thus, it is unlikely that CS chains of versican directly bind TGF-β and regulate its signaling, although they may affect joint formation, as *C4st1*$^{gt/gt}$ mice display an impaired segmentation of cartilage in digits (248).

Another candidate is the G1 domain, which specifically binds to HA. Conditional knockout mice of hyaluronan synthase 2 gene (*Has2*), *Prx1*-Cre/*Has2*$^{flox/flox}$, display dwarfism, abnormal digit patterning, impaired chondrocyte

maturation, and a defective synovial joint cavity (249). Their dwarfism and impaired chondrocyte maturation in the growth plate are likely due to the reduction of cartilage extracellular matrix containing HA and aggrecan. Although data of the mesenchymal condensation process of limb bud in these mice have not been presented, the result demonstrating that these mice undergo cartilage development even in the absence of HA in mesenchymal condensation areas suggests that HA is not essential for the initial stage of cartilage development. Because of a rather minor perturbation of mesenchymal condensation by the absence of HA, it is unlikely that the function of versican is via interaction with HA.

The C-terminal G3 domain interacts with fibulin-1, -2 (63, 65), and fibrillin-1 (66). Fibrillin-1 binds latent TGF-β binding protein (LTBP)-1 and 4, which bind and store TGF-β in the ECM. Recently, fibrillin-1 has been shown to be required for the appropriate matrix assembly of LTBPs, which is affected by fibulins (44). Versican may regulate balance deposition of fibrillin-1 and fibulins, and regulate the incorporation of LTBP into the ECM. Fibrillin-1 and -2 double knockout mice (250) and LTBP-1-null mice (251) exhibit no clear defects in cartilage development. Our western blot analysis showed similar levels of TGF-β deposition in the ECM of *Prx1*-Cre/*Vcan*$^{+/+}$ and *Prx1*-Cre/*Vcan*$^{flox/flox}$ micromass, which suggests that other ECM molecules contribute to its deposition even in the absence of versican. The mechanisms by which versican accumuluates TGF-β in the perinodular region of the ECM should be studied, in addition to LTBPs, fibulins, and fibrillin-1.

So far, we have not determined the functional domain of versican in regulation of TGF-β. Several ECM molecules have been shown to interact with each other and to TGF-β and its super family members, and the sequestration may involve the

supramolecular structure built up via interactions of various ECM molecules, and storage and action of signaling molecules may be regulated by alteration of the tertiary structure of the complex. Further studies that connect the structure of the ECM and its regulation on cell signaling remain to be performed.

4.2 Discussion for the effects of *A. galanga* on an inflammatory model of human synovial fibroblasts

Rheumatoid arthritis (RA) is an inflammatory disease marked by hyperplasia and chronic inflammation of the synovial membranes. The activated SFs in the lining layer of the synovial membrane invade deeply into the articular cartilage and bone and thus contributing to cartilage deterioration and joint destruction (21, 22, 23, 24). The progressive joint destruction by cytokines such as pro-inflammatory IL-1β, IL-6, and TNF-α released from macrophages, monocytes, and synovial fibroblasts is the crucial mechanism driving inflammation in RA (25). This occurs through induction of cytokine storming or up-regulation of MMPs, which are the major enzymes for erosion of bone and cartilage (26). Thus, inhibition of pro-inflammatory cytokine-induced MMPs secretion by synovial fibroblasts could be one possible mechanism by which to alleviate the symptoms in RA patients. Of particular interest, the study of *p*-hydroxycinnamaldehyde, the purified substance of the acetone extract of *A. galanga*, indicates the potential ability of *p*-hydroxycinnamaldehyde in suppression of the catabolic genes expression levels comprising MMP-3 and MMP-13, whereas increase the levels of anabolic genes of collagen II, SOX9 and aggrecan (226). Moreover, our previous studies have indicated that MMPs induction in chondrosarcomas: SW1353, synovial fibroblasts and cartilage explants is reduced by the hexane extract from *A.*

galanga, and the mRNA levels for the major cartilage degradation enzymes, MMP-1, -3, and -13 are inhibited in synovial fibroblasts. These data suggest that *A. galanga* extract can also block cartilage degradation by adjacent synovial membranes that are severally inflamed and commonly invade the articular cartilage (28). Therefore, *A. galanga* extracts can potentially reduce arthritis-associated inflammation of synovial membranes and MMPs production from the synovium.

In the present study, we focused on the anti-inflammatory effects of four fractions of hexane extract (fraction 1-4) obtained from the rhizomes of *A. galanga* on catabolic gene expression. In RA joints, MMPs destroy matrices of articular joints and activate other MMPs (26). Thus, we initially examined the effect of *A. galanga* extract on IL-1β-induced MMP-2 production by gelatin zymography. The results suggested that fraction 4 of *A. galanga* extract suppressed IL-1β-induced MMP-2 production. In contrast, there was no clear difference in down-regulation of the IL-1β-induced MMP-2 expression after treatment with *A. galanga* extracts. Though, the results indicated that human synovial fibroblasts spontaneously produce small amounts of pro-MMP2. However, when confluent synovial fibroblasts were treated with IL-1β (10 ng/ml) for 24 h, the level of MMP-2 in the conditioned medium showed a small increase compared to the untreated control cells. This might due to saturated activation of IL-1β treated SFs, therefore when compared the band density of MMP-2 with untreated control SFs, it was not showed the difference of MMP-2 level. The transcriptional and post-translational activation of this enzyme is tightly regulated (252). MMP-2 differs from several members of the MMP family in that its transcription is not induced in response to the pro-inflammatory cytokines TNF-α and IL-1β, and seems primarily regulated by post-translation (239, 253). As is

characteristic of most of the MMPs, MMP-2 is produced as a proenzyme, and requires activation by enzymatic cleavage. Evidence from several investigators has suggested that MMP-2 in its active form is bound in a trimolecular complex at the cell surface (254, 255, 256, 257). Pro-MMP-2 complexed with TIMP-2 binds to a TIMP-2 binding site on the membrane-bound MMP-14 receptor, and through a conformational change the catalytic domain becomes exposed forming the active enzyme complex. Upon dissociation from the MMP-14 receptor, TIMP-2 binds the catalytic site and suppresses the activity. Thus, the activity of MMP-2 is regulated locally by a delicate balance between MMP-14 activation and TIMP-2 inhibition. Therefore, it may be possible why MMP-2 expression was not activated when induced by IL-1β, leading to no obvious difference in down-regulation of the IL-1β-induced MMP-2 expression after treatment with *A. galanga* extracts.

Because of no *A. galanga* extracts treated SFs alone, it is still confounding whether the decrease of MMP-2 activity resulted from *A. galanga* extract itself. Thus, further study should be repeated and confirmed IL-1β-induced MMP-2 production by both gelatin zymography and gene expression and SFs treated *A. galanga* extracts alone should be performed. In addition, positive control, such as anti-arthritic drug using Diacerine might need as a control.

Usually, the phytochemicals possess toxic and anti-toxic properties by themselves. As previously mentioned, the decrease of cell viability by *A. galanga* extracts of hexane fractions 1-3 started at 10 μg/ml, whereas the extract of hexane fraction 4 at 50 μg/ml exhibited that of fractions 1-3. We speculate that less toxicity found in the extract of hexane fractions 4 may result from exclusion of the toxic extracts of hexane fractions 1-3.

Next, we examined other MMPs and the inflammation marker, Cox-2. We found that the expression of MMP-3,-13 and Cox-2 were down-regulated at 10 μg/ml of *A. galanga* extract fraction 4, and at 1 μg/ml of fraction 1. The expression of MMP-3 and -13 were also down-regulated at 1 μg/ml of fraction 2. Therefore, we speculated that *A. galanga* extract fraction 1 and 2 at 1 μg/ml may have the ability to down-regulate MMP-1, -3, -13 and COX-2 expression after induction by IL-1β, though high doses were toxic to the cells. IL-1β induced MMP-1 expression was barely increased; however, MMP-1 was suppressed by the extract of fraction 4, and fractions 1 and 2, at high doses and 1 μg/ml, respectively. Interestingly, *A. galanga* hexane extract fraction 4 yielded better results than the other fractions. Collectively, *A. galanga* hexane extract fraction 4 had a tendency to suppress IL-1β-induced gelatinases (MMP-2,-9) expression. It could also down-regulate collagenases (MMP-1, -3, -13), as well as Cox-2 expression, demonstrating its anti-inflammatory effect.

COX-2 is an inducible enzyme provoking in the production of prostaglandins that often cause the pain and swelling of inflammation and other painful conditions. Furthermore, selective inhibitor for Cox-2 is promising agent for drugs used in arthritis. In this study, *A. galanga* hexane extract of fraction 4 showed the inhibition effect on Cox-2 expression, hence, the selective inhibition of this fraction should additionally explored.

There were evidents suggested that the extract contained sterols and terpenoids possess anti-inflammatory activity in acute and certain aspects of chronic inflammation activity (258, 259). It might be that the anti-inflammatory activity of *A. galanga* hexane extracts derived from sterols and terpenoids content in the extracts,

however, the bioactive gradients containing in *A. galanga* hexane extracts is still left to be investigated.

In the pathogenesis of inflammation and joint damage associated with RA, SFs are considered as the cells that are responsible for marginal cartilage destruction. Forming pannus of the immoderate growth of rheumatoid synovial fibroblasts invades adjacent tissues and damages cartilage by synthesis and secretion of MMPs such as MMP-1, -3, -9, -10 and -13 (260, 261). However, the specific effects of distinct cytokines on the production of MMPs are highly variable and depend on the induced type of MMP, the cell type, and the transduction pathways. Accumulating lines of evidence have indicated that IL-1β is a potent activator of MAPK (262) and NF-κB (263) in the RA synovium. All three kinase families; extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38- (264), and NF-κB contribute to inflammation through the regulation of TNF-α, IL-6, IL-8, inducible nitric oxidase synthase (iNOS) and cyclooxygenase-2 (COX-2) (24, 265). The present study suggests that *A. galanga* hexane extracts decrease the expression of MMP-1, -3, -13 and COX-2 when induced by IL-1β. However the precise mechanism is still to be elucidated. Further studies should be investigated the active components of *A. galanga* hexane extract from fraction 4, the gene product of MMP-1, -3, -13, and investigate how the extract regulates and mediates intracellular cascades.

Versican is a component of the extracellular matrix, which interacts with several matrix and cell surface molecules. Versican plays central roles in tissue morphogenesis and homeostasis and is implicated in the development of numerous diseases. The expression of versican by multiple cell types is differentially regulated in a temporal and spatial manner in physiological and pathological processes (266). A number of reports have documented a significant involvement of versican in many diseases, such as atherosclerosis and restenosis (267). It has also suggested that versican is expressed in arthritis (268). Galectin-3, Versican, and Socs3 were previously implicated in RA and other immune and inflammatory disorders (268, 269, 270). All three genes were expressed to significantly greater extents in the arthritic animals than in the controls, correlating with inflammation and immune responses, and could be utilized to identify candidate blood biomarkers for RA.

In the present study, conditional knockout mice of versican revealed hypertrophic chondrocyte nodules, tilting and clefting of the joint surface, and a slight delay of cartilage development. Such studies, elaborating on the current work, will have important implications, both potentially from a clinical perspective, as joint disease is a major human health problem, as well as from the developmental biology perspective. Since mouse versican is identical to human versican (40), the anomaly seen in mouse models may display many of the same behavioral and pathological features seen in human. Thus, mice-lacked versican model could be further studied as a clinical model related to inflammatory joint diseases together with the treatment of *A. galanga* hexane extract of fraction 4.