Versican Facilitates Chondrocyte Differentiation and Regulates Joint Morphogenesis*‡

Kanyamas Choocheep§1, Sonoko Hatano‡, Hidekazu Takagi‡, Hiroki Watanabe‡, Koji Kimata‡, Prachya Kongtawelert§, and Hideto Watanabe‡2

From the §Institute for Molecular Science of Medicine, Aichi Medical University, Karimata 21, Yazako, Nagakute, Aichi 480-1195, Japan and the ‡Thailand Excellence Center for Tissue Engineering, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand

Versican/PG-M is a large chondroitin sulfate proteoglycan in the extracellular matrix, which is transiently expressed in mesenchymal condensation areas during tissue morphogenesis. Here, we generated versican conditional knock-out mice PrxI-Cre/Vcanfloxflox, in which Vcan is pruned out by site-specific Cre recombinase driven by the Prx1 promoter. Although PrxI-Cre/Vcanfloxflox mice are viable and fertile, they develop distorted digits. Histological analysis of newborn mice reveals hypertrophic chondrocytic nodules in cartilage, tilting of the joint, and a slight delay of chondrocyte differentiation in digits. By immunostaining, whereas the joint interzone of PrxI-Cre/Vcanfloxflox shows an accumulation of TGF-β, concomitant with versican, that of PrxI-Cre/Vcanfloxflox without versican expression exhibits a decreased incorporation of TGF-β. In a micro-mass culture system of mesenchymal cells from limb bud, whereas TGF-β and versican are co-localized in the perinodular regions of developing cartilage in PrxI-Cre/Vcanfloxflox, TGF-β is widely distributed in PrxI-Cre/Vcanfloxflox. These results suggest that versican facilitates chondrogenesis and joint morphogenesis, by localizing TGF-β in the extracellular matrix and regulating its signaling.

Versican/PG-M (1) consists of a core protein and chondroitin sulfate (CS)3 chains attached to the core protein. The core protein is composed of an N-terminal G1 and a C-terminal G3 globular domain and two CS-attachment domains CS-α and CS-β between the G1 and G3 domains. The N-terminal G1 domain comprises the A, B, and B’ subdomains and binds to both hyaluronan (HA) and link protein (2). The C-terminal G3 domain binds fibronectin (3), fibulin-1 and -2 (4, 5), tenascins (4, 6), and heparan sulfate proteoglycans (7). Versican exhibits four spliced variants: V0, V1, V2, and V3 (8–11), with different CS domains. The V0 variant contains all of the domains G1, CS-α, CS-β, and G3; V1 contains G1, CS-β, and G3; V2 contains G1, CS-α, and G3; and V3 contains only G1 and G3. Interestingly, V0 and V1 are expressed widely, V2 is restricted to the nervous system, and V3 has not been detected as a protein, although mRNA is detected. Thus, the number of CS chains required for the function of versican may vary among tissues. Versican is also characterized by two distinct expression patterns. In some adult tissues, such as heart, blood vessels, and brain, it is constitutively expressed, serving as a structural macromolecule of the ECM. In embryonic stages, it is transiently expressed in various developing tissues (12), including brain, hair follicles, developing heart, and mesenchymal condensation areas of cartilage primordium. Previous in vitro studies have revealed various effects of versican on cell behavior (13). For example, it inhibits cell adhesion of MG63 osteosarcoma cells and aggravates their malignant phenotype (14). It inhibits migration of neural crest cells and the outgrowth of motor and sensory axons (15). It is expressed in dermal papilla, a dense aggregate of dermis-derived stromal cells, suggesting its involvement in hair follicle formation (16). The fact that versican is transiently expressed at high levels in areas where cells are aggregating to a high density suggests that this proteoglycan plays a crucial role in cell aggregation that leads to tissue morphogenesis.

Cartilage development initiates from mesenchymal condensations where mesenchymal cells aggregate and further differentiate into chondrocytes. During mesenchymal condensation, there is an increased expression of ECM and cell surface molecules, such as versican, tenascin, syndecans, and N-CAM (17). With synchronized collaboration of these molecules, transforming growth factor-β (TGF-β) (18), bone morphogenetic protein (19), and GDF-5 (growth differentiation factor-5) (20) lead mesenchymal cells to commitment into the chondrocytic lineage and differentiation toward chondrocytes, which synthesize ECM molecules specific to cartilage, including type II collagen and aggrecan. During these processes, synovial joints are generated with packing and lining of mesenchymal cells at the future joint location termed the interzone (21–23). These mesenchymal cells are cells that are still undifferentiated (24) or derived from dedifferentiation of chondrocytes (25). Several signaling pathways are implicated in the molecular specification of the joint interzone. Wnt pathways mediated by Wnt4, Wnt-14, and Wnt-16 (26, 27) have been shown to participate in joint formation, and another study indicates that the Wnt/β-catenin canonical signaling pathway is necessary and sufficient
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for the induction of synovial joints in the limb (28). Other studies reported that CD44-HA signaling is involved in synovial joint cavitation (29). Analysis of conditional null mice of TGF-β receptor II (TβRII) has demonstrated an essential role of TGF-β signaling in joint morphogenesis (30). During the process of cartilage development and synovial joint formation, versican exhibits dynamic expression patterns. Its expression initiates at a high level in mesenchymal condensation areas. While the cells differentiate into chondrocytes, it remains in pericondensation areas. When the joint is formed by accumulation and lining up of mesenchymal cells, versican is expressed in the joint interzone. After the formation of the joint cavity, versican is present in the articular cartilage and synovial tissue, lining the inside margin of the cavity (31, 32). These characteristic expression patterns of versican and its effects on cell behavior observed in cell culture systems strongly suggest that versican regulates, in the ECM, the function of ligands that mediate signaling toward cartilage development and synovial joint formation. An in vitro analysis of limb mesenchyme from gene-trapped versican-deficient mouse hdf (heart defect) by microarray test indicated that versican is necessary for chondrocyte differentiation of mesenchymal cells (33). Our previous studies using N1511 chondrocytic cells demonstrated that versican is necessary for chondrocyte differentiation during chondrogenesis (34). However, the signaling pathways affected by versican have not been identified, and the mechanisms by which versican regulates their signal transduction toward cartilage development and joint formation remain to be elucidated.

To investigate the in vivo role of versican in cartilage development and joint formation, we generated versican conditional knock-out mice in the limb (35). Although Prx1-Cre/Vcanflox/flox mice are viable and fertile, they develop distorted digits. Histological analysis of newborn mice reveals 1) hypertrophic chondrocytic nodules in cartilage, 2) tilting of the joint, and 3) a slight delay of chondrocyte differentiation in limbs. We further observed decreased incorporation of TGF-β in the joint interzone and its altered distribution in micromass of Prx1-Cre/Vcanflox/flox. Our study provides evidence that versican regulates TGF-β-mediated signaling by localizing TGF-β in the ECM.

EXPERIMENTAL PROCEDURES

Generation of Conditional Vcan Knock-out Mice—To generate mice in which the Vcan gene is deleted in early limb mesenchyme, the conditional knock-out mice were generated by combining Cre/loxP with a Flp/FRT system as follows. A targeting vector harboring the Vcanflox allele was constructed by flanking exon 2 of the mouse Vcan gene with loxP sites in the combination of a PGK-neoR cassette flanked by the FRT sequence. Then mouse ES cells were electroporated with linearized targeting construct and cultured with G418 to positively select for clones that had integrated the targeting construct. Clones were screened by genomic PCR, and positive clones were confirmed by Southern blotting. After blastocyst injection and homologous recombination, chimeric mice whose genomic DNA contained the Vcanflox allele were obtained. Germ line transmission was attained by crossing these chimeric mice with C57BL/6 mice. Then, by crossing with CAG-flippase transgenic (Tg) mice, Vcanflox/+ mice whose genomic DNA lacks the PGK-neoR fragment were obtained. We crossed these mice with C57BL/6 to segregate the CAG-flippase transgene and back-crossed to C57BL/6 at least four times (n ≥ 4). Then by crossing Vcanflox/+ mice with Prx1-Cre Tg mice with a background of C57BL/6, Prx1-Cre/Vcanflox/+ male and female mice were crossed to obtain Prx1-Cre/Vcanflox/flox mice whose Vcan gene was removed by Cre-mediated excision. The genotyping of transgenic mice was performed by PCR using DNA template from proteinase K digestion of a tail biopsy. WT and floxed alleles were identified by PCR using a forward primer of Int1-1 sequence 5′-TGAGCTTACAGCGTTTACAG-3′ and a reverse primer of Kpn-1 sequence 5′-CGCATGACAGCATGGAGCGAG-3′, generating a PCR product of 3 kb in wild-type and 2 kb in floxed alleles. Genomic DNA was amplified for 25 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and elongation for 5 min at 72 °C in reaction buffer containing 2 mM MgCl2, 1× Ex Taq buffer (10× Ex Taq buffer, Takara), 0.25 mM dNTPs (dNTP mixture, Takara), 0.2 μM each primer. PCR for Cre transgene was performed using the primer set of Prx1-Cre forward (5′-CTCGGAAAAATGCTTCTGCGTTTGGC-3′) and Prx1-Cre reverse (5′-GAGTTGATAGCTGGCTGGCAG-3′), generating a PCR product of 620 bp.

Analysis of Prx1-Cre Activity—ROSA26 (Jackson Laboratory, Bar Harbor, ME) reporter mice were first crossed with the Prx1-Cre transgenic line to obtain the Prx1-Cre/R26R founders. Then Prx1-Cre/R26R were bred together to generate Prx1-Cre/R26R embryos at several stages. These embryos were then used for analysis.

X-ray Analysis and Histological Analysis—X-ray analysis was performed using a soft x-ray apparatus (Softex, Tokyo, Japan). Newborn mice or embryos from several embryonic stages were fixed in 10% neutral buffered formalin. Hind limbs were dissected and embedded in paraffin, 4- or 5-μm-thick paraffin sections were cut and mounted on Superfrost Mascot slides (Matsunami Glass Inc., Osaka, Japan), sections were stained in 1× Ex Taq buffer, Takara, 0.25 mM dNTPs (dNTP mixture, Takara), 0.2 μM each primer. PCR for Cre transgene was performed using the primer set of Prx1-Cre forward (5′-CTCGGAAAAATGCTTCTGCGTTTGGC-3′) and Prx1-Cre reverse (5′-GAGTTGATAGCTGGCTGGCAG-3′), generating a PCR product of 620 bp.

Cryosectioning and X-gal Staining—Hind limbs from various embryonic stages were dissected and put into cold 1× phosphate-buffered saline (PBS) containing 2 mM MgCl2, and then fixed in 4% paraformaldehyde at 4 °C for 2–3 h. They were then washed with PBS twice, soaked in the gradient of sucrose solution at concentrations of 15 and 30% at 4 °C overnight or until they sank. They were briefly rinsed with PBS and then embedded in optimal cutting temperature compound and kept at −80 °C. Cryosections were cut at a thickness of 15 μM. The slides were fixed in 4% paraformaldehyde at 4 °C for 10–15 min and then washed twice with PBS at 4 °C. Slides were stained in X-gal solution (1 mg/ml X-gal, 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 2 mM MgCl2, 0.01% deoxycholate, 0.02% Nonidet P-40, 0.5% potassium ferrocyanide, 0.5% potassium ferricyanide, 0.05% Triton X-100, 0.4 M NaCl, 0.025% sodium azide, 0.01% sodium sulfite).
P-40) at 37 °C overnight. After staining, they were washed in PBS and counterstained with eosin, dehydrated in ethanol, xylene, and mounted.

After 3 days of micromass culture (as described below), cultures were washed three times with PBS, fixed with 2% formaldehyde for 5 min, washed twice with PBS, and stained in staining solution (0.1 m citric acid, 0.2 m sodium phosphate, pH 6.0, 5 mm K2Fe(CN)6, 5 mm K3Fe(CN)6, 150 mm NaCl, 2 mm MgCl2) at 37 °C overnight.

Immunostaining and Hyaluronan Detection—Sections were deparaffinized in xylene, rehydrated in a gradient of ethanol, and briefly washed with PBS. Then endogenous peroxidase was inactivated through incubation in 3% H2O2 in methanol for 20 min. Pretreatment with chondroitinase ABC (5 milliunits/μl; Seikagaku, Tokyo, Japan) for 30–45 min was required for versican and aggrecan. Antigen retrieval with citrate buffer, pH 6.0, by autoclave for 20 min was also essential for CD44, β-catenin, and Cre immunostaining. Then they were treated with blocking solution (Dako) at room temperature for 1 h and incubated with primary antibodies, including anti-versican glycosaminoglycan (GAG) β-domain (Chemicon) at 1:1000, anti-aggrecan clone 1C6 at 1:20, anti-link protein clone 8A4 at 1:100, anti-CD44, β-catenin, and Cre immunostaining. Then their cell density was adjusted to 2 × 107 cells/ml, and 10 μl of the cell suspension was plated onto LabTek-II chamber slides (Nalge Nunc International, Tokyo, Japan) or 35-mm culture dishes. After cells were allowed to attach at 37 °C for 1 h, cultures were then flooded with Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (Sigma) containing 10% fetal bovine serum. Cultures were incubated at 37 °C in a CO2 incubator for 6 days with fresh conditioned medium added daily. Following incubation, cultures were processed for Alcian blue staining, HA staining, or immunostaining. For double immunofluorescent staining, the sample was co-incubated with rabbit anti-versican glycosaminoglycan (GAG) β-domain (1:1000; Chemicon) and mouse anti-TGF-β (1:20; R&D Systems) for 4 °C overnight and, after washing with PBS, with a mixture of Alexa Fluor 594 anti-rabbit IgG (1:400) and Alexa Fluor 488 anti-mouse IgG (1:400). The photos of micromass culture were taken by a confocal laser-scanning microscope (LSM 710 Carl Zeiss Microlmaging, Tokyo, Japan). To precisely evaluate the localizations of molecules, the Z-stack program was applied. Immunostaining for CS chains was performed using anti-CS (LY111, 1:200; Seikagaku) and Alexa Fluor 488 anti-mouse IgM (1:400) as primary and secondary antibody, respectively.

Western Blot Analysis—Three micromasses at day 6 were collected, and the sample was subjected to 15% SDS-PAGE under a non-reducing condition. The proteins were electro-transferred to a polyvinylidene difluoride membrane, and the membrane was soaked in 5% skim milk in TBS containing 0.1% Tween 20 (TBS-T) for blocking. The membrane was treated with mouse anti-TGF-β (R&D Systems, 1:250) for 1 h at room temperature. After washing three times with TBS-T, the membrane was treated with peroxidase-conjugated goat anti-mouse IgG (1:1000) for 1 h at room temperature. After washing three times as above, the signal was detected with Western Lightning™ Plus-ECL (PerkinElmer Life Sciences). The data were analyzed using an LAS 4000 mini, luminescent image analyzer (Fujifilm). For dilution of antibodies, Can Get Signal™ (ToyoBo Life Science, Osaka) was used. The membrane was treated with Restore™ Western blot stripping buffer (Pierce) and used for immunoblot analysis of actin. The band density was analyzed using ImageJ.

Alcian Blue Staining—Whole limbs from embryos at E13.5 and E15.5 days of gestation were fixed in 96% ethanol overnight and then placed in Alcian blue staining solution (0.1% Alcian blue, 70% ethanol, 1% HCl) for 2–3 days and dehydrated in 100% ethanol for 5 days, followed by maceration in 1% KOH for 3–4 days for close observation. After that, they were cleared in 25, 50, and 80% glycerol in distilled water for 1 day for each step and stored in 100% glycerol.

Alcian blue staining was carried out on day 6 of micromass culture as follows. Cultures were washed twice with cold PBS, fixed in 100% ethanol for 5 min, and incubated with Alcian blue solution overnight. Excess stain was washed off with deionized distilled water, and photos were taken using a Zeiss Stemi SV11 microscope with a Nikon CoolPix 995 digital camera or an Olympus SZX12 microscope with an Olympus DP12 camera. The staining density was analyzed using ImageJ.

RESULTS

Generation of Prx1-Cre/Vcan<sup>fllox/fllox</sup> Mice—We designed a conditional targeting vector for the versican gene Vcan, in
which exon 2 was flanked by the \( \text{loxP} \) sequence (Fig. 1A). The targeting vector contained a PGK-neo\( ^R \) cassette flanked by FRT sites. We transfected ES cells with the vector. After selection with G418 and screening by genomic PCR and Southern blot analyses, we obtained five ES cell clones with homologous recombination. After generation of chimeric mice by blas-
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tocyst injection, they were crossed with wild-type mice, and offspring mice with germ line transmission were obtained. Then we crossed them with CAG-flippase Tg mice and obtained Vcan<sup>+</sup>/floxed mice whose genomic DNA lacks the PGK-neo<sup>R</sup> cassette. These mice were back-crossed with C57BL/6 mice to segregate the CAG-flippase transgene and further back-crossed to C57BL/6 mice at least four times (n ≥ 4). Then, by crossing Vcan<sup>+</sup>/floxed male and female mice, we obtained Vcan<sup>flox/flox</sup> mice. Both Vcan<sup>+</sup>/floxed and Vcan<sup>flox/flox</sup> mice were healthy and fertile.

Versican is transiently expressed at a high level in the mesenchymal condensation areas of cartilage primordium. There, a transcription factor, Prx-1, is also expressed at a high level (35). We crossed Vcan<sup>+</sup>/floxed with Prx1-Cre Tg mice with the background of C57BL6 and obtained Prx1-Cre/Vcan<sup>+</sup>/floxed mice. Then, by crossing these mice, we obtained Prx1-Cre/Vcan<sup>flox/flox</sup> mice, which were supposed to lack versican expression in the mesenchymal condensation areas, where Prx1 promoter activity is present.

Genotyping of the mice was performed by genomic PCR, as described under “Experimental Procedures.” The bands at 3 and 2 kb represent the WT (Vcan<sup>++</sup>) and Vcan<sup>flox/flox</sup> allele, respectively (Fig. 1B).

Prx1-Cre/Vcan<sup>flox/flox</sup> Mice Exhibit Distortion of Digits—Prx1-Cre/Vcan<sup>flox/flox</sup> mice grew apparently normal by gross appearance and were fertile, but all of the Prx1-Cre/Vcan<sup>flox/flox</sup> mice displayed distorted digits in the hind limbs. No craniofacial anomalies were detected in Prx1-Cre/Vcan<sup>flox/flox</sup> mice, although Prx1 is expressed in craniofacial tissues and central nervous systems (35, 36). X-ray examination of adult mice revealed distortion of bones, especially in the proximal phalanges (Fig. 2, A (b)). By gross observation, the distortion became obvious as early as 1 week of age and was accompanied with limb shortening (Fig. 2A (d)). In contrast, distortion was not obvious in the forelimbs by gross appearance.

The distortion might have derived from abnormality of bone, cartilage, ligament, and tendon. To determine which region was responsible for the phenotype, we performed histological analyses. Newborn hind limbs of WT showed good alignment of metatarsus and phalanges, which contain a well organized structure of differentiating chondrocytes (Fig. 2B, a and c). In contrast, Prx1-Cre/Vcan<sup>flox/flox</sup> mice showed distorted digits, which contained nodules of hypertrophic chondrocytes surrounded by proliferative and prehypertrophic chondrocytes (Fig. 2B, b and d). The joint surface between phalanges was tilted (arrow), presumably due to altered orientation of chondrocyte columns. In addition, the proximal region of proximal phalanges, adjacent to the metatarsophalangeal (MP) joint, exhibited cleft formation (arrowhead). These abnormalities were observed with complete penetrance in more than 48 mutant mice examined. Although gross observations did not disclose abnormalities in forelimbs, histological analyses revealed aberrant nodules of hypertrophic chondrocytes in digits and tilting and clefting of the metacarpophalangeal joint, similar to that in hind limbs (supplemental Fig. S1). Because the deformity was more obvious in hind limbs, we decided to investigate hind limbs.

Prx1-Cre/Vcan<sup>flox/flox</sup> Digits Exhibit Tilted Joint Surface and Delayed Cartilage Development—To determine the initiation of the deformities, we performed histological analysis on embryonic stages. At E18.5, metatarsus of Prx1-Cre/Vcan<sup>++</sup> showed well organized chondrocyte columns in the growth plate and the primary ossification center with vascular invasion (Fig. 3A, a and c). In contrast, that of Prx1-Cre/Vcan<sup>flox/flox</sup> had an aberrant nodule of hypertrophic chondrocytes surrounded by prehypertrophic and proliferative chondrocytes in a concentric pattern, and it had not yet exhibited vascular invasion. At this stage, clefting of some metatarsophalangeal joints was seen (Fig. 3A, a and d).

At E15.5, Prx1-Cre/Vcan<sup>++</sup> digits exhibited a horizontally oriented interzone, forming a joint cavity (Fig. 3B, a and c), whereas Prx1-Cre/Vcan<sup>flox/flox</sup> digits exhibited a broader interzone of small mesenchymal cells, giving rise to a wedge-shaped or vertical/longitudinal cavity (Fig. 3B, b and d). In addition, their metatarsus contained no hypertrophic chondrocytes yet...
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FIGURE 3. Prx1-Cre/Vcan<sup>+/+</sup> digits exhibit tilted joint and delayed cartilage development. A, histological analysis of E18.5 Prx1-Cre/Vcan<sup>+/+</sup> (a and c) and Prx1-Cre/Vcanflox/flox (b and d) hind limbs by hematoxylin and eosin staining. Prx1-Cre/Vcan<sup>+/+</sup> digits demonstrate a well organized columnar structure of prehypertrophic chondrocytes with vascular invasion in metatarsus (m) and proximal phalanges (pp), whereas Prx1-Cre/Vcanflox/flox digits exhibit formation of hypertrophic chondrocyte nodules in a concentric pattern in proximal phalanges (pp) (arrows in b) and delayed endochondral ossification of metatarsus (d). In addition, clefing of some metatarsophalangeal joints is observed (b). Scale bars, 100 μm (a and b) and 40 μm (c and d). B, histological analysis of E15.5 Prx1-Cre/Vcan<sup>+/+</sup> and Prx1-Cre/Vcanflox/flox hind limbs by hematoxylin/eosin and Alcian blue staining. Prx1-Cre/Vcan<sup>+/+</sup> digits display horizontal stripes of the metatarsophalangeal joint interzones (a and c (as an enlarged image of the boxed area in d)). Prx1-Cre/Vcan<sup>+/+</sup> metatarsus shows prehypertrophic and hypertrophic chondrocyte layers (e). In contrast, Prx1-Cre/Vcanflox/flox digits display tilted joint interzones (b and d (as an enlarged image of the boxed area in b)). Prx1-Cre/Vcanflox/flox metatarsus shows prehypertrophic chondrocytes but not hypertrophic chondrocytes (f). Alcian blue staining of Prx1-Cre/Vcan<sup>+/+</sup> hind limb shows well aligned stripes of the interzone (g), whereas that of Prx1-Cre/Vcanflox/flox digits reveals tilting of the joint interzone (indicated by arrowheads in h). Scale bars, 150 μm (a and b), 30 μm (c and d), 50 μm (e and f), and 70 μm (g and h). The number of embryos analyzed was as follows: n = 2 each at E18.5; n = 3 and 5 for Prx1-Cre/Vcanflox/flox and Prx1-Cre/Vcan<sup>+/+</sup> at E15.5, respectively. At least two paraffin-embedded blocks were obtained, and their tissue sections were used for histological analysis.

FIGURE 4. Versican is absent in Prx1-Cre/Vcan<sup>lox/lox</sup> digits. A, immunostaining for versican of E15.5 Prx1-Cre/Vcan<sup>+/+</sup> and Prx1-Cre/Vcan<sup>lox/lox</sup> hind limbs. In Prx1-Cre/Vcan<sup>+/+</sup> digits, versican is strongly immunostained in the interzone and perichondrium and moderately immunostained in the proliferative zone of cartilage (a and c), whereas in Prx1-Cre/Vcan<sup>lox/lox</sup> digits, versican is immunostained neither in the interzone, the perichondrium, nor the proliferative zone of cartilage (b and d). Scale bars, 120 μm (a and b) and 70 μm (c and d). B, immunostaining for Cre enzyme of E15.5 Prx1-Cre/Vcan<sup>lox/lox</sup> hind limbs at low (top) and high (bottom) magnifications. Cre enzyme is immunostained in the perichondrium and in the tilted interzone. Scale bars, 100 and 50 μm, respectively. Two individual Prx1-Cre/Vcanflox/flox digits showed the same abnormalities. (Fig. 3B (b)), contrasting with Prx1-Cre/Vcan<sup>+/+</sup>, which already contained a hypertrophic cell mass (Fig. 3B (e)). At this stage, cleft formation was obvious in the proximal region of proximal phalanges (Fig. 3B, b and h (arrowhead)).

At E14.5, we observed the joint interzone, defined as the area that consists of compact and closely associated mesenchymal cells located along the presumptive joint location (24, 25), but not a clear joint cavity yet. Hematoxylin and eosin staining exhibited no obvious differences between Prx1-Cre/Vcan<sup>+/+</sup> and Prx1-Cre/Vcan<sup>lox/lox</sup> in the joint interzone, although there were no clear joint interzone stripes in Prx1-Cre/Vcan<sup>lox/lox</sup>, compared with Prx1-Cre/Vcan<sup>+/+</sup> embryos (supplemental Fig. S2A). Immunostaining for Ki67, a protein expressed in the growing phase, showed a similar level of proliferation in cartilage between Prx1-Cre/Vcan<sup>+/+</sup> and Prx1-Cre/Vcan<sup>lox/lox</sup>. Taken together, Prx1-Cre/Vcan<sup>lox/lox</sup> digits exhibited two abnormalities, delayed chondrocyte differentiation, as observed by a decrease in hypertrophic cells, and tilting of joint surface, which initially appear at E15.5. Formation of hypertrophic chondrocyte nests at E18.5 is probably due to a shift of the columnar axis by tilting of the joint surface.

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Versican Distribution in Mice Digits—Next, we examined expression patterns of versican at E15.5, when abnormalities initiated. Whereas versican was strongly immunostained in the joint interzone and perichondrium and moderately immunostained in the proliferative zone of cartilage in Prx1-Cre/ Vcan<sup>+/+</sup> (Fig. 4A, a and c), it was not immunostained in the interzone, perichondrium, and cartilage of Prx1-Cre/ Vcan<sup>flox/flox</sup> (Fig. 4A, b and d). The regions lacking versican specifically in Prx1-Cre/Vcan<sup>flox/flox</sup> were supposed to be the regions where Cre enzyme was or had been expressed. When immunostained, Cre enzyme was observed in the perichondrium and the interzone in Prx1-Cre/Vcan<sup>flox/flox</sup> (Fig. 4B). By X-gal staining at E14.5 of Prx1-Cre/R26R, β-galactosidase activity was found in chondrocytes as well as in perichondrium (supplemental Fig. S2B). These observations support the lack of versican expression in Prx1-Cre/Vcan<sup>flox/flox</sup>

Distribution of Hyaluronan and Its Binding Molecules in the Joint—Whereas HA is bound to both aggrecan and link protein and profoundly incorporated as the proteoglycan aggregate in cartilage, it is also accumulated in the joint interzone, which contributes to joint formation. Recent studies suggested that the accumulated HA mediates signals via CD44 toward joint cavity formation (29). Because our immunostaining demonstrated the presence of versican in the interzone at E15.5 of Prx1-Cre/Vcan<sup>+/+</sup> and its absence in Prx1-Cre/Vcan<sup>flox/flox</sup>, we speculated that versican is necessary for adequate levels of HA accumulation and HA-mediated signaling toward joint formation and that its absence caused the joint abnormality. By the detection method using biotinylated HABP, accumulation of HA was observed at a similar level in the interzone of both Prx1-Cre/Vcan<sup>+/+</sup> and Prx1-Cre/Vcan<sup>flox/flox</sup> (Fig. 5, A and B). Immunofluorescent staining displayed the presence of versican in the interzone, future articular surface, and perichondrium in Prx1-Cre/Vcan<sup>+/+</sup> and its absence in Prx1-Cre/Vcan<sup>flox/flox</sup> (Fig. 5, C and D). Immunofluorescent staining for aggrecan that clearly demarcates cartilage tissue from the interzone revealed clefting of the future joint surface in Prx1-Cre/ Vcan<sup>flox/flox</sup> (Fig. 5, E and F). Whereas link protein was colocalized with aggrecan in Prx1-Cre/Vcan<sup>+/+</sup>, it was also present in the interzone in Prx1-Cre/Vcan<sup>flox/flox</sup> (Fig. 5, G and H). These observations suggest that whereas versican retains HA in the interzone, link protein does so in the absence of versican. CD44 was not detected at E15.5, eliminating involvement of CD44 in the joint formation at this embryonic stage (Fig. 5, I and J).

TGF-β Signaling Is Attenuated in Prx1-Cre/Vcan<sup>flox/flox</sup> Interzone—When compared with animals that exhibit joint malformation, the abnormalities found in Prx1-Cre/ Vcan<sup>flox/flox</sup> embryos resembled conditional knock-out mice of TβRII generated by crossing Prx1-Cre transgenic and Tgfbr2<sup>flox/flox</sup> mice, which exhibit the failure of joint interzone development (30). We speculated that TGF-β signaling is altered in Prx1-Cre/Vcan<sup>flox/flox</sup>. When the expression patterns of TGF-β and its related molecules were investigated by immunostaining, whereas TGF-β was localized in Prx1-Cre/Vcan<sup>+/+</sup> interzone, it was not detected in Prx1-Cre/Vcan<sup>flox/flox</sup> (Fig. 6, A and B). TβRII was broadly immunostained in interzone, perichondrium, and chondrocytes of both Prx1-Cre/Vcan<sup>+/+</sup> and Prx1-Cre/Vcan<sup>flox/flox</sup> digits (Fig. 6, C and D). Phospho-Smad2/3 was detected in the nuclei of cells in the Prx1-Cre/Vcan<sup>+/+</sup> interzone (Fig. 6F). In contrast, it was not detected in Prx1-Cre/Vcan<sup>flox/flox</sup> (Fig. 6F). These results indicate that TGF-β signaling is substantially diminished in Prx1-Cre/Vcan<sup>flox/flox</sup>, although the expression of its receptor is unaffected.

Impaired Mesenchymal Condensations and Altered TGF-β Signaling in Prx1-Cre/Vcan<sup>flox/flox</sup> Mice Lead to Delayed Chondrocyte Differentiation—Prx1-Cre/Vcan<sup>flox/flox</sup> digits showed a delay in chondrocyte differentiation at E15.5 (Fig. 3B). Interestingly, whole mount Alcian blue staining demonstrated a decrease in areas positive for Alcian blue in Prx1-Cre/ Vcan<sup>flox/flox</sup> hind limbs compared with Prx1-Cre/Vcan<sup>+/+</sup> (supplemental Fig. S3), suggesting that delay of chondrocyte differentiation took place prior to E13.5. To obtain insight into
the mechanisms of delayed chondrocyte differentiation in Prx1-Cre/Vecan^fl/+ digits, we applied a high density micromass culture system of limb bud mesenchymal cells. At day 3 of micromass culture, Prx1-Cre/Vecan^+/+ contained some cartilaginous nodules stained with Alcian blue (Fig. 7A). At day 6, the number of the nodules positive for Alcian blue increased considerably (Fig. 7C). When compared with Prx1-Cre/Vecan^+/+, Prx1-Cre/Vecan^fl/+ micromass at day 3 contained a small number of cartilaginous nodules (Fig. 7B). At day 6, the number of the nodules positive for Alcian blue increased, but it remained smaller than Prx1-Cre/Vecan^+/+ (Fig. 7D). At day 9, their number increased, but the nodules remained smaller than Prx1-Cre/Vecan^+/+ micromass (Fig. 7F). Quantitatively, the percentage of area positive for Alcian blue was 21 ± 2 and 18 ± 4.2% at day 3, and 35 ± 2.6 and 32 ± 5.6% at day 6 in Prx1-Cre/Vecan^+/+ and Prx1-Cre/Vecan^fl/+ micromass (from two different littersmates), respectively. These observations indicate that Prx1-Cre/Vecan^fl/+ cells in the mesenchymal condensation showed a delay in chondrocyte differentiation.

Then we investigated expression of versican in the micromass. At day 3 in Prx1-Cre/Vecan^+/+, versican was localized in the center of the condensation areas of future cartilaginous nodules (supplemental Fig. S4A), confirming its transient high expression in mesenchymal condensation areas. At day 6, it was found in areas surrounding the cartilaginous nodules, designated perinodular regions (Fig. 8A). In contrast, versican was not observed in micromass of Prx1-Cre/Vecan^fl/+; which suggests that the Cre enzyme driven by Prx1 promoter successfully abrogated versican expression in all of the mesenchymal cells in the micromass (Fig. 8B). This was further confirmed by X-gal staining of micromass obtained from Prx1-Cre/R26R limb buds (supplemental Fig. S4B).

TGF-β signaling is known to facilitate chondrocyte differentiation because the chondrocyte differentiation medium of bone marrow mesenchymal stem cells contains TGF-β (37). As shown in Fig. 6, TGF-β was substantially decreased in the Prx1-Cre/Vecan^fl/+ micromass culture, which caused the delay in chondrocyte differentiation. By immunofluorescent staining, TGF-β was localized in the perinodular regions in Prx1-Cre/Vecan^+/+ micromass (Fig. 8C), similar to versican. In contrast, it was observed diffusely in the extracellular matrix at a substantially lower intensity in Prx1-Cre/Vecan^fl/+ micromass (Fig. 8D). When merged, both versican and TGF-β were well co-localized in Prx1-Cre/Vecan^+/+ micromass (Fig. 8E).

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**FIGURE 8.** TGF-β signaling is altered in the micromass of Prx1-Cre/Vcan\(^{+/+}\) and Prx1-Cre/Vcan\(^{flox/flox}\) micromass. Immunofluorescent staining at day 6 of culture, for versican (A and B), TGF-β (C and D), both merged (E and F), TßRII (G and H), and phospho-Smad2/3 (I and J) of Prx1-Cre/Vcan\(^{+/+}\) (A, C, E, G, and I) and Prx1-Cre/Vcan\(^{flox/flox}\) (B, D, F, H, and J) are shown. Versican is immunostained strongly in the perinodular region and moderately in the internodular region in Prx1-Cre/Vcan\(^{+/+}\) (A), whereas it is immunostained very faintly in Prx1-Cre/Vcan\(^{flox/flox}\) (B). TGF-β is mainly localized in the perinodular regions in Prx1-Cre/Vcan\(^{+/+}\) similar to versican (C), whereas it is immunostained diffusely at a substantially lower intensity in Prx1-Cre/Vcan\(^{flox/flox}\) (D). When merged, both versican and TGF-β are well co-localized in the perinodular region (E). TßRII is immunostained mainly in the perinodular region in both Prx1-Cre/Vcan\(^{+/+}\) (G) and Prx1-Cre/Vcan\(^{flox/flox}\) (H) micromass. In addition, it is immunostained rather diffusely in Prx1-Cre/Vcan\(^{flox/flox}\) (H). Phospho-Smad2/3 is immunostained in the nuclei of perinodular cells strongly and in chondrocytes in the nodules moderately in Prx1-Cre/Vcan\(^{+/+}\) micromass (I). In contrast, it is immunostained moderately in the nuclei of chondrocytes in the nodules and weakly in the perinodular cells in the Prx1-Cre/Vcan\(^{flox/flox}\) micromass (J).

In addition, it was immunostained rather diffusely in Prx1-Cre/Vcan\(^{flox/flox}\) (Fig. 8H). Phospho-Smad2/3 was immunostained strongly in the nuclei of perinodular cells and moderately in the nuclei of chondrocytes within the nodules in the Prx1-Cre/Vcan\(^{+/+}\) micromass (Fig. 8I). In contrast, it was immunostained in the nuclei of chondrocytes in the nodules moderately and in those of the cells weakly in the perinodular regions of the Prx1-Cre/Vcan\(^{flox/flox}\) micromass (Fig. 8J). These results strongly suggest that versican accumulates TGF-β in the extracellular matrix of the perinodular regions and facilitates its signaling. The diffuse patterns of TGF-β in the extracellular matrix and the positive immunostaining for phospho-Smad2/3 in chondrocytes indicate that TGF-β signaling functions toward the differentiation even in the absence of versican. By Western blot analysis, Prx1-Cre/Vcan\(^{flox/flox}\) micromass exhibited comparable expression levels of TGF-β (supplemental Fig. 5E).

Co-localization of versican and TGF-β in Prx1-Cre/Vcan\(^{+/+}\) micromass and diffuse patterns of TGF-β in the absence of versican in the Prx1-Cre/Vcan\(^{flox/flox}\) micromass strongly suggest a direct binding of versican to TGF-β or its complex. Versican contains at least three functional domains: the G1 domain that binds hyaluronan, the G3 domain that binds various ECM molecules, and CS chains. To investigate whether CS chains are necessary for localizing TGF-β in the ECM, we treated Prx1-Cre/Vcan\(^{+/+}\) micromass with chondroitinase ABC for 48 h before immunostaining. In the micromass treated with chondroitinase ABC, versican remained in the areas surrounding the nodules (Fig. 9, A and B), confirming that CS chains are not essential for incorporation of versican in the ECM. TGF-β also similarly remained in the areas surrounding the nodules (Fig. 9, C and D). When merged, these molecules were well co-localized in the perinodular region (Fig. 9, E and F), even after ablation of CS chains (Fig. 9H). These observations indicate that versican without CS chains may retain the function of localizing TGF-β.

**DISCUSSION**

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 of the joint surface, and a slight delay of cartilage 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 of the joint surface, and a slight delay of cartilage development.

Immunofluorescent staining was visualized by Alexa Fluor 594. Scale bars, 13 μm. Immunofluorescent staining was performed at least twice, with essentially the same results. Immunostaining was performed at least three times from individual micromass for Prx1-Cre/Vcan\(^{+/+}\) and Prx1-Cre/Vcan\(^{flox/flox}\), with essentially the same immunostaining patterns.
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misaligned 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 (24, 25). 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/Vcanflox/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 phospho-Smad2/3 were markedly diminished. Recently, TGF-β type II receptor gene (Tgfrb2) conditional knock-out mice under the control of the 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 (30). The absence of versican in the joint interzone of Prx1-Cre/Vcanflox/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/Vcanflox/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 dedifferentiation of chondrocytes. Because chondrocytes were absent in the interzone of Prx1-Cre/Vcanflox/flox as evaluated by immunostaining for aggrecan, the process of their dedifferentiation to mesenchymal cells is probably intact, even with considerable reduction of TGF-β signaling.

HA and its receptor CD44 have been proposed to play key roles in joint cavitation (38–40). We have shown that unincorporated HA in the joint interzone of both Prx1-Cre/Vcan+/+ and Prx1-Cre/Vcanflox/flox is accumulated at similar levels in the joint interzone. In addition, the expression of CD44 in the joint was not detected until E18.5 (supplemental Fig. S5A). These observations may exclude direct involvement of HA- and CD44-mediated signaling in the joint abnormalities of Prx1-Cre/Vcanflox/flox digits.

The Wnt/β-catenin canonical signaling pathway has been reported to be necessary and sufficient for the induction of synovial joints in the limb (28). 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/Vcanflox/flox (supplemental Fig. S5B), although more mesenchymal cells were observed in the interzone. Together with the fact that the joints themselves are formed in Prx1-Cre/Vcanflox/flox, versican is unlikely to have direct effects on Wnt signaling.

It is intriguing that the abnormalities of Prx1-Cre/Vcanflox/flox appeared only in autopods, although versican is expressed in both proximal and distal joint interzone, articular cartilage, and synovia of limbs (28). 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

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knock-out 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.

Versican Accumulates TGF-β to Perinodular Regions in Mesenchymal Condensation—Because versican was identified in mesenchymal condensation areas of chick limb bud, this proteoglycan has been assumed to play an important role in cartilage development. Our previous study using N1511 chondrocyteoglycan has been assumed to play an important role in cartilage development. Our previous study using N1511 chondrocyteoglycan has been assumed to play an important role in cartilage development. Our previous study using N1511 chondrocyteoglycan has been assumed to play an important role in cartilage development. Our previous study using N1511 chondrocyteoglycan has been assumed to play an important role in cartilage development. Our previous study using N1511 chondrocyteoglycan has been assumed to play an important role in cartilage development. Our previous study using N1511 chondrocyteoglycan has been assumed to play an important role in cartilage development. 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