Evidence of vasculature and chondrocyte to osteoblast transdifferentiation in craniofacial synovial joints: Implications for osteoarthritis diagnosis and therapy

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Abstract
Temporomandibular joint osteoarthritis (TMJ OA) leads to permanent cartilage destruction, jaw dysfunction, and compromises the quality of life. However, the pathological mechanisms governing TMJ OA are poorly understood. Unlike appendicular articular cartilage, the TMJ has two distinct functions as the synovial joint of the craniofacial complex and also as the site for endochondral jaw bone growth. The established dogma of endochondral bone ossification is that hypertrophic chondrocytes undergo apoptosis, while invading vasculature with osteoprogenitors replace cartilage with bone. However, contemporary murine genetic studies support the direct differentiation of chondrocytes into osteoblasts and osteocytes in the TMJ. Here we sought to characterize putative vasculature and cartilage to bone transdifferentiation using healthy and diseased TMJ tissues from miniature pigs and humans. During endochondral ossification, the presence of fully formed vasculature expressing CD31+ endothelial cells and α-SMA+ vascular smooth muscle cells were detected within all cellular zones in growing miniature pigs. Arterial, endothelial, venous, angiogenic, and mural cell markers were significantly upregulated in miniature pig TMJ tissues relative to donor matched knee meniscus fibrocartilage tissue. Upon surgically creating TMJ OA in miniature pigs, we discovered increased vasculature and putative chondrocyte to osteoblast transformation dually marked by COL2 and BSP or RUNX2 within the vascular bundles. Pathological human TMJ tissues also exhibited increased vasculature, while isolated diseased human TMJ cells exhibited marked increase in vasculature markers relative to control 293T cells. Our study provides evidence to suggest that the TMJ in higher order species are in fact vascularized. There

Abbreviations: ACAN, aggrecan; BMSCs, bone marrow stromal cells; BSP, bone sialoprotein; CCs, condylar chondrocytes; EC, endothelial cells; ECM, extracellular matrix; FCSC, fibrocartilage stem cells; H&E, hematoxylin and eosin; HZ, hypertrophic zone; MCC, mandibular condylar cartilage; MZ, maturation zone; OA, osteoarthritis; PZ, polymorphic zone; SB, subchondral bone; SZ, superficial zone; TMJ, temporomandibular joint; VEGF, vascular endothelial growth factor; vSMCs, vascular smooth muscle cells.
Hyaline articular cartilage lines joint surfaces in adults and is primarily comprised of chondrocytes that maintain a relatively low rate of metabolic turnover under hypoxic conditions.1,2 Chondrocytes are embedded within an avascular, extracellular matrix (ECM) enriched in proteoglycans and non-collagenous proteins that are fixed in a complex network of collagens.3-5 The ECM provides diverse functions in articular cartilage, including providing biomechanical strength, anchorage for chondrocytes, and regulation of intercellular and ECM communication.6-8 Osteoarthritis (OA) is characterized by progressive degeneration of the articular cartilage ECM, as well as changes to the surrounding joint tissues.4,9-11 Given that it is well established that cartilage is avascular,12,13 the current OA pathological paradigm supports the idea that chondrocyte phenotypic stability plays a pivotal role in maintaining the delicate balance of tissue and ECM homeostasis.1,6,7,14,15 During OA pathogenesis, the functional demands of the articular cartilage surpass the chondrocytes’ ability to repair the ECM, leading to changes in chondrocyte phenotype and cartilage degradation.4,10,16

While these concepts are well-established for hyaline articular cartilages lining the appendicular skeleton, little is known about chondrocyte stability in the fibrocartilage craniofacial synovial joint called the temporomandibular joint (TMJ). The TMJ is comprised of the temporal bone and mandible separated by a fibrocartilaginous disc and is critical for eating and speaking.17 Unlike hyaline articular cartilage of the axial skeleton, the TMJ mandibular condyle is secondary fibrocartilage, derived from periosteum, and has a distinctive dual nature acting as both a site for jaw bone growth through endochondral ossification and as a permanent articular cartilage.18-20 Given these distinct differences between hyaline articular cartilage and craniofacial articular cartilage, it is likely that the processes regulating TMJ homeostasis and osteoarthritis are different.21

Recent evidence using powerful lineage tracing experiments has transformed the central dogma of endochondral ossification. Mouse genetics has provided evidence of the direct differentiation of chondrocytes into osteoblasts and osteocytes during endochondral bone formation in the TMJ condyle, as well as in the long bone growth plate and during bone fracture healing.22-25 However, the mechanisms governing the chondrocyte to osteoblast/osteocyte transformation in the TMJ are not well defined. Furthermore, whether the direct transformation of chondrocytes to osteoblasts and osteocytes contributes to TMJ OA and other TMJ and jaw pathologies in humans is unknown. Given that endothelial cells and invading vasculature have been shown to provide critical environmental cues that trigger the cartilage to bone transformation during bone fracture healing,26 we hypothesize that the vasculature also plays a vital role in the cartilage to bone transformation in the TMJ.

Here we characterized the putative vasculature and chondrocyte to osteoblast transformation using both healthy and osteoarthritic TMJ tissues and cells from a preclinical, miniature pig model, and in human patients. We discover for the first time the presence of fully formed vasculature within the TMJ condylar cartilage in both healthy and diseased TMJs in miniature pigs and humans. Our data further reveal the presence of transitional cells that co-express both cartilage and bone markers embedded within vascular fibrocartilaginous bundles and cartilage ECM, which may represent a critical cell fate phenotypic change from chondrocytes to osteoblasts. These studies represent a paradigm shift in cartilage and TMJ biology, where higher order species have vascularized craniofacial articular joints that may drive the critical phenotypic switch from chondrocyte to osteoblast fate specification. Our work may alter the clinical management of TMJ OA patients and help define new therapeutic targets that both inhibit angiogenesis and cartilage to bone transformation.

## MATERIALS AND METHODS

### 2.1 Animals

All animal procedures were performed using equal number of male/female 28-week-old Yucatan miniature pigs with approval from the Institution of Animal Care and Use Committee at the Medical University of South Carolina...
(2017-00047). About 12-week-old miniature pigs and 28-week-old Mongrel canines were donated through cadaver tissue sharing services at the Columbia University Irving Medical Center.

2.2 TMJ injury model

TMJ osteoarthritis was surgically induced in Yucatan miniature pigs using a disc perforation model as previously described.27,28 An oblique incision was created superior to the zygomatic process. Tissue was elevated and retracted to access the TMJ superior joint space and the disc was retracted posteriorly. A periosteal elevator was placed under the disc to protect the condyle from injury. A punch biopsy was used to create a 5.0 mm perforation in the posterior-lateral portion of the TMJ disc and the perforated disc tissue was excised (Supplemental Figure 1). No disc attachments were severed and the disc reduced to its normal anatomical location upon the release of disc retraction. Sham surgery was performed where the TMJ superior joint space was accessed and the disc was not perforated. Miniature pigs were euthanized 3-4 weeks after surgery for histological evaluation.

2.3 Human samples

Human TMJ tissue samples were collected and analyzed from patients who were undergoing TMJ arthroscopy, arthrocentesis or joint replacement surgery for TMJ treatment. The study was approved by the Institutional Review Board of Columbia University Irving Medical Center (AAAQ8195) and the Institutional Review Board of Weill Cornell New York Presbyterian (1608017486-A002). All subjects were provided with written informed consent prior to study enrollment. All experiments were performed in accordance with relevant guidelines and regulations.

2.4 Cell isolation and culture

TMJ fibrocartilage, condyle, disc, subchondral bone, and knee meniscus were dissected from miniature pigs. Pig and human tissues, derived from patients undergoing TMJ surgery, were digested with dispase II/collagenase I (4 mg/mL, 3 mg/mL) and single-cell suspensions of cells were cultured (5% CO2, 37°C) in basal medium consisting of DMEM (Invitrogen 11885-092) supplemented with 20% lot-selected fetal bovine serum (FBS, Gibco ES Cell FBS, 10439-024), glutamax (Invitrogen 35050-061), penicillin-streptomycin (Invitrogen 15140-163), and 100 mM 2-mercaptoethanol (Gibco) for 4-6 days. Cells were detached with trypsin-EDTA (GIBCO) and plated at P1 for the in vitro experiments. 293T was acquired from (ATCC) and maintained in IMDM (Gibco) + 10 % HI-FBS + 1× Penn/Strep.

2.5 Histology & immunohistochemistry

Tissue samples were fixed in 4% paraformaldehyde, decalcified in EDTA, and prepared for either paraffin or frozen embedded sections. Serial tissue sections were stained hematoxylin and eosin (H&E). For immunohistochemistry, tissue sections were enzymatically treated with Chondroitinase ABC (Fisher C3667-10UN), blocked with 3% BSA for 1 hour, and immunolabeled with primary antibodies at 4°C overnight (Supplemental Table 1). Treatment with secondary antibody (Alexa Fluor 488, 1:1000 or Alexa Fluor 546, 1:2000) for 1 hour at room temperature was used to detect immunoreactivity. Isotype-matched negative control antibodies were used under the same conditions. ProLong Gold antifade reagent with DAPI (Invitrogen, P36931) was used for the nuclear counterstain.

2.6 In situ hybridization

RNAscope in situ hybridization (ISH, Advanced Cell Diagnostics, #323100) was performed with 5 μm sections of fixed frozen pig TMJ tissues. Tissue samples were pretreated by boiling in Target Retrieval solution for 5 minutes followed by Protease III treatment for 30 minutes at 40°C. Subsequently, the tissue samples were processed with the RNAscope Multiplex Fluorescent Assay according to manufacturer protocols. We designed the probe for Pig Sox9 (NM_212843.2).

2.7 RNA isolation and qRT-PCR

Total RNA was purified from cells or whole tissue (Invitrogen 12183018A) and treated with DNase I (Ambion AM2222) to remove genomic DNA. RNA quantity and purity were determined using NanoDrop. RNA samples (260/280 ≥ 1.8) were used to obtain cDNA (Bio-Rad AM2222). Quantitative RT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems4309155) and presdesigned primers (Supplemental Table 2) for both human (Hs) and pig (Ss) (Integrated DNA Technologies). Gene expression levels were normalized to the housekeeping gene Gapdh.

2.8 ELISA

The VEGF (Boster EK0539) concentrations in human TMJ cells were measured using the quantitative sandwich enzyme
immunoassay technique following the manufacturer’s instructions. Briefly, standard or sample (100 μL) was added to each well and incubated for 2 hr at 37°C, followed by treatment with primary antibody using either biotinylated anti-human VEGF antibody for 1 hr at 37°C. For antibody detection, 100 μL of HRP-Avidin was for 1 hr at 37°C. After washing, TMB substrate was added to each well for 15-30 min at 37°C, followed by the addition of 50 μL of Stop Solution. The optical density (OD) of each well was determined using a microplate reader at 450 nm. The VEGF concentrations were calculated by comparing the OD of each sample to the standard curve.

2.9 | Histomorphometry

In order to distinguish the superficial zone (SZ) from the maturation zone (MZ), we performed immunohistochemistry on pig tissue sections using antibodies against type I collagen (COL1A1) to mark the SZ and type II collagen (COL2A1) to mark the MZ. The thickness of the type II collagen expressing MZ versus the type I collagen expressing SZ was measured in both the Sham and Injury samples. All measurements were quantified using Olympus cellSens Dimension imaging software.

2.10 | Statistical analysis

All statistics were calculated using Prism 7 GraphPad Software. The statistical significance between two groups was determined using paired Student’s t test assuming Gaussian distribution. The normality of distribution was confirmed using the Kolmogorov–Smirnov test and the resulting two-tailed P value ≤ .05 was regarded as a statistically significant difference. Among the two groups, one-way ANOVA followed by Tukey’s post hoc test was used for statistical comparisons. For multiple comparisons, a two-way ANOVA followed by Tukey’s post hoc was used for statistical comparisons.

3 | RESULTS

3.1 | Miniature pig craniofacial synovial joint harbors non-lateral cellular zones of maturation interrupted by vascularized fibrocartilaginous bundles

During rodent jaw development, lateral cellular zones of maturation mark the TMJ mandibular condylar cartilage, including the superficial zone (SZ) harboring fibrocartilage stem cells (FSCCs), polymorphic zone (PM) containing heterogeneous proliferating cells, maturation zone (MZ) harboring chondrocytes, hypertrophic zone (HZ) harboring terminally differentiated hypertrophic chondrocytes, and the erosive zone (EZ) where cartilage is resorbed by osteoclasts and osteoprogenitors form bone. While rodent mammals have provided significant insight into mammalian TMJ morphogenesis, large animal models provide significant advantages over rodents, including similarity to human physiology and pathology. Unlike rodent TMJs, pig TMJs are more similar to humans given pig condyles articulate with the articular eminence as opposed to the fossa, have bilateral occlusion, facilitate translational mechanics, have similar disc anatomy, and have analogous biomechanics. Therefore, miniature pigs are well-accepted as an ideal preclinical, large animal species for studying TMJ most similar to humans. To gain insight into the preclinical pig TMJ model, we collected samples from growing 12-week-old Yucatan miniature pigs and assessed the expression of the fibrocartilage marker, type I collagen (COL1); cartilage markers, aggrecan (ACAN), type II collagen (COL2A1), and Sox9; and bone markers, bone sialoprotein (BSP) and RUNX2, by immunohistochemistry and in situ hybridization. Histological evaluation of H&E sections demonstrated that, unlike rodents, the miniature pig TMJ condyle exhibits non-lateral cellular zones of maturation that were distinct morphologically (Figure 1A). The expression pattern of the disc and condyle differed. The disc expressed both fibrocartilage and cartilage markers, including COL1 and ACAN respectively, but did not express COL2A1, BSP, RUNX2, and Sox9 (Figure 1B). Distinct expression profiles defined the cellular zones of maturation within the miniature pig TMJ mandibular condyle. Sox9, COL2A1, BSP, and RUNX2 were expressed in the maturation and hypertrophic zones (Figure 1C), suggesting that chondroprogenitors (Sox9+), chondrocytes (COL2A1+), and bone cells (BSP+, RUNX2+) occupied these zones. COL1 was expressed in the fibrocartilaginous superficial zone (Figure 1C).

To confirm our in vivo findings, we performed qRT-PCR using RNA isolated from whole TMJ miniature pig tissue. qRT-PCR also showed fibrocartilage marker Col1 significantly upregulated in the SZ and disc relative to condylar cartilage (CC) tissue, whereas cartilage marker Col2a1 was significantly upregulated in CC relative to the SZ and disc tissue (Figure 1D). Most strikingly COL1 positive cellular bundles were also in areas surrounding mature chondrocytes (COL2A1+) and contained vascular-like epithelium (arrows, Figure 1A,C), which spanned within all of the cellular zones of maturation. These data suggest for the first time the presence of vascularized cartilage within miniature pig craniofacial synovial joint.

3.2 | Evidence of vasculature within type I collagen fibrocartilage bundles in miniature pig craniofacial synovial joints

To confirm the presence of vasculature within the TMJ mandibular condylar cartilage in miniature pigs, we characterized
FIGURE 1  Miniature pig TMJ mandibular condyle harbor non-lateral cellular zones of maturation interrupted by fibrocartilaginous type I collagen bundles. A, H&E of 12-week-old miniature pig TMJ mandibular condyle depicting the cellular zones if maturation, including superficial zone (SZ) polymorphic zone (PM), maturation zone (MZ), and hypertrophic zone (HZ). Scale bar, 500 μm. B, Miniature pig TMJ disc H&E, in situ hybridization for Sox9, and immunohistochemistry for aggrecan (ACAN), runt-related transcription factor 2 (RUNX2), type II collagen (COL2A1), bone sialoprotein (BSP), and type I collagen (COL1A1). Scale bar, 100 μm. C, Miniature pig TMJ condylar cartilage H&E, in situ hybridization for Sox9, and immunohistochemistry for aggrecan (ACAN), runt-related transcription factor 2 (RUNX2), type II collagen (COL2A1), bone sialoprotein (BSP), and type I collagen (COL1A1). Scale bar, 100 μm. D, qRT-PCR of miniature pig SZ tissue relative to condylar cartilage tissue (PM+MZ+HZ). Data represented are mean fold change normalized to GAPDH; n = 3; ****P ≤ .0001; Student’s t test.
FIGURE 2  Evidence of vasculature in miniature pig TMJ mandibular condyle and disk A-C, H&E and immunohistochemistry for the cluster of differentiation 31 (CD31) and alpha-smooth muscle actin (α-SMA) in 12-week-old miniature pig TMJ disc. Scale bar, 100 μm. D-H, H&E and immunohistochemistry for CD31 and α-SMA in 12-week old miniature pig TMJ mandibular condyle within the superficial zone (SZ) (F), polymorphic zone (PZ) (G), and maturation zone (MZ) (H). Scale bar, 100 μm. I-K, H&E and immunohistochemistry in 12-week-old pig meniscus for CD31 and α-SMA. Scale bar, 100 μm. L-P, qRT-PCR using RNA from whole tissue derived from miniature pig TMJ fibrocartilage (FC), subchondral bone (SB), condylar cartilage (CC), and disc relative to the knee meniscus. Data represented are mean fold change normalized to GAPDH; n = 3; *P ≤ .05, **P ≤ .01, ***P ≤ .001, ****P ≤ .0001; Student’s t test.
two cell types found in functional blood vessels, including endothelial cells (ECs) and vascular mural cells, which comprise pericytes and vascular smooth muscle cells (vSMCs)\(^\text{42,47}\) (Figure 2). To determine the distribution of endothelial cells, which form the inner vessel wall, and mural cells, which surround the endothelial cell tube for structural support and regulation of diameter and blood flow, we examined the presence of CD31\(^+\) ECs\(^\text{44}\) and alpha-smooth muscle actin (\(\alpha\text{-SMA}\))\(^+\) vSMCs by immunohistochemistry (Figure 2A-H). The anterior and posterior horn of the knee meniscus has been reported to contain a vascular supply\(^\text{45-48}\) and was used as positive control tissue for confirming CD31\(^+\) ECs and \(\alpha\text{-SMA}\)\(^+\) vSMCs immunostainings (Figure 2I-K). Disk \(\alpha\text{-SMA}\)\(^+\) cells were associated with low expression CD31\(^+\) ECs (Figure 2A-C). The expression pattern within the knee meniscus tissue was similar to that of the disc, where \(\alpha\text{-SMA}\)\(^+\) vSMCs were associated with low expression of CD31\(^+\) ECs (Figure 2J,K). Within the mandibular condyle SZ and PM zones, CD31\(^+\) ECs were associated with surrounding \(\alpha\text{-SMA}\)\(^+\) vSMCs (Figure 2D-G). CD31\(^+\) ECs forming an inner vessel wall was associated with surrounding \(\alpha\text{-SMA}\)\(^+\) vSMCs embedded within the COL1A1 collagen bundles in the MZ zone containing mature COL2A1\(^+\) chondrocytes (Figure 2D,E,H).

To corroborate our in vivo data demonstrating the presence of blood vessels in the miniature pig TMJ, we evaluated gene transcripts of endothelial cell markers (\(CD31, \text{VE-cadherin})\)\(^\text{44,49}\), mural cell marker (\(Pdgfr\beta)\)\(^\text{50,51}\), arterial vessel markers (\(Ephrinb2, \alpha\text{-SMA})\)\(^\text{43,52,53}\), venous marker (\(Ephb4)\)\(^\text{42,43,53}\), and markers for angiogenetic mediators (\(Vegfa, Vegfr-2)\)\(^\text{54-57}\) by qRT-PCR using RNA derived from whole TMJ tissues (Figure 2L-P). We isolated RNA from miniature pig TMJ fibrocartilage (FC) containing the SZ, condylar cartilage (CC) harboring the PZ, MZ and HZ, subchondral bone (SB) harboring the EZ, and the TMJ disk\(^\text{28,31}\).

As a control, we compared all vascular transcripts in TMJ tissues relative to donor-matched vascularized knee meniscus tissue\(^\text{45-48}\). Similar to our in vivo data, the expression of endothelial cell markers (\(CD31, \text{VE-cadherin})\)\(^\text{44,49}\) was significantly upregulated in the TMJ fibrocartilage, condylar cartilage and disk in comparison to the knee meniscus tissue (Figure 2L), while the mural cell marker (\(Pdgfr\beta)\)\(^\text{50,51}\) was upregulated in the TMJ condylar cartilage in comparison to knee meniscus (Figure 2M). The expression of arterial vessel markers (\(Ephrinb2, \alpha\text{-SMA})\)\(^\text{43,52,53}\) (Figure 2N) and the venous marker (\(Ephb4)\)\(^\text{42,43,53}\) (Figure 2O) were all significantly upregulated in the TMJ fibrocartilage (SZ) and the condylar cartilage (PZ, MZ, HZ), suggesting that both arteries and veins span throughout all of the cellular zones in the TMJ mandibular condyle and also in the TMJ disc. Moreover, we found that the mediators of angiogenesis (\(Vegfa, Vegfr-2)\)\(^\text{54-57}\) were also significantly upregulated in the TMJ fibrocartilage, condylar cartilage, and disk relative to the knee meniscus, suggesting active angiogenesis in TMJ (Figure 2P). Strikingly, endothelial cell markers (Figure 2L), arterial markers (Figure 2N), and venous marker (Figure 2O) were more significantly upregulated in the TMJ fibrocartilage and condylar cartilage than in subchondral bone tissue when compared to knee meniscus tissue, suggesting that blood vessels may be more abundant in cartilage than in the underlying bone. While miniature pig TMJs exhibit vasculature, it is generally accepted that smaller species like rodents do not. Given that, unlike rodents, miniature pig TMJs undergo sliding or translational joint movements, we further investigated whether joint biomechanics may play a role in the presence of vasculature by evaluating the TMJs of dogs with mainly hinge or rotational movement\(^\text{58}\) (Supplemental Figure 2). Immunohistochemistry showed that canine TMJ disk had \(\alpha\text{-SMA}\)\(^+\) vSMCs that were also associated with the expression of CD31\(^+\) ECs (Supplemental Figure 2A-C). However, canine condyle exhibited fibrocartilaginous SZ tissue with \(\alpha\text{-SMA}\)\(^+\) vSMCs that were associated with low expression of CD31\(^+\) ECs, and no cellular zones of maturation (Supplemental Figure 2D-F). These data suggest that translational mechanics present in pigs may be critical for the presence of more complex vasculature. Taken together, these data reveal for the first time the presence of blood vessels within miniature pig TMJ. However, whether the presence of blood vessels alters TMJ injury or pathology in the miniature pig is unknown.

### 3.3 Increased expression of bone-associated proteins is coupled with altered vasculature architecture in injured miniature pig mandibular condylar cartilage

To determine the putative role of the vasculature in miniature pig TMJ pathology, we created a surgically induced TMJ disc perforation animal model (Figure 3). We have previously published that surgically creating a perforation in the TMJ discs of rabbits leads to secondary TMJ condylar degeneration and OA\(^\text{27,28}\). We aimed to recapitulate the TMJ disc perforation model in miniature pigs and characterize the vasculature phenotype. A punch biopsy was used to create a 5.0 mm perforation in the posterior-lateral TMJ disc and the perforated tissue was excised (Supplemental Figure 1).\(^\text{27,28}\) Sham surgery control was performed where the TMJ superior joint space was accessed but the disc was not perforated. The pigs were euthanized 4 weeks after the surgery for analysis.

Histological evaluation showed the disk in both conditions was positive for COLIa1 and ACAN expression and negative for COL2a1, BSP, and RUNX2 (Supplemental Figure 3A). Histological analysis further demonstrated that alterations in the thickness of the condylar cartilage and cellular zones of maturation in the injury model relative to the sham control (Figure 3A,B, Supplemental Figure 3B,C).
FIGURE 3  Development of TMJ disk perforation injury model in miniature pigs. A 5.0 mm perforation was surgically created in 28-week-old miniature pig TMJ disk, while sham operation served as a comparison control. TMJ disks and condyles were analyzed 4 weeks after surgery.

A, H&E of sham-operated and injured (B) miniature pig TMJ mandibular condyle depicting the zones maturation, including superficial zone (SZ) polymorphic zone (PZ), maturation zone (MZ), and hypertrophic zone (HZ). Scale bar, 500 μm. C, Histomorphometric analysis of the superficial zone (SZ) and maturation zone (MZ) thickness in the sham control and injury models; ***P ≤ .001; Student’s t test. D, H&E and immunohistochemistry in sham-operated pig TMJ condyle for aggrecan (ACAN), runt-related transcription factor 2 (RUNX2), type II collagen (COL2A1), bone sialoprotein (BSP), and type I collagen (COL1). Scale bar, 100 μm. E, H&E and immunohistochemistry in the injured pig TMJ condyle for aggrecan (ACAN), runt-related transcription factor 2 (RUNX2), type II collagen (COL2A1), bone sialoprotein (BSP), and type I collagen (COL1). Scale bar, 100 μm
Histomorphometric analysis revealed that the COL1a1+ SZ was significantly thicker in the injury when compared to the sham controls (Figure 3C), suggesting an expansion of the fibrocartilaginous condylar cartilage tissue. However, the COL2+ MZ thickness significantly decreased upon disc perforation injury when compared to the sham controls (Figure 3C), suggesting a loss of mature COL2a1+ chondrocytes, which are critical for cartilage function.1,14 Immunohistochemical analysis confirmed a loss of COL2a1 expression in the injured TMJ condyle relative to sham controls (Figure 3D,E). Most notably, the injured TMJ condyle demonstrated an expansion of bone-related proteins in the MZ and HZ, including BSP and RUNX2, relative to sham controls, suggesting that the loss of COL2a1+ chondrocytes was coupled with a gain of bone-like cells (Figure 3D,E). These data reveal that the TMJ disk perforation injury model causes a loss of cartilage thickness and COL2a1+ chondrocytes, which is coupled with an expansion of fibrocartilage tissue and bone-like cells.

To investigate the vascular epithelium, including ECs and vSMCs, in miniature pig TMJ upon pathological injury, we performed immunohistochemistry for CD31+ ECs and α-SMA+ vSMCs (Figure 4) in sham (Figure 4A-H) and injured miniature pigs (Figure 4I-P). The sham-operated disk (Figure 4A-C), injured disk (Figure 4I-K), and the sham mandibular condylar cartilage (Figure 4D-H), including the SZ (Figure 4F), PZ (Figure 4G), and MZ (Figure 4H), showed α-SMA+ vSMCs vessels with no CD31+ EC expression. However, immunohistochemical analysis of the injured condylar cartilage SZ (Figure 4N), PZ (Figure 4O), and MZ (Figure 4P) showed CD31+ECs surrounded by α-SMA+ vSMCs. Interestingly, the vascularized collagen bundles within the MZ zone (Figure 4P) harbored CD31+ ECs associated with α-SMA+ cells. Thus, the vascular architecture is altered upon TMJ injury. Furthermore, the increased number of CD31+ ECs localized within the injured miniature pig TMJ condylar cartilage, suggests that angiogenesis may play a critical role in disease and/or repair upon TMJ pathology. In addition, the number of vascular bundles in the SZ of the injured mandibular condyle was more abundant in comparison to the sham condyle. However, the role of new blood vessel formation in TMJ disease and/or pathology is unclear.

3.4 | Evidence of cartilage to bone transformation in injured miniature pig mandibular condylar cartilage and in pathological human mandibular condylar cartilage

Recent studies in mouse genetics have revealed that in the murine TMJ mandibular condyle, chondrocytes directly differentiate into osteoblasts and osteocytes during endochondral bone formation in the TMJ condyle, as well as in fracture healing in the long bone.22-25 Moreover, signals from the blood vasculature have been shown to direct the cartilage to bone transformation during fracture healing.26 Our data reveals that there is an expansion of bone-associated proteins BSP and RUNX2 (Figure 3), which is coupled with an increase in CD31+ ECs and alterations in blood vessel structure in the mandibular condylar cartilage upon TMJ injury (Figure 4). Thus we investigated putative cartilage to bone transformation in our miniature pig injury model (Figure 5). Sham-operated miniature pig TMJ condyles showed no evidence of transitional cells co-expressing RUNX2+/COL2A1+ or BSP+/COL2A1+ along vascular bundles (Figure 5A-D). Localized adjacent to fibrocartilaginous vascular bundles (Figure 5E,F, black arrows), immunohistochemistry showed putative transitional cells that expressed both bone and cartilage markers, RUNX2+/COL2A1+ cells (Figure 5G, white arrows) and BSP+/COL2A1+ cells (Figure 5H, white arrows). Interestingly, RUNX2+/COL2A1+ and BSP+/COL2A1+ were embedded within COL2A1 enriched ECM (Figure 5G,H). Interestingly, the presence of RUNX2+/COL2A1+ and BSP+/COL2A1+ transdifferentiated cells in the injured miniature pig condyle is coupled with an overall loss of a cartilage ECM enrich in COL2a1, and transition into a bone-like ECM phenotype (Figure 3). These data are similar to other studies showing the co-localization of chondrocyte marker with osteoblast/osteocyte markers during cartilage to bone transformation in jaw growth and bone fracture healing,18,22,23,26 and provide evidence of cartilage to bone transformation in our miniature pig TMJ injury model.

We further investigated whether cartilage to bone transformation in the TMJ mandibular condyle plays a critical role in mediating TMJ pathology in human patients. Condylar hyperplasia59-61 is diagnosed radiographically and characterized by excessive mandibular condyle bone growth that usually presents unilaterally, resulting in facial asymmetry.62 In a patient with condylar hyperplasia, we examined the evidence of chondrocyte transdifferentiation into osteoblasts by immunohistochemistry (Figure 5I-K). Our data revealed the presence of RUNX2+/COL2A1+ cells (white arrows, Figure 5J) and BSP+/COL2A1+ cells (white arrows, Figure 5K) embedded within a COL2a1 enriched ECM, providing the evidence of putative cartilage to bone transformation in this patient. These data implicate that chondrocyte to osteoblast/osteocyte differentiation may play a crucial role during the progression of human TMJ pathology, including condylar hyperplasia.

3.5 | Evidence of vasculature in pathological human TMJ tissues

Given that we discovered that the injured miniature pig showed an increased expression of bone markers (Figure 3) that was
FIGURE 4  Alterations in vascular architecture in miniature pig TMJ injury model. A-C, H&E and immunohistochemistry, in 7-month-old sham miniature pig disc, for the cluster of differentiation 31 (CD31, green) and alpha-smooth muscle actin (α-SMA, red). Scale bar, 100 μm. D-H, H&E and immunohistochemistry, in 7-month-old sham miniature pig TMJ mandibular condyle, for CD31 and α-SMA within the superficial zone (SZ) (F), polymorphic zone (PZ) (G), and maturation zone (MZ) (H). Scale bar, 100 μm. I-K, H&E and immunohistochemistry, in injured miniature pig disc for CD31 (green) and α-SMA (red). Scale bar, 100 μm. L-P, H&E and immunohistochemistry, in injured miniature pig TMJ mandibular condyle, for CD31 (green) and α-SMA (red) within the superficial zone (SZ) (N), polymorphic zone (PZ) (O), and maturation zone (MZ) (P). Scale bar, 100 μm
FIGURE 5 Evidence of cartilage to bone transformation in injured miniature pig mandibular condylar cartilage and in pathological human mandibular condylar cartilage. A,B,E,F, H&E and immunohistochemistry (C,D,G,H) of RUNX2 (green), COL2A1 (red), and bone sialoprotein (red, BSP) in sham-operated miniature pig (A-D) and injured miniature pig (E-H) TMJ mandibular condyle. Scale bar (A,E), 200 μm. Scale bar (B-D, F-H), 100 μm. (I) H&E and (J,K) immunohistochemistry of RUNX2 (green), COL2A1 (red), and bone sialoprotein (red, BSP) in the mandibular condylar cartilage of a human patient with condylar hyperplasia. Scale bar (I), 200 μm. Scale bar (J,K), 100 μm
FIGURE 6  Vasculature present in human OA tissue. A, B, Immunohistochemistry of a patient’s disc tissue with osteoarthritis (OA) stained for the cluster of differentiation 31 (CD31) and alpha-smooth muscle actin (α-SMA). Scale bar, 100 μm. C, D, Immunohistochemistry of a patient’s synovium tissue with osteoarthritis (OA) stained for CD31 and α-SMA. Scale bar, 100 μm. E-I, qRT-PCR of human fibrocartilage stem cells (FCSCs), subchondral bone cells (SBCs), and condylar cartilage cells (CCCs), relative to 293T cells. Data represented are mean fold change normalized to GAPDH, n = 3 human diseased tissue; Student’s t test. J, ELISA was used to measure VEGF, a protein produced by cells that stimulate the formation of blood vessels, in human diseased FCSCs, SBCs, and CCCs relative to 293T cells. Data represented are mean fold change normalized to GAPDH, n = 3 human samples/tissue type.
coupled with alterations in the vasculature architecture (Figure 4) and evidence of cartilage to bone transformation (Figure 5), we investigated whether blood vessels were associated with pathological human TMJ tissues (Figure 6). Similar to miniature pig TMJ discs, immunohistochemistry showed that osteoarthritic human TMJ discs expressed COL1A1, ACAN, and COL2A1 (Supplemental Figure 4A,B), but did not express bone markers BSP or RUNX2 (Supplemental Figure 4C,D). Pathological osteoarthritic human TMJ synovium tissue expressed COL1A1 and ACAN, but not COL2A1, BSP or RUNX2 (Supplemental Figure 4E,H). We examined ECs by CD31 expression and the vSMCs by alpha-smooth muscle actin (α-SMA) expression using immunohistochemistry in human osteoarthritic disc and synovium (Figure 6). Both the disc (Figure 6A,B) and synovium (Figure 6C,D) contain CD31+ ECs that are also surrounded by α-SMA+ cells, where α-SMA+ expression is discontinuous and intermittent, suggesting that the vasculature has a leaky architecture. To corroborate these in vivo findings, we examined the expression of arterial (Ephrinb2, α-SMA), endothelial (CD31, VE-cadherin), venous (Ephb4), angiogenesis (Vegfa, Vegfr-2), and mural cell (Pdgfrβ) markers by qRT-PCR using isolated osteoarthritic human primary fibrocartilage stem cells (FCSCs), subchondral bone marrow stromal cells (BMSCs), and condylar chondrocytes (CCs) derived from their respective human TMJ tissues. 293T cells served as a comparative negative control. We found a significant increase in gene transcripts for α-SMA and endothelial cells (VE-cadherin) in all osteoarthritic TMJ cell types, while mural cell marker Ephrinb2 was significantly decreased in comparison to control 293T cells (Figure 6). However, there were significant increases in CD31 expression in FCSCs and BMSCs, Ephb4 in FCSCs, Vegfa, and Vegfr-2 in FCSCs and CCs, and Pdgfrβ in BMSCs and CCs (Figure 6). These data suggest that in a diseased state both TMJ FCSCs and CCs maintain their vascular properties and angiogenetic potential. To further confirm the presence of blood vessels in the TMJ mandibular condyle, we measured secreted vascular endothelial growth factor (VEGFA) protein levels in conditioned media from human osteoarthritic FCSCs, BMSCs, and CCs relative to control 293T cells (Figure 6J). All TMJ cell types isolated from osteoarthritic human TMJ showed elevated secreted VEGFA levels relative to 293T cells (Figure 6J), suggesting that pathological TMJ cells have potent angiogenetic properties.

Taken together, our data show that higher order species have vascularized craniofacial articular joints that may drive the critical phenotypic switch from chondrocyte to osteoblast fate during TMJ pathology in both injured miniature pigs and in osteoarthritic human TMJ tissues. These data suggest that pharmacologically blocking angiogenesis may ameliorate TMJ OA. To test this hypothesis, we treated porcine FCSCs and osteoarthritic human FCSCs with propranolol, an anti-angiogenic drug and non-selective β-adrenergic blocking drug used to control the growth of infantile hemangiomas and tumors. Genes associated with angiogenesis (Vegfa and Vegfr2) and bone (Runx2) were analyzed by qRT-PCR in porcine FCSCs and osteoarthritic human FCSCs treated with 100 μm propranolol for 24 hours (Supplemental Figure 5). Both miniature pig (Supplemental Figure 5A) and OA human FCSCs (Supplemental Figure 5B) treated with 100 μm propranolol showed significantly lower the transcript levels of Vegfa, Vegfr2, and Runx2 relative to vehicle control cells. These results suggest that drugs that block angiogenesis, such as propranolol, may also reduce cartilage to bone transformation.

### 4 DISCUSSION

The temporomandibular joint (TMJ) is the synovial joint in the mammalian craniofacial complex that is critical for prey capture, food intake, and speech. TMJ osteoarthritis can lead to permanent tissue degradation, jaw and facial asymmetry, dental malocclusion, pain, and severely impairs life quality. Therapies are directed toward either symptom management or highly invasive joint replacement surgeries with high failure rates. Here we show for the first time that the TMJ disk and condylar cartilage are vascularized in miniature pigs, a well-established large animal that emulates human TMJ physiology and anatomy, and also in osteoarthritic human TMJ tissues. In osteoarthritic joints the vasculature architecture changes and is coupled with increased bone and evidence of cartilage to bone transformation, a phenomenon only shown until now in the TMJs of healthy growing rodents. Our studies reveal a plausible paradigm shift in the treatment of TMJ OA, where pharmacological agents, such as propranolol, that either suppress angiogenesis and/or bone formation could be used to ameliorate TMJ OA pathology in humans. However, it is unclear why blood vessels are present in the TMJs of higher order species, but not in smaller species such as rodents.

Mammalian jaw evolution may provide clues as to the biological necessity of vascularized TMJs in pigs and humans and the cellular origin and mechanisms regulating TMJ morphogenesis. The primary or old jaw joint found in early vertebrates and modern-day reptiles is composed of an upper quadrate bone and a lower articular bone derived from endochondral ossification of Meckel’s cartilage. However, it is speculated that the evolution of the new jaw joint arouses from the need to adapt to more complex jaw movements, a stronger bite-force and improved hearing. Present-day mammalian TMJ is formed independent of Meckel’s cartilage, while the posterior segments of Meckel’s cartilage evolved to form the auditory ossicles and sphenomandibular
ligament. While rodent mammals have provided insight into mammalian TMJ morphogenesis, large animal models give more significant advantages over rodents, including similarity to human TMJ physiology and pathology. Unlike rodents, pigs are similar to humans given pig condyles articulate with the articular eminence as opposed to the fossa, have bilateral occlusion, facilitate translational mechanics, have similar disc anatomy, and have analogous biomechanics. Therefore, miniature pigs are well-accepted as an ideal large animal species for studying TMJ. Additional advantages in using miniature pigs as opposed to rodents in modelling human disease are that they grow to a similar size as humans, and have similar organ systems, physiology and metabolism as humans. Relative to rodents, the pig immune response is genetically and functionally analogous to humans making pig an ideal species to model complex human diseases, such as TMJ osteoarthritis, and for predicting human response to joint therapies.

Mammalian TMJ development is distinct from appendicular joints and thus may also indicate the requirement of vascularized joints. The TMJ is comprised of the glenoid fossa of the skull base, articular disc, ligaments, and the mandibular condyle of the jaw bone. Similar to flat craniofacial bones, mandibular bone is produced by intramembranous ossification, where mesenchymal cells derived from the ectomesenchymal neural crest cells directly differentiate into osteoblasts. Moreover, TMJ mandibular condyle is formed by endochondral ossification and eventually unites with the intramembranous mandibular bone to form the jaw. However, the mechanisms regulating how these two bony fronts in the vertebrate jaw unite are unknown. Unlike appendicular joints formed by a single primordium, the TMJ is formed by two different mesenchymal condensations, including the glenoid blastema undergoing intramembranous ossification and condyle blastema primarily developing through endochondral ossification appearing at E13.5 in mice and 8th gestational week in humans. While the precise cellular origin of the TMJ is unclear, cranial neural crest cells and mandible bone periostial cells are speculated to initially form the condyle blastema. The articular disc is formed from a condensation of cells that separates from the surface of the condyle blastema that divides the joint space into the inferior and superior joint cavities. The inner mass of cells in the condyle blastemal undergoes endochondral ossification, which is lined by perichondrium-like tissue. Remnants of this perichondrium-like tissue persist throughout the life span of mammalian adults, lines the condylar cartilage, and is classified as fibrocartilage. In post-natal TMJ, this fibrocartilaginous tissue provides tensile strength for joint biomechanics and serves as a niche harboring fibrocartilage stem cells with potent chondrogenic and osteogenic capabilities.

Our data show that mammalian TMJ in higher order species may have similar properties to vascularized perichondrium tissue. During limb bud development the initiation of endochondral ossification involves a complex and dynamic interplay among cells in the perichondrium, vascular endothelium, and cartilage. Mesenchymal cells condense and differentiate into chondrocytes, while the surrounding cells elongate to form the perichondrium. Perichondrium tissue is the source of osteoblasts that establish the bone periosteum and also endothelial cells that commence the angiogenesis of the primary and secondary ossification centers. The cartilage templates forming adjacent axial bones are interrupted and separated by the interzone tissue comprised of avascular mesenchymal cells at each prospective joint site, which marks the first stages of synovial joint formation. Both long bone and synovial joint development are tightly coordinated events regulated by complex intrinsic and extrinsic factors.

While these developmental processes have been delineated in the axial skeletal, much less is known about the jaw and synovial joint morphogenesis in the craniofacial complex. Here we show for the first time that the TMJ in miniature pigs and humans is vascularized similar to perichondrium tissue. In fact similar endochondral ossification during bone fracture healing, we observed the evidence of lineage reprogramming, whereby chondrocytes directly differentiate into osteoblasts/osteocytes during TMJ injury and disease. Taken together, these studies provide novel insight into new therapeutic targets for the treatment of TMJ OA.
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**SUPPORTING INFORMATION**
Additional supporting information may be found online in the Supporting Information section.

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