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Anterior cruciate ligament-derived cells have high chondrogenic potential

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ABSTRACT

Anterior cruciate ligament (ACL)-derived cells have a character different from medial collateral ligament (MCL)-derived cells. However, the critical difference between ACL and MCL is still unclear in their healing potential and cellular response. The objective of this study was to investigate the mesenchymal differentiation property of each ligament-derived cell. Both ligament-derived cells differentiated into adipogenic, osteogenic, and chondrogenic lineages. In chondrogenesis, ACL-derived cells had the higher chondrogenic property than MCL-derived cells. The chondrogenic marker genes, Sox9 and α1(II) collagen (Col2a1), were induced faster in ACL-derived pellets than in MCL-derived pellets. Sox9 expression preceded the increase of Col2a1 in both pellet-cultured cells. However, the expression level of Sox9 and a ligament/tendon transcription factor Scleraxis did not parallel the increase of Col2a1 expression along with chondrogenic induction. The present study demonstrates that the balance between Sox9 and Scleraxis have an important role in the chondrogenic differentiation of ligament-derived cells.

Introduction

Anterior cruciate ligament (ACL) and medial collateral ligament (MCL) are major stabilizers of the knee. ACL injury can result in early development of osteoarthritis without reconstructive surgery because of its poor healing property [1,2]. On the other hand, MCL injury has a great capacity to heal [2]. Several authors have reported the difference in cellular potential between ACL and MCL. ACL includes more chondrocytic cells, identified as fusiform, ovoid, and spheroid cells, compared with MCL [3]. Fusiform and spheroid cells packaged in the ligament-to-bone interface can produce a cartilage specific extracellular matrix (ECM), type II collagen [4]. ACL fibroblasts show lower proliferation and migration potential compared with MCL fibroblasts [4–6]. Repair-associated molecules including transforming growth factor (TGF)-β and type III collagen do not increase in rabbit ACL injury, unlike MCL injury [7]. We have previously demonstrated that growth factor-stimulated cellular migration is slower in ACL than in MCL fibroblasts [8]. In addition, we have identified chondroblastic interface cells derived from the ACL-to-bone insertion [9]. However, the critical difference between ACL- and MCL-derived cells is still unclear. We hypothesized that the higher chondrocytic feature of ACL could cause disadvantage of ligament repair compared with MCL. In this study, we investigated the property of chondrogenic differentiation in ACL- and MCL-derived cells using a pellet culture procedure.

Mesenchymal stem cells (MSCs) are multipotent cells that differentiate into chondrogenic, osteogenic, and adipogenic lineages [10]. Several tissue-derived MSCs, such as bone marrow [10], synovium [11], adipose [12], ligament [13], cartilage, and meniscus [14] have identified as a source of regeneration for damaged tissue. However, attached colony forming cells are commonly characterized as tissue-derived MSCs. Colony forming rate of tissue-derived cells is quite different among various mesenchymal tissues [14]. In blastema-induced limb regeneration, undifferentiated progenitors keep a memory of their tissue origin and have restricted potential without complete dedifferentiation to a pluripotent state [15]. Tendon progenitors that align between the differentiating cartilage and muscle are maintained by the recruitment of adjacent MSCs in the presence of TGF-β signaling [16]. We hypothesized that ligament-derived cells might have mesenchymal redifferentiation potential, and that recruitment of progenitors should influence ligament cell fate. The present study demonstrated that ligament-derived cells differentiated into mesenchymal lineages, and that chondrogenic property was higher in ACL-derived cells than in MCL-derived cells.

Materials and methods

Cells and cell culture: Institutional Review Board Approval was obtained before beginning all animal studies. Ligament-derived cells were isolated from ACL and MCL of 10-week-old Japanese white rabbits ($n = 4$) (Shimizu Laboratory Supplies). The synovial sheath and fat tissue of ligaments were removed. The midsubstance cores of

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ACL and MCL were digested using collagenase (Sigma) as described [8]. Attached cells (passage 0) were maintained with Dulbecco's modified Eagle's medium (DMEM, Wako) containing fetal bovine serum (HyClone) and penicillin/streptomycin (Sigma) at 37 °C in a humidified atmosphere of 5% CO_2. Cells were subcultured at a density of 5000 cells/cm^2 to avoid colony formation. Ligament-derived cells between passage 3 and 6 were used.

In vitro mesenchymal differentiation and histology: To induce adipogenic differentiation, confluent cells were cultured with adipogenesis induction and maintenance media for 3 weeks according to the manufacturer's protocol (Millipore). Lipid vacuoles were stained with oil red O solution. Osteogenic induction was performed using Mesenchymal Stem Cell Osteogenesis Kit (Millipore) and 1 ng/ml of recombinant human bone morphogenetic protein (BMP)-2 (kindly provided from Wyeth). Calcium deposition was visualized by von Kossa staining. For chondrogenesis, pellet-cultured cells (5×10^5 cells/pellet) were maintained in the chondrogenic induction medium (CIM) supplemented with 10 ng/ml of BMP-2 and/or recombinant human TGF-β3 (R&D Systems) as described [17]. Pellets were observed with hematoxylin–eosin (HE) and safranin O staining [18]. Immunohistological analyses were performed using anti-collagen type I or II antibodies (Merck Chemicals).

RT-PCR and quantitative real-time PCR: RNA samples (500 ng) were reverse-transcribed with ReverTra Ace (Toyobo). The cDNAs underwent PCR amplification in the presence of specific primers using Taq DNA polymerase (TaKaRa). For all the RT-PCR fragments, the reaction was allowed to proceed for 28–32 cycles. Quantitative real-time PCR analyses were performed as described [9]. Mesenchymal differentiation was assessed using the following primers: Sry-type HMG box 9 (Sox9) and α1(II) collagen (Col2a1) [5′-gca ccc atg gac att gga ggg-3′ and 5′-gac acg gag tag cac cat cg-3′] for chondrogenesis, Scleraxis (Scx) for ligament/tendon differentiation, peroxisome proliferators-activated receptor γ (Pparγ) for adipogenesis, alkaline phosphatase (Alp) for osteogenesis, α1(I) collagen (Col1a1), and glyceraldehyde-3-phosphate dehydrogenase (G3pdh) [19,20]. Relative expression levels were normalized with the level of each CIM-treated pellet.

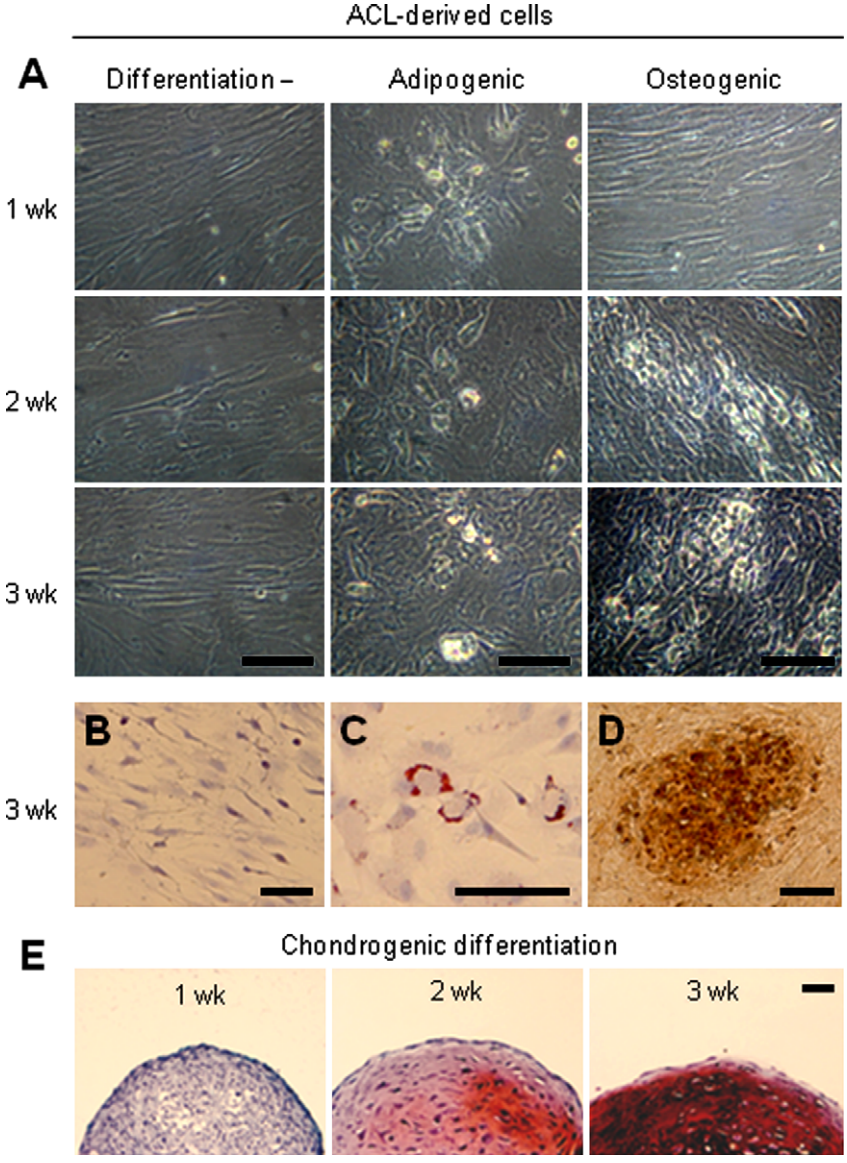

Fig. 1. Mesenchymal differentiation of ACL-derived cells. (A) Phase-contrast microscopic images of ACL-derived cells during adipogenic and osteogenic induction. (B) Undifferentiated fibroblastic cells were observed by hematoxylin staining. (C) Lipid vacuoles were observed by oil red O staining after adipogenic differentiation. (D) Osteogenic calcium deposition was visualized using von Kossa staining. (E) Safranin O-stained proteoglycans were increased along with chondrogenic differentiation (1–3 weeks) in the presence of TGF-β3 (10 ng/ml). Bars, 100 μm.

Statistical analysis: All experiments were repeated in triplicate and similar results were obtained. Data were expressed as means ± SD. Differences among groups were compared using the Mann–Whitney *U*-test. Significance was established at $p < 0.05$.

Results

Ligament-derived cells have a potential to differentiate into mesenchymal lineages

ACL-derived cells differentiated into adipogenic, osteogenic, and chondrogenic lineages (Fig. 1). MCL-derived cells also had a multilineage potential (Fig. 2). Adipogenic induction was indicated by the accumulation of lipid vacuoles (Figs. 1C and 2C). Pparγ, the master transcription factor in adipogenesis, expression was induced in both ACL- and MCL-derived cells (Fig. 3). Osteogenic calcium deposition was induced under the osteogenic condition (Figs. 1D and 2D). The expression of Alp was also detected in both osteogenic-differentiated cells (Fig. 3). Chondrogenic-differentiated pellets in the presence of TGF-β3 (10 ng/ml) were stained by safranin O dye (Figs. 1E and 2E). The intensity of safranin O-stained proteoglycans was higher in ACL-derived pellets than in pellet-cultured cells of MCL origin (Figs. 1E and 2E, 2 and 3 weeks). The inductions of chondrogenic genes, Sox9 and Col2a1, were faster in ACL-derived cells than in MCL-derived cells (Fig. 3). These results indicated that adipogenic and osteogenic differentiation was similarly activated in both ACL- and MCL-derived cells. However, the chondrogenic potential was higher in ACL-derived cells than in MCL-derived cells.

ACL-derived cells have the higher chondrogenic property than MCL-derived cells

To compare the chondrogenic potential of ACL- and MCL-derived cells, we investigated the balance between two transcription factors, Sox9 and Scx, in the presence of BMP-2 and/or TGF-β3. TGF-β3 treatments increased Col2a1 expression in 2-week-cultured ACL pellets (Fig. 4 B and C). BMP-2 enhanced the Col2a1

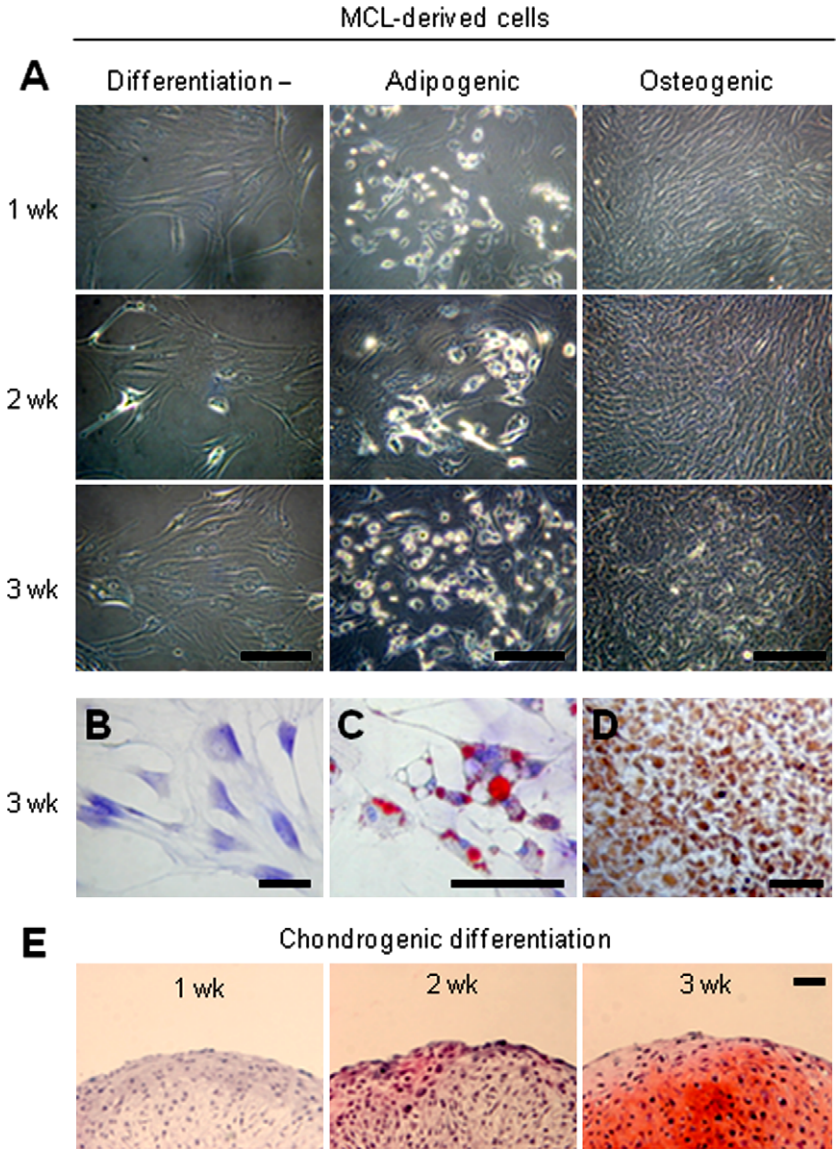

Fig. 2. Mesenchymal differentiation of MCL-derived cells. (A) Phase-contrast microscopic images of MCL-derived cells along with adipogenic and osteogenic induction. (B) Hematoxylin-stained fibroblastic cells. (C) Oil red O staining after adipogenic differentiation. (D) Osteogenic-differentiated cells. (E) Chondrogenic induction was slower in MCL-derived cells than in ACL-derived cells (compared with Fig. 1E, safranin O staining). Bars, 100 μm.

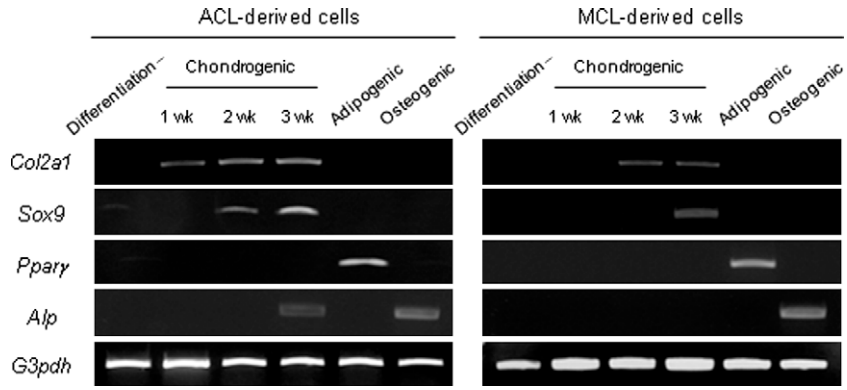

Fig. 3. ACL-derived cells have the higher chondrogenic potential than MCL-derived cells. RT-PCR analyses revealed that Col2a1 and Sox9 expressions were gradually increased by chondrogenic induction in the presence of TGF-β3 (10 ng/ml). The expression of chondrogenic marker genes was activated earlier in ACL-derived pellets than in MCL-derived pellets. Pparγ and Alp expressions were similarly induced in both cells. Alp expression was also detected in 3-week-pellets of ACL-derived cells.

expression of ACL pellet in the presence of TGF-β3 (Fig. 4C). On the other hand, Col2a1 expression of MCL-derived pellet was insufficiently activated by TGF-β3 (Fig. 4 B and D). Sox9 expression preceded the increase of Col2a1 in both pellet-cultured cells (Fig. 4B–D). Interestingly, the expression balance between Sox9 and Scx was reversed under a mature chondrogenic stage in ACL-derived pellets (Fig. 4B). The expression of Scx was equally detected in the presence or absence of BMP-2 and TGF-β3 (Fig. 4B). In immnohistological analyses, type II collagen was observed in TGF-β3-treated ACL pellets (Fig. 4E, Col II). However, we could not detect the positive staining for type II collagen in MCL-derived pellets (Fig. 4E). These findings suggest that the prior expression of Sox9, triggered by BMP-2 and/or TGF-β3, and the balance between Sox9 and Scx have key roles in chondrogenic induction of ligament-derived cells.

Discussion

Ligaments are dense and stress-resisting fibers composed of rich collagenous ECM. Remodeling and maintenance of ligament tissues are influenced by several factors including cellular response, ECM structure, vascularization, and growth factors [21,22]. However, the difference in chondrogenic property between ACL- and MCL-derived cells has not been elucidated. The present study demonstrated that ligament-derived cells differentiated into mesenchymal (chondrogenic, adipogenic, and osteogenic) lineages. In addition, ACL-derived cells had the higher chondrogenic property than MCL-derived cells.

In this study, we prepared ligament-derived cells without forming MSC-originated colonies. These ligament-derived cells might contain undifferentiated progenitors or MSCs. However, there is no appropriate method to discriminate ligament cells and MSCs, except for cell electrophoresis [23]. Longtime culture of ACL-derived cells (passage 6) under 30% confluent conditions produced a few colonies (Supplemental Fig. A). Approximately 10 colonies (per 75-cm^2 flask) were observed in ACL-derived cells at passage 6 (5000 cells/colony). The aged ligament cells (passage 7) with the exclusion of colony forming cells did not show sufficient chondrogenic differentiation (Supplemental Fig. B). Indirect co-culture system with tenocytes stimulates the expression of ligament/tendon-related genes in bone marrow-derived MSCs [24]. These findings suggest that the interaction between ligament cells and undifferentiated MSCs might have a key role to induce mesenchymal differentiation. Further investigation will be required to understand the cross-talk between ligament cells and tissue-derived MSCs in chondrogenic redifferentiation.

Chondrogenic differentiation is cooperatively regulated by several transcription factors, such as Sox9, Sox5, and Sox6 [25,26]. Our results indicated that the expression of chondrogenic master gene (Sox9) and major ECM molecule during chondrogenesis (Col2a1) was induced faster in ACL-derived pellets, rather than in MCL-derived pellets (Fig. 3). In addition, Col1a1 expression, the major component in ligament ECM, was transiently suppressed during chondrogenic induction (Fig. 4B). Col1a1 expression was then recovered at matured chondrogenic stage along with Sox9 reduction (Fig. 4B, BMP-2/TGF-β3-treated ACL pellets). The key transcription factor for ligament/tendon development (Scx) was also reinduced in CIM-treated pellets (Fig. 4B). Several authors have indicated that common mesenchymal precursor cells expressing both Sox9 and Scx are involved in the coordinated development of cartilages, tendons, and ligaments [27,28]. In the early phase of embryonic development, the expressions of Scx and Sox9 are overlapped in sclerotome. However, Scx is reduced in the center of Sox9-expresssed sclerotome during the following development [27]. On the other hand, Scx expression is transiently increased in the early stage of embryonic stem cell-derived chondrogenesis [29]. We have previously demonstrated that Scx stimulates the Sox9-dependent transcription by forming transcriptional complex with E47 and p300 in early chondrogenesis [20]. These findings suggest that the balance between Sox9 and Scx have an important role in the chondrogenic induction and maturation of ligament-derived cells.

The TGF-β superfamily including TGF-β and BMP is a multifunctional growth factor for many cellular responses such as proliferation and differentiation [30,31]. In chondrogenesis, TGF-β stimulation is necessary for chondrogenic differentiation derived from MSCs [10]. We have previously described that TGF-β signal Smad3 promotes the early chondrogenesis through the activation of Sox9 [17]. TGF-β-regulated Smad3 also activates the Sox9-dependent transcription on chromatin without influencing Sox9 expression itself [32]. In the present study, TGF-β3 and/or BMP-2 treatments stimulated Col2a1 expression during chondrogenic differentiation of ligament-derived cells (Fig. 4B). However, the expression level of Sox9 and Scx did not parallel the increase of Col2a1 expression (Fig. 4B–D). In the limb buds, diversification of cartilage and ligament/tendon lineages is regulated by antagonism between BMP and fibroblast growth factor (FGF) signaling pathways [33]. BMP-2 not only promotes chondrogenesis, but also inhibits ligament/tendon development, while FGF-4 has the opposite effect [34]. The BMP inhibitor Noggin represses the expression of Sox9 while inducing the Scx expression [35]. In micromass cultures of undifferentiated mesodermal cells, TGF-β/Smad signaling is a direct inducer of Sox9 and Scx, but transcriptional repressors

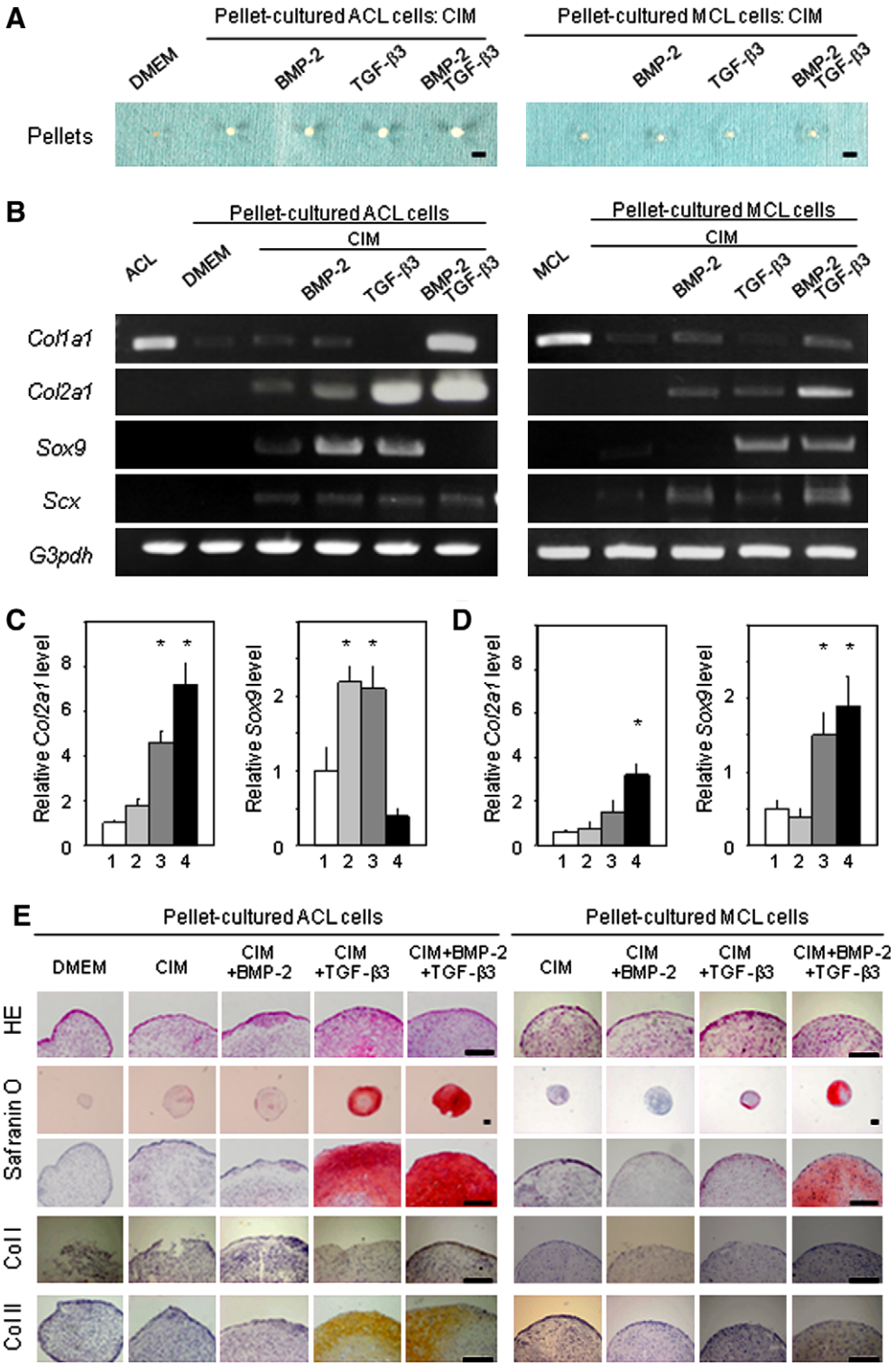

Fig. 4. Sox9-dependent chondrogenic differentiation in ACL- and MCL-derived cells. Pellet cultures were performed for 2 weeks in different conditions: 1, CIM; 2, CIM + BMP-2; 3, CIM + TGF-β3; 4, CIM + BMP-2/TGF-β3; growth factor concentration, 10 ng/ml. (A) ACL-derived pellets were larger than pellet-cultured cells derived from MCL. Bars, 2 mm. (B–D) TGF-β3 treatments increased Col2a1 expression up to a 4.6-fold level of CIM control in ACL-derived pellets. BMP-2/TGF-β3 treatments enhanced the Col2a1 expression up to 7.2- and 3.2-fold levels of control in ACL- and MCL-derived pellets, respectively. Sox9 expression was also increased to a 2.1-fold level of control by TGF-β3 in ACL-derived pellets. The prior induction of Sox9 was observed along with the progress of chondrogenesis. The expression of Scx was equally detected among each group. *Statistical significances ($p < 0.05$) compared to each CIM-treated pellet were observed in quantitative real-time PCR analyses. Error bars, SD. (E) Safranin O-stained proteoglycans and type II collagen (Col II) were increased in TGF-β3-treated ACL pellets, rather than MCL pellets. Type I collagen (Col I) was also detected by BMP-2/TGF-β3 treatments in ACL-derived pellets. Bars, 200 μm.

of TGF-β signaling (Tgif1 and SnoN) modulate the expression of Sox9 and Scx [36]. These findings suggest that TGF-β superfamily modulate the balance between Sox9 and Scx according to each differentiation stage of chondrogenesis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.12.044.

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