ANIMAL MODELS

The in Vivo Effect of Prophylactic Subchondral Bone Protection of Osteoarthritic Synovial Membrane in Bone-Specific Ephb4-Overexpressing Mice

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Osteoarthritis (OA) is characterized by progressive joint destruction, including synovial membrane alteration. EphB4 and its ligand ephrin-B2 were found in vitro to positively affect OA subchondral bone and cartilage. In vivo in an experimental mouse model overexpressing bone-specific Ephb4 (TgEphB4), a protective effect was found on both the subchondral bone and cartilage during OA. We investigated in the TgEphB4 mouse model the in vivo effect on synovial membrane during OA. Knee OA was surgically induced by destabilization of the medial meniscus (DMM). Synovial membrane was evaluated using histology, histomorphometry, IHC, and real-time PCR. Compared to DMM-wild-type (WT) mice, DMM-TgEphB4 mice had a significant decrease in synovial membrane thickness, vascular endothelial growth factor, and the profibrotic markers fibrin, type 1 procollagen, type 3 collagen, connective tissue growth factor, smooth muscle actin-α, cartilage oligomeric matrix protein, and procollagen-lysine, and 2-oxoglutarate 5-dioxygenase 2. Moreover, factors known to modulate transforming growth factor-β signaling, transforming growth factor receptor 1/ALK1, phosphorylated Smad-1, and heat shock protein 90β were significantly decreased in DMM-TgEphB4 compared with DMM-WT mice. Ephb4 overexpression also exhibited a protective effect on synovial membrane thickness of aged (24-month-old) mice. Overexpression of bone-specific Ephb4 clearly demonstrated prevention of the development and/or progression of fibrosis in OA synovial membrane, reinforcing the hypothesis that protecting the subchondral bone prophylactically and during OA reduces the pathologic changes in other articular tissues. (Am J Pathol 2015, 185: 335–346; http://dx.doi.org/10.1016/j.ajpath.2014.10.004)

Osteoarthritis (OA) is the most common form of arthritis and has a higher prevalence in the elderly population. Although considerable advancement has been made toward a better understanding of the pathophysiology of the disease process, no therapy has yet been approved as a disease-modifying osteoarthritis drug by regulatory agencies. There is thus a serious need to identify new candidates that will arrest the destruction of the joint tissues.

OA is a debilitating disease that results from a complex degradative mechanism in the joint. The final common pathway is joint tissue destruction due to failure of tissues or cells to maintain a balance between matrix synthesis and degradation. Because OA is now recognized to be an organ disease that involves degenerative processes not only in the articular cartilage but also in the subchondral bone and the synovial membrane, it is therefore extremely important to identify therapeutic candidates whose effects influence the joint globally.

The ephrin system includes members of the erythropoietin-producing hepatocellular (Eph) receptors, which is a subfamily of membranous tyrosine kinases, and their ephrin ligands. Ephs are grouped into two subclasses (A and B) according to their primary structure and function. Support was obtained from a grant from the Chair in Osteoarthritis of the University of Montreal, Montreal, QC, Canada.

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their ephrin ligand (A or B) specificity. Although the Eph receptors and ephrins are involved in different tissues and organs and in various phenomena, a major common role is controlling the remodeling of the extracellular matrix (ECM). Ephrins are known to play key roles in the development of several tissues and organs. In the musculoskeletal system, EphB4 and its specific ligand ephrin-B2 were recently found to be involved in the postembryonic control of bone homeostasis.\(^1\) The role of ephrins in OA is an emerging field, in which our group was the first to report the presence of ephrin-B2/EphB4 in human adult subchondral bone and cartilage and the in vitro beneficial effects of ephrin-B2 activation of EphB4 on OA osteoblasts and chondrocytes.\(^4,5\) A further in vivo study using the bone-specific Ephb4-overexpressing mouse model\(^6\) found a significant reduction in knee OA progression not only in the subchondral bone but also in the cartilage. These findings indicate that protecting the subchondral bone prophylactically and during the OA process could impart a protective effect on structural changes in another articular tissue. However, it is important to complement the effect on this mouse model with data on OA synovial membrane. We therefore further investigated the in vivo effect of bone-specific Ephb4 overexpression on synovial membrane alterations during OA and in aging mice.

Materials and Methods

Bone-Specific Ephb4-Overexpressing Mice

The model used in this study was a transgenic mouse in which Ephb4 is overexpressed under the control of the mouse Col1 promoter,\(^1\) which contains a 2.3-kb osteoblastic-specific promoter region for the mouse type I procollagen-\(\alpha\)-1 [pro-\(\alpha\)-1(1)] collagen gene. This promoter is widely used in the bone field for its robust promoter activity and transgene expression specificity to mature osteoblast.\(^1\) This transgenic mouse model (TgEphB4), in which the zygosity and the transgene overexpression protein production were confirmed, was recently used and described by our group.\(^6\)

All procedures that involved animals were performed according to regulations of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the University of Montreal Hospital Centre. All mice were kept in a 12-hour light/dark cycle. Food and water were available \textit{ad libitum} as previously described.\(^6\)

Surgically Induced OA Mouse Model

OA was surgically induced for 12 weeks in 10-week-old TgEphB4 homozygous and wild-type (WT) male mice by destabilization of the medial meniscus (DMM) of the right knee as previously described.\(^6,8\) The mice were anesthetized with isoflurane and O\(_2\), and the destabilization of the right knee joint was induced by transection of the anterior attachment of the medial meniscus to the tibial plateau. A sham operation, which involves a similar incision to the knee without compromising the joint capsule, was also performed on the right knee of the 10-week-old WT and TgEphB4 mice. Mice were observed daily to verify healing and to ensure that they were using their right limbs.

Aged Animals

To evaluate the effects of bone-specific Ephb4 overexpression on aged animals, the knee joints of 6-, 12- and 24-month-old male TgEphB4 and WT mice not operated on were further processed for histologic evaluation.

Histology, Histomorphometry, and Immunohistology Determinations

Histology

Mice undergoing DMM operations and sham operations at 12 weeks after surgery and controls not undergoing operations at an equivalent age of 22 weeks (or at 6, 12, or 24 months old) for the aging experiments were euthanized. The right knee joints were dissected free of tissue, fixed for 16 hours at 4°C in 4% paraformaldehyde, rinsed in phosphate-buffered saline, decalcified in RDO Rapid Decalciﬁer (Apex Engineering, Plainfield, IL), and embedded in paraffin as previously described.\(^6\) Sections of 5 \(\mu\)m were deparaffinized in xylene followed by a graded series of alcohol washes and stained with Safranin-O (Sigma-Aldrich, Oakville, ON, Canada). For fibrin, evaluation was performed using the Martius, Scarlet, and Blue method as described.\(^9,10\)

Histomorphometric Quantitative Analysis

Histomorphometric quantitative analysis of the anterior and posterior synovial membrane thickness was performed on Safranin-O sections. Images were captured at 63× with a Leitz Diaplan microscope (Leica Microsystems, Wetzlar, Germany) coupled to a personal computer and histomorphometric data determined with Bioquant OSTEO Image Analysis Software version 12.5.60 MIR (Bioquant, Nashville, TN); data are expressed as micrometers. The anterior and posterior medial synovial membrane lining hyperplasia was graded on a scale of 0 to 2, with 0 indicating absence; 1, hyperplasia of lining <50% of the surface; and 2, hyperplasia of lining >50% of the surface, as previously described.\(^11\) Two independent observers (G. V. F. and F. Paré) blinded to group allocation graded the severity of the OA synovial membrane. Three sections were made from each block, each slide was examined, and the final score was a consensus between the two observations.

Immunohistochemical Analysis

Immunohistochemical (IHC) analysis was performed on 5-\(\mu\)m paraffin sections as previously described.\(^12\) Briefly, the slides were heated at 65°C for 20 minutes in 10 mmol/L citrate buffer (pH 6.0), then preincubated with 0.3% Triton X-100 for 30 minutes at room temperature and with 2% H\(_2\)O\(_2\) for 15 minutes at room temperature. They were further
incubated for 60 minutes with 2% normal serum (Vector Laboratories, Burlingame, CA) and overlaid with the primary antibodies for 18 hours at 4°C in a humidified chamber. The antibodies were a goat polyclonal anti-EphB4 (dilution 1:100; R&D Systems, Minneapolis, MN), rabbit polyclonal anti—proliferating cell nuclear antigen (dilution 1:200; Abcam, Cambridge, MA), goat polyclonal anti—pro-COL1A1 for cell staining (N-17, dilution 1:200) and for ECM staining (dilution 1:2000), rabbit polyclonal anti-COL3A1 (S17, dilution 1:100) (both from Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti—procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (Plod2) (dilution 1:300; Cedarlane, Burlington, NC), phosphospecific rabbit polyclonal anti-Smad1 (Ser465) (dilution 1:100; Abcam), mouse anti—heat shock protein (Hsp) 90β (dilution 1:10,000; Enzo Life Sciences, New York, NY), and a rabbit polyclonal anti—vascular endothelial growth factor (VEGF) (dilution 1:1000; Abcam). Each slide was washed 3 times in phosphate-buffered saline (pH 7.4) and incubated with a secondary antibody using the Vectastain ABC kit (Vector Laboratories) following the manufacturer’s instructions. The color was developed with diaminobenzidine that contained H2O2 with nickel, except for Smad1 phosphospecific and pro-COL1A1, and slides were counterstained with eosin and for the nuclei with hematoxylin.

Control procedures were performed according to the experimental protocol as follows: omission of the primary antibody and substitution of the primary antibody with a nonspecific IgG from the same host as the primary antibody (Santa Cruz Biotechnology). For each synovial membrane specimen, positive cells were quantified according to a previously described method. In brief, for each section, three microscopic fields (200×) were assigned, using the medial meniscus as a point of reference, and cells staining positive were quantified following the determination of the total number of cells and of those staining positive for the antigen. The final results were expressed as the percentage of cells staining positive for the antigen, with the maximum score being 100%. Each slide was examined by two independent observers (G.V.F. and F. Paré) and scored blindly.

The pro-COL1A1 stain detected in the ECM was graded on a scale of 0 to 3, with 0 indicating no staining; 1, minor staining; 2, marked staining; and 3, maximal staining, as previously described.14

PCR Analysis

Synovial Membrane Isolation

To avoid other tissue contamination, the synovial membrane was isolated in vivo by a board certified veterinary surgeon (B.L.) using the following technique with the assistance of an inverse microscope (Motic Life Science, Causeway Bay, Hong Kong) and under aseptic conditions. Briefly, a medial arthroscopy was first performed from the tibial plateau to the proximal part of the patella. The patellar tendon was transected and a lateral arthroscopy was performed, thus isolating and elevating the quadriceps femoris and the patella from the knee joint. This resulted in the exposure of the joint capsule and synovial membrane on the femur. Then, a double reversed horseshoe-shaped incision was made around the trochlea femoralis with a number 12 scalpel blade. The delineated area was then gently detached from the femur using blunt dissection, isolating the femoral section of the synovial membrane. The isolated synovial membrane was grasped with fine-toothed forceps and transferred to a vial that contained TRIzol reagent (Invitrogen, Carlsbad, CA).

RNA Extraction, Reverse Transcription, and Real-Time PCR

Total cellular RNA from normal and OA synovial membranes were extracted with TRIzol reagent (Invitrogen) according to the manufacturer’s specifications and treated with the DNA-free DNase Treatment and Removal Kit (Ambion, Austin, TX) to ensure complete removal of chromosomal DNA. The RNA was quantitated with the RiboGreen RNA Quantitation Kit (Molecular Probes, Eugene, OR). The reverse transcription reactions were primed with random hexamers and real-time quantitation performed in the GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA) with QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA), used according to the manufacturer’s specifications. The primer sequences were as follows for sense and antisense, respectively: for α-smooth muscle actin (α-SMA), 5′-GGATCCGACGCTGAGAAG-TA-3′ and 5′-GGTCATGGTCTGTCACA-3′; for cartilage oligomeric matrix protein (COMP), 5′-GATAAGTGG-GGAGATGCTCTG-3′ and 5′-CGGGGACAGTTGTCACGTA-3′; for connective tissue growth factor (CTGF), 5′-CCACCCGAGTTACCAATGAC-3′ and 5′-CCTTGCGGA-TTTAGGTGTC-3′; for interleukin-1β (IL-1β), 5′-AAGG-GTCTGTTCCAAACCTGTCGAC-3′ and 5′-ATACGCTGCCGCTGAAACCTTGTG-3′; for matrix metalloproteinase (MMP)-9, 5′-CTGTCGCGCTTTGTCAGCT-3′ and 5′-AG-ACTGACATAAGGCCATCC-3′; for transforming growth factor (TGF)-β1, 5′-GGAGAGCCTGTACCAA-3′ and 5′-AAGTGCGATGTAGCCCT-3′; for TGF-β receptor 1 (TGF-βR1)/Alk5, 5′-CTGCTCTACGTTGCTTTC-3′ and 5′-GCAAAGACCATTCTGTCACA-3′; for TGF-βR1/Alk1, 5′-GTCAGCACAATACTCCAGAG-3′ and 5′-ACCTCTCTCACTCTCCTAC-3′; and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CAACAGCTCAT-GGCACT-3′ and 5′-GATCCACGACAGCAGGAA-3′.

The data were collected and processed using Rotor-Gene RG-3000A software version 6.1 (Corbett Research, Mortlake, VIC, Australia) and given as a threshold cycle (Ct). Plasmid DNAs that contained the target gene sequences were used to generate the standard curves, and the values for each sample were calculated as the ratio of the number of molecules of the target gene to the number of molecules of the housekeeping gene GAPDH and expressed as arbitrary units. The primer efficiencies for the test genes were the same as for the GAPDH gene.
Statistical Analysis

Data are expressed as means ± SEM. Statistical significance was assessed by the Mann-Whitney test or the Fisher’s exact test where appropriate. \( P \leq 0.05 \) was considered significant.

Results

To confirm that the promoter used to generate the TgEphB4 mice was osteoblast specific, we first looked at the level of EphB4 in the synovial membrane comparing the WT with the TgEphB4 controls (22-week-old nonoperated on mice) and DMM mice. Data revealed an increased EphB4 level in the synovial membrane of DMM mice compared with their controls (Figure 1, A–E). However, as expected, a similar level of EphB4 between control WT and TgEphB4, and DMM-WT and -TgEphB4 mice was found (Figure 1E).

DMM-TgEphB4 Mice Exhibit a Protective Effect on Synovial Membrane Remodeling

Histomorphometric evaluation of the synovial membrane (Figure 2A) comparing OA-DMM with controls not undergoing operation or undergoing sham operations revealed an increase in synovial membrane thickness in DMM-WT (Figure 2, B–D and H) and DMM-TgEphB4 (Figure 2, E–H) mice. However, the synovial membrane thickness of the DMM-TgEphB4 mice was significantly reduced compared with the DMM-WT mice (Figure 2H). The synovial lining layers (Figure 2I) were also found to be significantly elevated in the DMM-WT mice compared with the mice not undergoing operation or undergoing sham operation. A slight reduction was found for the DMM-TgEphB4 mice compared with the DMM-WT mice (Figure 2I); however, this finding did not reach statistical significance.

To ascertain that the decreased synovial membrane thickness in the DMM-TgEphB4 mice was not associated with a decrease in cells, we further investigated the level of cell proliferation using the proliferating cell nuclear antigen antibody. Data revealed no significant differences in cell proliferation between DMM-WT and DMM-TgEphB4 mice (data not shown).

Because angiogenesis is a process that reflects loss of the synovial membrane integrity, we further looked at the level of VEGF, a marker of this process. VEGF and the number of blood vessels were markedly elevated in the DMM mice but markedly decreased in the DMM-TgEphB4 mice compared with the DMM-WT mice (Figure 2, J and K).

DMM-TgEphB4 Mice Exhibit Reduced OA Synovial Membrane Fibrosis Markers

The greater thickness of the synovial membrane of the DMM mice then suggested the presence of fibrosis. We therefore investigated factors associated with the persistence of fibrosis in OA synovial membrane. First, we looked at the presence of fibrin (Figure 2, L–R), a well-recognized inductor of synovial cell activation and an ECM protein involved in synovial tissue remodeling. Data revealed the presence of fibrin in the DMM-WT mice (Figure 2O), whereas almost no zone of fibrin was found in the DMM-TgEphB4 mice (Figure 2R) \( P < 0.02 \), Fisher’s exact test). Second, using IHC, we evaluated the production of two collagen types present in the synovial membrane, pro-COL1A1, and COL3A1. Compared with the DMM-WT mice, the synovial membrane of the DMM-TgEphB4 mice revealed a statistically significant decrease in pro-COL1A1 in the matrix and in the percentage of positive synoviocytes (Figure 3A). The latter was also found for COL3A1 (Figure 3B). Because collagen cross-linking is an important factor involved in collagen fiber degradation, we further evaluated the levels of a modulator of collagen cross-linking, Pld2. Pld2 is an enzyme that catalyzes the hydroxylation of lysyl residues in collagen-like peptides and is therefore a critical factor for the stability of
intermolecular cross-links. Data also revealed a significant decrease in Plod2 in DMM-TgEphB4 mice compared with DMM-WT mice (Figure 3C).

Determination of the expression levels of factors known to be involved in synovial membrane fibrosis (Figure 4, A–C), such as CTGF, α-SMA, and COMP, revealed a significant decrease in all the factors in the DMM-TgEphB4 compared with the DMM-WT mice.

Also known to be involved in synovial membrane remodeling and inflammation are certain MMPs and ILs. Assessment of MMP-9 and IL-1β (Figure 4, D and E) revealed that although a lower level of expression was seen for the DMM-TgEphB4 mice, this finding did not reach statistical significance.

Furthermore, we looked at one of the major contributors to fibrosis, TGF-β. Data revealed that although the
DMM-TgEphB4 synovial membrane had a lower TGF-β1 gene expression level than the DMM-WT mice, the difference was not statistically significant (Figure 5A). We then further looked at the signaling activities of TGF-β. We first determined the levels of the TGF-β receptors in the DMM-WT and DMM-TgEphB4 mice. TGF-β1 effects are mediated by the binding of TGF-β1 to its specific receptor, TGF-βR2, which recruits the type I receptor activin-like kinase 1 (TGF-βR1/ALK1) or type I receptor activin-like kinase 5 (TGF-βR1/ALK5). Data revealed that although Tgf-βR2 was not significantly modulated (data not shown) between DMM-WT and DMM-TgEphB4 mice, the TGF-βR1/ALK1, but not the TGF-βR1/ALK5, was significantly decreased in the DMM-TgEphB4 mice (Figure 5, B and C). This result was further confirmed by reduced levels of phosphorylated Smad-1 observed in the synovial membrane of the DMM-TgEphB4 mice (Figure 5D).

In addition, Hsp90β, which is known to facilitate the folding and stabilization of many intracellular signaling molecules and to play a critical role in TGF-β signaling by stabilizing TGF-β receptors,18 was also markedly and significantly reduced in the DMM-TgEphB4 mice compared with the DMM-WT mice (Figure 5E). Together, these findings suggest that TGF-β signaling is down-regulated in OA-DMM TgEphB4 mice.

**TgEphB4 Mice Exhibit a Protective Effect on Aged Synovial Membrane**

Previous studies have found that C57BL/6 mice exhibit spontaneous OA characteristics during aging, revealing a
high incidence of OA changes in the knee joint from approximately the 17th month of life. We therefore looked at the effects in the bone-specific Ephb4-overexpressing mouse model on the integrity of the synovial membrane of 6-, 12-, and 24-month-old mice (Figure 6A-C). Histologic evaluation of the synovial membrane of control (WT) mice revealed significant differences in synovial membrane thickness between 6-, 12-, and 24-month-old mice (Figure 6D) as previously observed. In addition, synovial membrane of WT mice at 24 months of age also exhibited an increase in synovial membrane lining layers (Figure 6E) compared with TgEphB4 mice; however, this finding did not reach statistical significance. Importantly, at 24 months of age, the TgEphB4 mice had a decrease in the synovial thickness and the lining layers, with significance reached for the thickness at 24 months of age.

Discussion

From the previous data indicating that in vitro Ephb4 activation has beneficial effects on the OA cartilage and subchondral bone and in vivo evidence that its bone overexpression prophylactically protects the OA alterations not only in the subchondral bone but also the cartilage, we hypothesized that, during the OA process, protecting the subchondral bone would minimize the evolution of the disease with the severity of OA abnormalities being reduced in other articular tissues. Because, in addition to subchondral bone remodeling and cartilage destruction, alterations in the synovial membrane are a key phenotypic characteristic associated with the development of OA, we further looked at the effects of bone-specific Ephb4 overexpression on OA synovial membrane.

The present study found that protecting the subchondral bone in vivo prophylactically and during the OA process protects the synovial membrane architecture by decreasing the development of the synovial membrane thickness, fibrosis, and its associated markers. Our study confirmed that the beneficial effect observed in the DMM-TgEphB4 synovial membrane is not due to an overexpression of EphB4 in this tissue but associated with a protective effect on the subchondral bone. Indeed, the synovial membrane of both WT and TgEphB4 mice had similar levels of EphB4. In turn, this finding also confirmed the bone specificity of the Ephb4 cDNA that was subcloned upstream of the osteoblast-specific promoter region of the mouse proα(1) collagen gene. Moreover, the increased levels of EphB4 in the DMM mice compared with controls agree with previous findings that EphB4 is up-regulated during the OA process.

Fibrosis is characterized by excessive accumulation of ECM in the affected tissue that often results in destruction of its normal architecture, leading to a loss of function. Stiffening of the joint is a feature of knee OA reported to be, at least in part, due to the synovial membrane alterations, of which fibrosis is a major event. The protective effect of the bone-specific Ephb4 overexpression on the synovial membrane architecture and fibrotic markers is reflected in the DMM-TgEphB4 mice by a marked reduction of the OA features, including thickening, hyperplasia of the lining layer,
fibrin deposition, and vascularity, as well as the classical fibrotic markers CTGF, α-SMA, and COMP, known to play a major role in fibrotic diseases,\textsuperscript{26–28} and more specifically in synovial membrane fibrosis.\textsuperscript{29–32} Moreover, in fibrotic tissues, the excessive deposition of macromolecules in the ECM involves primarily collagen. Although type 1 collagen is the major component in the process of building up fibrosis, type 3 collagen is also increased in such diseased tissues,\textsuperscript{33} and these two types of collagen are up-regulated in experimental OA synovial membrane.\textsuperscript{33,34} We found that both types 1 and 3 collagen were also markedly decreased in the DMM-TgEphB4 mice, indicating that the increased collagen deposition during fibrosis may result from an imbalance between the synthesis and degradation of these macromolecules. The latter findings also agree with the significant decrease in Plod2 in the DMM-TgEphB4 mice. Hence Plod2 is a factor known to induce the formation of pyridinoline cross-links in collagen,\textsuperscript{35} and collagen that contains more pyridinoline cross-links reduces its susceptibility to enzymatic degradation and in turn will lead to collagen accumulation.\textsuperscript{36–38}

Also explored was the effect on two inflammatory markers, MMP-9 and IL-1β. However, only a moderate reduction in these factors was observed, indicating that they were not major players in the beneficial effect of Ephb4 bone overexpression. It is noteworthy that in this DMM mouse model, inflammation of the synovial membrane is
not the major feature and the inflammatory process in this model could be mostly due to the surgery occurring at the beginning of the intervention, whereas the data of the current study were collected 12 weeks after surgery.

TGF-β is another factor that plays a central role in fibrosis, especially in noninflammatory progressive fibrosis, which is the case in the synovial membrane of this OA model. During fibrosis, TGF-β signaling promotes ECM deposition and regulates the expression of several profibrotic genes, such as Ctgf, aSMA (Acta2), and Comp, that were decreased in the OA synovial membrane of the bone-specific Ephb4-overexpressing mice. Surprisingly, although TGF-β was decreased in the DMM-TgEphB4 mice, the difference did not reach statistical significance. However, although many fibrotic disorders are characterized by elevated levels of TGF-β, additional mechanisms could contribute to the increased TGF-β signaling in fibrosis. TGF-β is secreted and stored in the ECM in inactive form (latent), and spatial and temporal activation are essential before transmitting its effects. Although many factors have been implicated in the activation of TGF-β, the exact mechanisms in different tissues remain to be determined. Interestingly, clinical and experimental evidence reveals excessive activation of TGF-β in the development of fibrosis. Thus, it could be that in the DMM-TgEphB4 specimens, it is not the latent form of TGF-β that is significantly diminished but its signaling activities. Because the response of cells to TGF-β depends on the expression of specific receptors, we further explored its receptor levels in the synovial membrane of this mouse model. TGF-β was found to be able to signal through TGF-βR1 not only via the Alk5-induced Smad2/3 phosphorylation but also via Alk1-induced Smad1/5/8 phosphorylation. During the OA process, TGF-β signaling pathways are dysregulated, with differential expression of its receptors, in which there is a shift of the dominant use of the receptor Alk5 to an enrichment of Alk143,50,51. Activation of Alk5/Smad2/3 phosphorylation is reported to be associated with inhibition of cell proliferation and migration as well as other features consistent with induction of the quiescent phenotype, whereas signaling via Alk1/Smad1/5/8 phosphorylation leads to activation of catabolic responses.51,52 However, although the Alk-1/Smad1/5/8 signaling pathway has been described mainly for angiogenesis, there is evidence of this receptor’s involvement in the ECM regulation and fibrosis.53–58 Data from the present study revealed a significant reduction in Alk1 but not Alk5, as well as a subsequent

Figure 6 Representative sections of synovial membrane stained with Safranin-O and histologic evaluations of wild-type (WT) and bone-specific Ephb4-overexpressing mice (TgEphB4) at 6, 12, and 24 months of age. A: Boxed area represents the enlargement of the knee joint for WT (B) and TgEphB4 (C) at 24 months of age. Histograms of synovial membrane thickness (D) and lining layers (E) of 6-month-old (WT, n = 15; TgEphB4, n = 8), 12-month-old (WT, n = 12; TgEphB4, n = 8), and 24-month-old (WT, n = 3; TgEphB4, n = 4) WT and TgEphB4 mice, respectively. Brackets indicate the synovial membrane thickness (B and C). Data are expressed as means ± SEM. *P < 0.05, Mann-Whitney U-test. Scale bar = 100 μm. Original magnification: ×63 (A); ×250 (B and C). Ca, capsule; SM, synovial membrane.
decrease in phosphorylated Smad-1 in the DMM-TgEphB4 mice. These data also agree with the nonsignificant differences in cell proliferation between DMM-WT and DMM-TgEphB4 mice and the decreased level of VEGF and number of blood vessels in the latter.

In addition, TGF-β receptors could also be modulated by a chaperone protein, Hsp90b, which facilitates the folding and stabilization of the TGF-β receptors. Indeed, recent studies identified TGF-β receptors as Hsp90β client proteins and found that inhibition of Hsp90β accelerates ubiquitination and increases proteasomal degradation of TGF-β receptors.59,60 Moreover, in preclinical models of fibrosis, Hsp90β amplified the profibrotic effect of TGF-β, and its pharmacologic inhibition decreased fibrosis occurring through TGF-βR1.18 As confirmatory evidence of the decrease in Tgf-β receptor activities, we found that Hsp90β is also markedly decreased in DMM-TgEphB4 mice. Together these findings suggest a down-regulation of the TGF-β canonical signaling in the DMM-TgEphB4 mice.

Data from this study concur with the hypothesis that preserving subchondral bone leads to a protective effect on other OA joint structural alterations, including cartilage6 and synovial membrane. Currently, it cannot be determined whether the synovial membrane preservation occurs via a direct effect of the subchondral bone and/or the preservation of the cartilage2 because studies have revealed a cross-talk of factors among all these articular tissues. Hence, it could have occurred by a reduced mechanical overloading, biological factors, and/or degradation products from the subchondral bone-cartilage unit.

With respect to the biological factors, although speculative, TGF-β could be a candidate because high levels of active TGF-β have been reported in the synovial fluid of OA patients42,61,62 and in other articular tissues, particularly in the bone and subchondral bone of OA patients.63–65 Interestingly, inhibition of TGF-β in the subchondral bone in the anterior cruciate ligament transection mouse model of OA improved the bone architecture and attenuated the cartilage degeneration.66 Another factor of interest is IL-6. Hence, in human OA subchondral bone osteoblasts, ephrin-B2 (ligand of EphB4) treatment down-regulates IL-6.4 Moreover, in addition to IL-6 being found elevated in the synovial fluid of OA patients67,68 and implicated in the cross-talk between bone and cartilage,69 IL-6 in combination with other cytokines was found to switch osteoblasts from a normal to a sclerotic phenotype.70 Finally, IL-6 also plays important roles in endothelial cell dysfunction and fibrogenesis.70,71 However, more research should be performed to elucidate the precise mechanism underlying the effect of IL-6 on the synovial membrane during the OA process as well as the effect of Ephb4 on this cytokine in this tissue.

Previous studies have found that C57BL/6 mice exhibit spontaneous OA characteristics during aging.72,73 We thus further looked at the synovial membrane of the aged TgEphB4 mice who did not undergo operation. The findings also suggested a protective effect of the bone-specific Ephb4-overexpression on synovial membrane as data revealed in the 24-month-old TgEphB4 mice a reduction in synovial membrane thickness.

**Conclusion**

We found that in OA, *in vivo* protection of the subchondral bone is associated with preservation of the OA synovial membrane architecture, with less fibrosis associated with a marked reduction in fibrotic markers and TGF-β signaling. To the ongoing discussion of the effect of subchondral bone remodeling during the OA process, this study provides evidence that the protection of this tissue during the disease process has an extended effect on other knee joint tissues, cartilage and synovial membrane, thus stressing the *in vivo* importance of subchondral bone remodeling playing a key role in the genesis of OA joint degeneration.

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