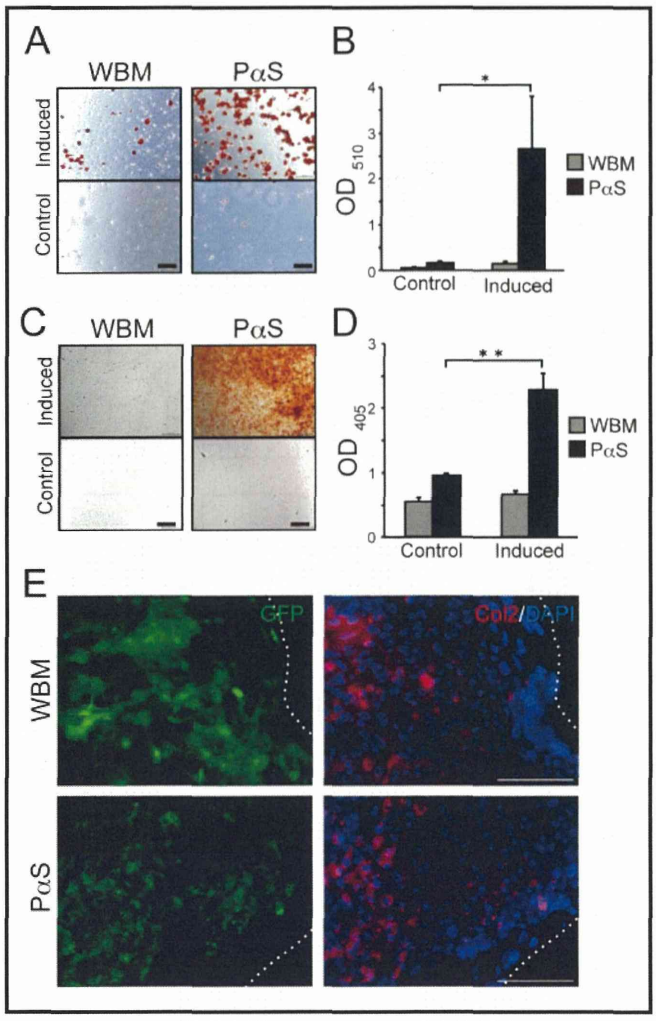

Fig.2 PαS-MSCs show high differentiation potency for the mesenchymal lineage

(A, B) Adipogenically-differentiated WBM and PαS cells were stained with Oil red O, and adipocyte formation was analyzed by using absorption spectrometry at 510 nm.

(C, D) Osteogenically-differentiated WBM and PαS cells were stained with alizarin red, and osteoblast formation was analyzed by absorption 30 spectrometry at 405 nm.

(E) Chondrogenically differentiated WBM and PαS cells were immunofluorescently stained with an antibody against Col2.

The scale bar is 500 μm in (A) and (C), 15 μm in (E). Data in (B) and (D) indicate the means ± the SEM. *p<0.05, **p<0.01.

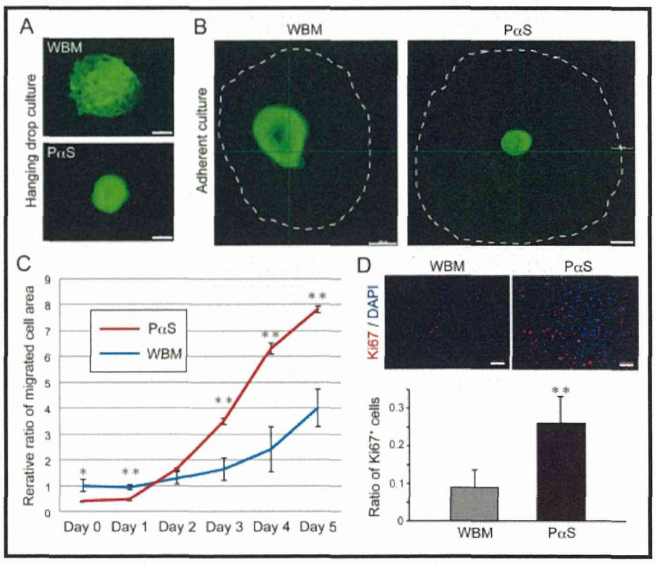

Fig.3 PαS cells have high growth capacity *in vitro*

(A) WBM and PαS sphere were cultured by the hanging drop method for 3 days and visualized under a fluorescence microscope.

(B) GFP$^+$ 8 MSCs derived from hanging drop culture were grown on glass slides for 5 days. Representative neurosphere cultures are shown in the four panels. The scale bar is 500 μm in A and B, and 50 μm in D.

(C) The radii of the aggregates were measured every day for 5 days (blue line, WBM cells; red line, PαS cells).

(D) Double fluorescence staining with anti-Ki67 antibody (red) and DAPI (blue). The ratio of the Ki67$^+$ area to the DAPI$^+$ area is shown.

Data in (C) and (D) are given as the means ± the SEM (n=3, p<0.01).

at 510 nm of Oil red O-stained cells, revealing a significantly higher adipogenic differentiation potential for PαS vs. WBM cells (Fig. 2B). Osteogenic differentiation potential, as assessed by alizarin red staining, was also enhanced in PαS vs. WBM cells (Fig. 2C). Spectrometric quantification of the alizarin red-stained cells at 405 nm revealed 2.3-fold higher staining intensity for the PαS cells (Fig. 2D). Lastly, chondrogenic differentiation was analyzed by sectioning and staining of cell pellets with anti-Col2 antibody. PαS and WBM cells had differentiation ability to chondrocyte at the same level (Fig. 2E). Taken together, these results suggest that PαS cells have an augmented differentiation potential for the mesenchymal lineage relative to WBM-derived MSCs.

3)Cell migration and proliferation *in vitro*

Cell migration and proliferation ability are important for the proper functioning of MSCs. A cell migration assay revealed significantly greater motility for PαS-derived spheres compared with WBM-MSC-derived spheres (Fig. 3A), with greater spreading on plastic dish (Fig. 3B). No difference was found for cell motility between the groups within 3 days of culture. After 5 days in culture, cell migration was significantly increased in PαS cells (2-fold) (Fig. 3C). Ki67 (proliferation marker) immunohistological staining indicated a higher proliferation in PαS cells compared with WBM cells (Fig. 3D).

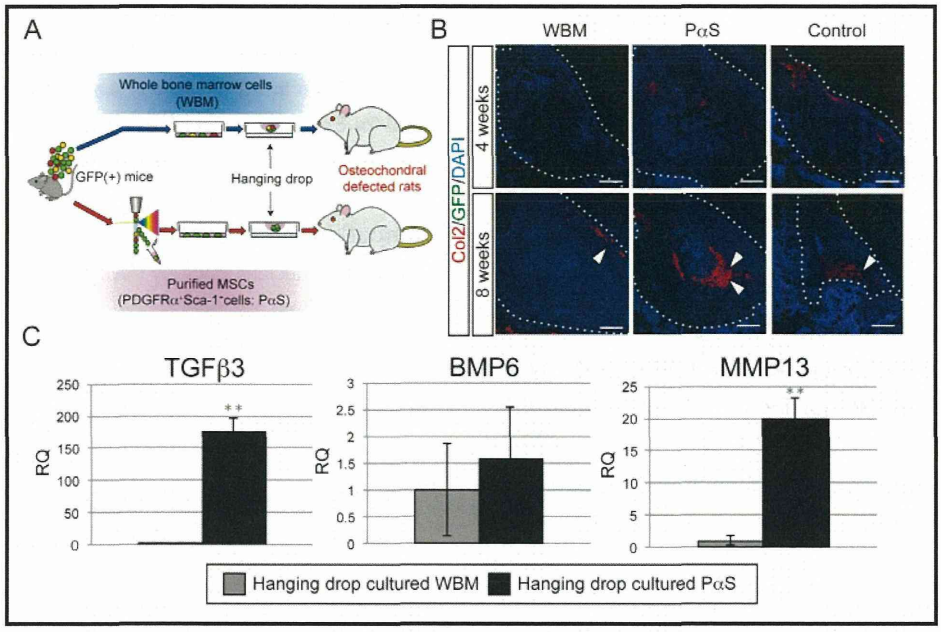

Fig.4 PαS cells promote cartilage reconstitution *in vivo*

(A)Scheme of experiment MSCs were transplanted into a rat cartilage defect model.
(B) Double fluorescence staining is shown for the chondrogenic marker, Col2 (red), and GFP (green). Chondrocyte-like cells are indicated by the white arrowheads. The scale bar is 250 μm.
(C) Quantitative analysis of mRNA expression of TGFβ3, BMP6, and MMP13 of hanging drop cultured WBM-MSCs and hanging drop cultured PαS-MSCs. The mRNA expression of each gene was normalized using hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA expression. The gene expression of hanging drop cultured WBM-MSCs was set as 1.0. $^{**}p<0.01$.

4)Cartilage regenerating effects of MSCs *in vivo*

To analyze the regenerative potential of PαS and WBM cells *in vivo*, we separately transplanted both cell types into a rat cartilage defect model (Fig. 4A). Cultured MSCs (100 × 10^4 cells in four aggregates) were transplanted into each defect, and the wounds were surgically closed. After 4 or 8 weeks, the cartilage was surgically removed and analyzed by immunohistochemistry. Immunohistochemical staining with Col2 antibody demonstrated that PαS MSCs generated markedly improved cartilage relative to WBM-MSCs *in vivo*, especially at 8 weeks after transplantation (Fig. 4B). These findings are strongly indicative of PαS cell-promoted cartilage regeneration *in vivo*. Nonetheless, we failed to identify GFP^+ cells in the defect site, even though the stem cells were derived from CAG-EGFP mice. It is likely that PaS-MSCs exert a paracrine effect on surrounding tissues to stimulate chondrogenic regeneration. We quantitatively analyzed mRNA expression of the endogenous cytokines in hanging drop cultured WBM-MSCs and hanging drop cultured PaS-MSCs (Fig.4C). PaS-MSCs express significantly higher TGFβ3 and matrix metalloproteinase 13 (MMP13), and somewhat higher BMP6 than WBM-MSCs.

Discussion

This study compared the basic properties and therapeutic aptitude of prospectively-isolated vs. plastic-adherent MSCs. Our data showed that prospectively-isolated MSCs exhibit enhanced growth, differentiation, and regenerative potential. These data are not altogether unexpected. Although flushed WBM undoubtedly contains immature MSCs, we previously demonstrated that other cell populations or "contaminating cells" can dramatically affect the fundamental characteristics of human MSCs in culture[12]. These contaminating actions appear to be independent of cell density. In this report, we also showed that an unpurified population of mouse WBM-MSCs expresses lower levels of the PDGFRα and Sca-1 antigens than PαS cells, and also contains $CD45^+$ hematopoietic cells. By contrast, prospectively-isolated MSCs are free from contaminating cells, revealing the true therapeutic potential of these multi-potent stem cells.

Traditional MSC isolation by plastic adherence requires prolonged time in culture to exclude contaminating cells.

Unfortunately, this culture step diminishes the "stemness" of MSCs[13]. The optimal culture conditions to expand MSCs while maintaining their undifferentiated state has not yet been established. Previous work indicated that aggregation of MSCs into three-dimensional spheroids enhances their stem cell potency, especially their CFU-F capacity and differentiation ability[14]. The current data showed that PαS cell aggregates can spread to a greater extent *in vitro* than WBM-MSC aggregates, and also have greater proliferative capacity *in vitro* and chondrogenic regenerative potency *in vivo*. Possible explanations for these findings include the negative effect of contaminating cell populations on MSC properties and the presence of additional plastic-adherent cells (e.g., fibroblasts and macrophages) that lack stem cell-like properties.

Although the transplanted PαS cells significantly improved chondrogenic regeneration in the present animal model, we could not identify GFP$^+$ transplanted cells in the defect area. Therefore, the difference in cartilage regeneration between PαS cells and WBM-MSCs cannot merely be explained by long-term engraftment. Indeed, previous data demonstrated that the number of transplanted MSCs diminishes with time after transplantation[15]. It is likely that PαS-MSCs exert a paracrine effect on surrounding tissues to stimulate the chondrogenic regeneration when we transplanted the cell. TGFβ and BMP6 are necessary for chondrogenic differentiation[16-20], and MMP13 is important for wound healing[21]. If Elevation of TGFβ3, BMP6, and MMP13 may therefore be a possible mechanism by which PαS cells mediate their pro-chondrogenic effects.

In conclusion, our findings imply that transplanting purified MSCs might improve chondrogenic regeneration via the production growth factors, although this hypothesis requires further investigation. In clinical applications, evaluation of the production of autocrine and paracrine factors by transplanted stem cells is essential to long-term success, because the therapeutic efficacy of MSCs depends on both autocrine and paracrine molecular and cellular events. Overall, however, our work shows that prospectively-isolated MSCs, which are free of contaminating cell populations, have enhanced stemness and augmented capacity for bone repair relative to plastic-adherent, WBM-derived MSCs.

Acknowledgment and Source of funding

We thank S. de Vega and M. Ojima for technical support; D.D. Houlihan for advise. This study was supported by a Grant-in-Aid for Scientific Research (Grant No. 25505001) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to C.A., as well as by a Project for Health and Labour Sciences Research Grant of Japan to I.S. This study was supported in part by a Grant-in-Aid for Encouragement of Young Scientists from the President of Tokyo Medical and Dental University (to Y.M.).

Competing interests

The authors declare that they have no competing financial interests in association with this manuscript.

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Teneurin-4, a Transmembrane Protein, Is a Novel Regulator That Suppresses Chondrogenic Differentiation

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Received 19 September 2013; accepted 24 February 2014
Published online 20 March 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jor.22616

ABSTRACT: Teneurin-4 (Ten-4), a transmembrane protein, is expressed in the nervous systems and the mesenchymal tissues, including the cartilage. However, the Ten-4 function in cartilage development remains unknown. Here, we showed that Ten-4 is a novel regulator of chondrogenesis. In situ hybridization analysis revealed that Ten-4 was highly expressed in the mesenchymal condensation area of the mouse femur at embryonic day (E) 13.5, while its expression was decreased in the growth plate of the femur at E18.5. Using the cartilage-like pellet culture of human synovial mesenchymal cells, Ten-4 expression was induced and peaked 7 days after induction of differentiation, while a production of type II and X collagens was increased after Day 14. In the cartilage-like pellet, Ten-4 was highly expressed in the less differentiated region. In the chondrogenic cell line ATDC5, knockdown of Ten-4 expression significantly increased the alcian blue staining and expression levels of aggrecan and type II and X collagens. Further, an elevated expression of Sox6, Sox9, and Runx2 and an attenuation of the ERK activation were observed in the Ten-4-knockdown ATDC5 cells. These results suggested that Ten-4 suppresses chondrogenic differentiation and regulates the expression and activation of the key molecules for chondrogenesis. © 2014 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 32:915–922, 2014.

Keywords: teneurin-4; chondrogenic differentiation; suppressor; human synovial mesenchymal cell; ATDC5

During endochondral ossification, cartilage tissues are initially formed by the mesenchymal cell condensation. The mesenchymal cells differentiate to chondrocytes, while the peripheral cells become perichondrial cells. Chondrocytes proliferate and express extracellular matrix proteins and proteoglycans, including type II collagen and aggrecan, and subsequently, they exit the cell cycle and terminally differentiate into post-mitotic hypertrophic chondrocytes accompanying with the expression of type X collagen.[1] Besides the extracellular matrix proteins and proteoglycans, transcription factors, Sox5, Sox6, Sox9, and Runx2, are also expressed and required for the chondrocyte differentiation.[1,2] Since these differentiation processes are essential for proper development of cartilage, gene mutations of critical molecules, such as Sox9, cause chondrodysplasia.[3] In addition, extracellular-signal-regulated kinase (ERK) is one of the crucial signaling molecules in chondrogenesis. Inhibition and promotion of ERK activation increases and decreases proteoglycan synthesis, respectively, in human chondrocytes and the mouse chondrogenic cell line ATDC5, indicating that the ERK signaling negatively regulates chondrocyte differentiation.[4–6]

Teneurin (Ten-m/Odz) is a family of type II transmembrane proteins and highly conserved from invertebrates to mammals. In mammals, 4 teneurin members have been identified (Ten-1 to -4). All teneurin members are highly expressed in the nervous systems, however, their expression is also observed in non-neural tissues, including mesenchymal tissues.[7] Recently, Murakami et al. showed that Ten-3 is expressed in the cartilage tissues during mouse development. Ten-3 expression is observed in the fibrous layer of the mandibular condylar cartilage, the perichondrium of the growth plate cartilage, and proliferative chondrocytes of the both cartilage tissues, but not in hypertrophic chondrocytes.[8] Ben-Zur et al.[9] analyzed Ten-4 expression in whole mouse embryos and found high expression of Ten-4 in the joint between humerus and radius/ulna, which seems to be the cartilage tissue, at embryonic day (E) 12.5, when the mesenchymal condensation for the cartilage growth plate formation occurs. In addition, the reduced expression of Ten-4 is observed in the growth plate of the tibia at E18.5.[9] We have recently demonstrated that Ten-4 is required for myelination in the central nervous system, due to dysfunction of oligodendrocytes.[10] However, the detailed expression pattern and the biological function of Ten-4 in cartilage development have not been elucidated yet.

In this study, we found that Ten-4 was highly expressed in the less differentiated regions of the mouse cartilage tissues and of the cartilage-like pellet from human synovial mesenchymal cells. In ATDC5 cells, knockdown of Ten-4 expression promoted differentiation, and it increased and reduced the expression levels of Sox6, Sox9, and Runx2 and the phosphorylation of ERK, respectively. Taken together, Ten-4 is a novel negative regulator of chondrogenic differentiation.

Grant sponsor: Ministry of Education, Culture, Sports, Science, and Technology (MEXT) in Japan; Grant number: 25505001; Grant sponsor: Ministry of Health, Labor and Welfare of Japan; Grant number: 2230201.
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Table 1. Primer Sequences for RT-PCR

Gene	Species	Sequence
Ten-4 (Odz4)	Human	Forward: 5′-CTTTCATCACACAGGAGCACACC-3′
		Reverse: 5′-AGGACAGATTTGTAGCCAGGGGTC-3′
	Mouse	Forward: 5′-GTGGACAAGTTTGGGCTCATTTAC-3′
		Reverse: 5′-GGGTTGATGGCTAAGTCTGTGG-3′
Aggrecan (Acan)	Mouse	Forward: 5′-TGGAGCATGCTAGAACCCTCG-3′
		Reverse: 5′-GCGACAAGAAGACACCATGTG-3′
Type II collagen (Col2a1)	Human	Forward: 5′-TCACGTACACTGCCCTGAAG-3′
		Reverse: 5′-TGCAACGGATTGTGTTGTTT-3′
	Mouse	Forward: 5′-TTGAGACAGCACGACGTGGAG-3′
		Reverse: 5′-AGCCAGGTTGCCATCGCCATA-3′
Type X collagen (Col10a1)	Human	Forward: 5′-AATGCCCACAGGCATAAAAG-3′
		Reverse: 5′-AGGACTTCCGTAGCCTGGTT-3′
	Mouse	Forward: 5′-AGCCCCAAGACACAATACTTCATC-3′
		Reverse: 5′-TTTCCCCTTTCCGCCCATTCACAC-3′
Sox5	Mouse	Forward:5′-CCCCACATAAAGCGTCCAATG-3′
		Reverse: 5′-TCTCCAGGTGCTGTTTGCTGAG-3′
Sox6	Mouse	Forward: 5′-AGGGAAAAGGAAGAGGGCAGTG-3′
		Reverse: 5′-CAGGCTTATGTGGTGAGGTAGACG-3′
Sox9	Mouse	Forward: 5′-TCTCCTAATGCTATCTTCAAGGCG-3′
		Reverse: 5′-TGCTCAGTTCACCGATGTCCAC-3′
Runx2	Mouse	Forward: 5′-CAGGCGTATTTCAGATGATGACAC-3′
		Reverse: 5′-ATTCGTGGGTTGGAGAAGCG-3′
HPRT	Human	Forward: 5′-TGAGGATTTGGAAAGGGTGT-3′
		Reverse: 5′-AATCCAGCAGGTCAGCAAAG-3′
	Mouse	Forward: 5′-TCAGTCAACGGGGGACATAAA-3′
		Reverse: 5′-GGGGCTGTACTGCTTAACCAG-3′
	Mouse	Forward: 5′-GTTAAGCAGTACAGCCCCAAA-3′
		Reverse: 5′-AGGGCATATCCAACAACAAACTT-3′
Ten-1 (Odz1)	Human	Forward:5′-TTAGCAAAGGGAACAGGGGG-3′
		Reverse: 5′-TGGTGGATAGTAATCTGGAAACGC-3′
Ten-2 (Odz2)	Human	Forward:5′-GCGATGAAACAGGATGGACG-3′
		Reverse: 5′-ACTGAAGAGAGGTTGGTGATGACAG-3′
Ten-3 (Odz3)	Human	Forward:5′-TTGTTCAAGAGCCGCCTGTC-3′
		Reverse: 5′-ACACTGTGGGTCAATACACTGGG-3′

METHODS

In Situ Hybridization

Frozen sections of E13.5 and E18.5 mouse hindlimbs were prepared, and in situ hybridization using probes for Ten-4 and type II collagen was performed, as described previously.[10–12]

Human Synovial Mesenchymal Cell Culture

Human synovial mesenchymal cell culture was prepared and differentiated as described previously.[13] This study was approved by an institutional review board, and informed consent was obtained from all subjects (approved No. 1030). Human synovium was harvested from donors during anterior cruciate ligament reconstruction surgery for ligament injury and digested with 3 mg/ml of collagenase D (Roche Applied Science, Penzberg, Germany) in α-minimal essential medium (αMEM; Life Technologies, Carlsbad, CA) at 37°C. The digested cells were cultured with complete culture medium: αMEM containing 10% fetal bovine serum (FBS; Life Technologies), 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (Life Technologies). After a 14-day-culture, cells were passaged and cultured for another 14 days. Then, cells were harvested and used for the experiments. Cells (cell number: 2.5×10^5) cultured as monolayer were pelleted by trypsinization and centrifugation. The pellets were cultured in 400 μl chondrogenic medium consisting of high-glucose Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 1 μg/ml BMP-7 (Stryker Biotech, Boston, MA), 10 ng/ml transforming growth factor-β3 (R&D Systems, Inc., Minneapolis, MN), 100 nM dexamethasone (Sigma–Aldrich, St. Louis, MO), 50 μg/ml ascorbate-2-phosphate, 40 μg/ml proline, 100 μg/ml pyruvate, and 1:100 diluted ITS + Premix (6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenious acid, 1.25 mg/ml bovine serum albumin, and 5.35 mg/ml linoleic acid; BD Biosciences, San Jose, CA). The medium was changed every 3–4 days. Total RNA was prepared from four different subjects for RT-PCR, and two pellets from two different sources were embedded in paraffin and sectioned for immunostaining.

RT-PCR

Total RNA was prepared with TRI reagent (Sigma–Aldrich) as described previously.[10] The quantitative RT-PCR was performed using the StepOne real-time PCR system (Life Technologies). cDNA was amplified with 40 PCR cycles at 95°C for 3 s, 60°C for 30 s using FAST SYBR Green Master Mix (Life Technologies) and gene-specific primers (Table 1).

Immunostaining

To immunostain sections of the human synovial mesenchymal cell pellets, the paraffin-embedded samples were deparaffinized, and then were treated with 3% H_2O_2 in methanol to fix and inactivate endogenous peroxidase. After that, the samples were blocked with Power Block Universal Blocking Reagent (BioGenex Laboratories, Fremont, CA). Proteins were detected by primary and secondary antibodies, as follows: Ten-4 (R&D systems), type II collagen (Daiichi Fine Chemical, Toyama, Japan), sheep IgG-HRP (R&D Systems), biotinylated mouse IgG (Vector Laboratories, Burlingame, CA), and DAB staining kit (Thermo Scientific, Rockford, IL) or VECTASTAIN ABC reagent (Vector Laboratories).

ATDC5 Cell Culture

The mouse chondrogenic cell line ATDC5 (RIKEN BRC, Tsukuba, Japan) was cultured in DMEM containing 5% FBS (GE Healthcare, Little Chalfont, UK), 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies) (Proliferation medium) under 5% CO_2. For differentiation, ATDC5 cells were cultured in Proliferation medium supplemented with insulin-transferrin-sodium selenite (Roche Applied Science).

shRNA

For knockdown of Ten-4, the pGIPZ shRNAmir vector, which contains the targeting sequence of Ten-4 (Thermo Scientific), was used. The control was non-silencing-GIPZ shRNAmir (Thermo Scientific). The vectors were transfected into ATDC5 cells with Neon™ Transfection System (Life Technologies). The transfected ATDC5 cells were cultured in the presence of 3 μg/ml puromycin (Sigma–Aldrich) until all of non-transfected cells died. The selected cells with puromycin were used for each experiment. Three independent experiments were performed and gave similar results.

Western Blotting

Protein samples from ATDC5 cells were analyzed by Western blotting as described previously.[10] The following antibodies were used to detect proteins: Ten-4 (R&D Systems), α-tubulin (Sigma–Aldrich), phospho-ERK1/2 (Thr202/Tyr204), ERK1/2 (Cell Signaling Technology, Danvers, MA), sheep IgG-HRP (R&D Systems), rabbit IgG-HRP (Sigma-Aldrich), and mouse IgG-HRP (GE Healthcare).

Alcian Blue Staining

For alcian blue staining of the ATDC5 cell culture, cells were fixed with methanol for 10 min at −20°C. After fixation, staining was performed with 0.1% alcian blue 8GX (Sigma–Aldrich) in 0.1 M HCl for 2 hr at room temperature. To quantify the intensity of the staining, the dye was dissolved with 6 M guanidine/HCl for 8 hr at room temperature and the optical density 650 nm was measured.

RESULTS

We first examined the Ten-4 expression pattern in the rudiment of the femur at E13.5 (Fig. 1A), where and when mesenchymal cells are condensed and initiate to differentiate to chondrocytes, and in the growth plate of the femur at E18.5 in mice (Fig. 1D). In situ hybridization analysis using the Ten-4 anti-sense probe revealed that Ten-4 was highly expressed in the

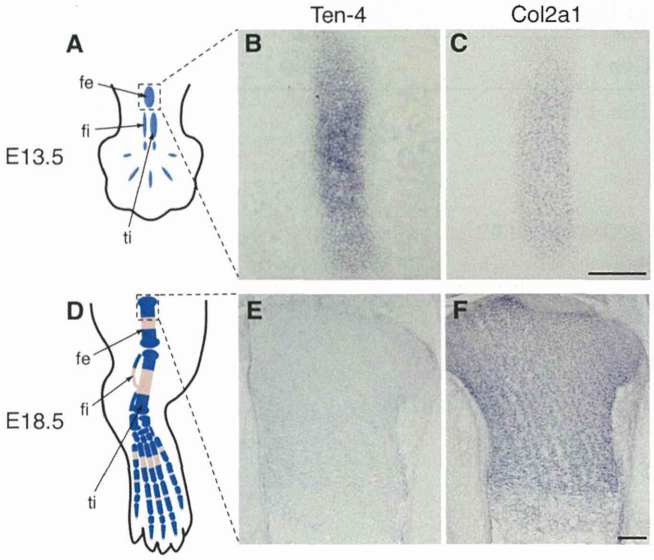

Figure 1. Ten-4 expression in the mouse hindlimb cartilage tissues. In situ hybridization of Ten-4 (B,E) and type II collagen (Col2a1) (C,F) in the rudiment of the femur at E13.5 and the growth plate of the femur at E18.5 was carried out. (A) and (D) represent regions shown in (B,C) and (E,F), respectively. fe: femur; fi: fibula; ti: tibia; Light blue area in (A): rudiment; Blue area in (D): cartilage; Light pink area in (D): bone; Scale bar, 100 μm.

rudiment of the femur at E13.5, compared with that in the growth plate of the femur at E18.5 (Fig. 1B,E). There was no signal using the sense probe of Ten-4 (data not shown). Type II collagen was weakly expressed in the rudiment at E13.5, but its expression was substantially increased in the growth plate at E18.5 (Fig. 1C,F). These results suggested that Ten-4 is highly expressed prior to that the expression of type II collagen becomes intense during in vivo mouse chondrogenesis.

We next examined the expression pattern of Ten-4 in the human synovial mesenchymal cell culture that is established to analyze chondrogenesis, as well as the culture of the bone marrow mesenchymal cells.[13,14] After induction of chondrogenic differentiation, the mesenchymal cells differentiate to chondrocytes and form cartilage-like pellets with a production of differentiation markers, including type II and X collagens (Fig. 2A,B).[13] As a result of quantitative RT-PCR, the peak of Ten-4 mRNA expression was observed on Day 7, while the mRNA expression of type II and X collagens was increased between 14 and 21 days after induction of differentiation (Fig. 2B). In addition, immunostaining of Ten-4 revealed that the expression level of Ten-4 protein was higher in the pellet on Day 7, compared with that in the Day 14-pellet (Fig. 2C: a, b), which is consistent with the RT-PCR data (Fig. 2B). In the pellets, the type II collagen expression and trousine blue staining were less intense in the center area than the outer area, indicating that the center region was less differentiated, compared with

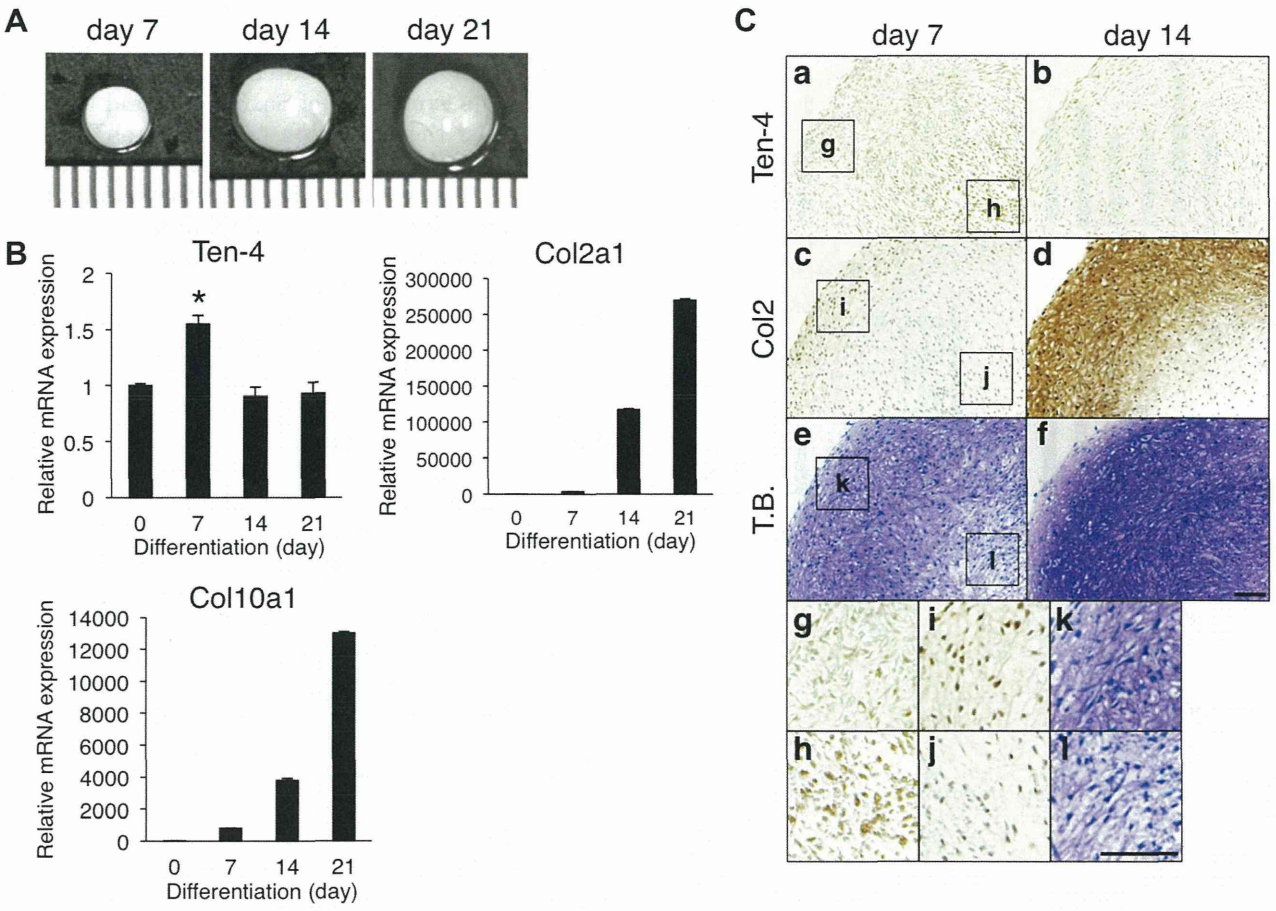

Figure 2. Ten-4 expression in the human synovial mesenchymal cell culture during its chondrogenic differentiation. (A) Morphology and sizes of pellets of the cell culture. Scale, 500 μm. (B) Quantitative analysis of mRNA expression of Ten-4, type II collagen (Col2a1), and type X collagen (Col10a1) during chondrogenic differentiation of the mesenchymal cells. The mRNA expression of each gene was normalized using hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA expression. The normalized expression of each gene on Day 0 was set as 1.0. The expression level of Ten-4 on Day 7 was significantly higher than those on Day 0, 14, and 21 ($^*p < 0.05$). Error bars, s.e.m. (C) Immunostaining of Ten-4 and type II collagen (Col2) in the cell pellets. Hematoxylin staining was performed with the Col2 immunostaining. Toluidine blue staining (T.B.) was used as a control. In (g,h), (i,j), and (k,l), enlarged images of boxes with black lines in (a), (c), and (e), respectively, are shown. Scale bar, 100 μm.

the outer region (Fig. 2C: c–f). We found that Ten-4 protein was highly expressed in the less differentiated region of the pellet on Day 7 (Fig. 2C: g–l). Similar expression pattern of Ten-4 was observed during the cartilage-like pellet formation of the human bone marrow mesenchymal cells (Supplementary Fig. 1). These findings suggested that Ten-4 is dominantly expressed in less differentiated cells during chondrogenesis, and agreed with the in situ hybridization data (Fig. 1).

To elucidate the function of Ten-4 in chondrogenesis, we used the mouse chondrogenic cell line ATDC5. In the presence of insulin-transferrin-selenium, ATDC5 cells can be differentiated to chondrocyte-like cells accompanying with an increase of the chondrocyte differentiation markers' expression (Fig. 3A).[15] When Ten-4 expression was examined by quantitative RT-PCR during the differentiation of ATDC5 cells, Ten-4 mRNA expression was induced at the beginning of differentiation, and the peak of Ten-4 expression was observed 8 days after induction of differentiation (Fig. 3A). The peak of Ten-4 expression on Day 8 was also detected in its protein expression level by Western blotting (Fig. 3B). The mRNA expression of the chondrocyte differentiation markers, aggrecan and type II and X collagens, was increased 12 days after induction of differentiation and the peaks of their expression were found between Day 16 and 24 (Fig. 3A). These observations indicated that Ten-4 expression is induced and peaked at the early stage of ATDC5 differentiation, before the expression of chondrocyte differentiation markers becomes high. These data are consistent with the Ten-4 expression data in mouse cartilage tissues and in the human mesenchymal cell cultures (Figs. 1 and 2; Supplementary Fig. 1).

We further analyzed Ten-4's function in ATDC5 differentiation using knockdown of Ten-4 expression by shRNA. Ten-4-knockdown ATDC5 cells (shTen-4 cells) were established by stable transfection with

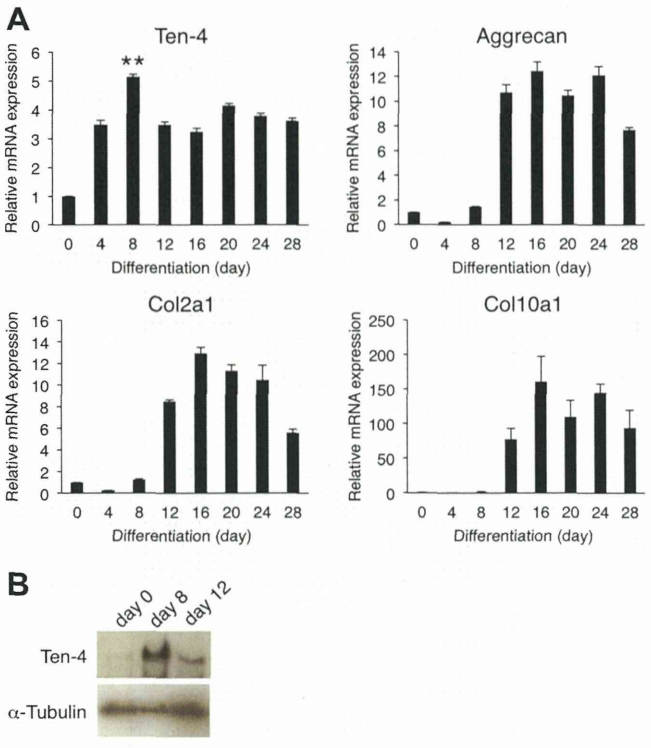

Figure 3. Ten-4 expression in the chondrocytic cell line ATDC5. (A) Quantitative analysis of mRNA expression of Ten-4, aggrecan, type II collagen (Col2a1), and type X collagen (Col10a1) in ATDC5 cells during differentiation. The mRNA expression of each gene was normalized using HPRT mRNA expression. The normalized expression of each gene on Day 0 was set as 1.0. The expression level of Ten-4 on Day 8 was statistically higher than those on the other days ($^{**}p < 0.01$). Error bars, s.e.m. (B) Western blotting of Ten-4 in ATDC5 cells on Day 0, 8, and 12. The expression of α-tubulin was used as the control.

shRNA for Ten-4, and its cell differentiation was examined. Quantitative RT-PCR showed that 8 and 12 days after induction of differentiation, Ten-4 mRNA expression in shTen-4 cells was reduced to approximately half of that in control-shRNA-transfected ATDC5 cells (shCont cells) (Fig. 4A). The expression level of Ten-4 protein was also significantly decreased in shTen-4 cells, compared with that in shCont cells (Fig. 4B). We found that alcian blue staining was significantly increased in the shTen-4 cell culture on Day 8 and 12, in comparison with the shCont culture, whereas no significant difference was observed on Day 6 (Fig. 4C). In addition, mRNA expression levels of aggrecan and type II and X collagens were dramatically increased in shTen-4 cells 8 and 12 days after induction of differentiation (Fig. 4D). The knockdown of Ten-4 by another shRNA construct with a different sequence yielded similar results (data not shown). From these results, Ten-4 suppresses the differentiation of ATDC5 cells.

Since the transcription factors, Sox5, Sox6, Sox9, and Runx2, are critically involved in chondrocyte differentiation,[1,2] we examined their expression levels in shTen-4 cells. In shTen-4 cells on both Day 8 and 12, the expression levels of Sox6, Sox9, and Runx2 were elevated, while Sox5 expression was normal (Fig. 5). This result indicated that Ten-4 negatively regulates the expression of Sox6, Sox9, and Runx2. We also assessed the activation of ERK in shTen-4 cells. The phosphorylation levels of ERK were significantly reduced on 8 and 12, compared with those in shCont cells (Fig. 6). The ERK phosphorylation level in shTen-4 cells was slightly attenuated on Day 6 (Fig. 6), prior to that the Ten-4 expression was peaked on Day 8 (Fig. 3). Together, our data showed that the expression of the transcription factors and the ERK pathway are regulated by the Ten-4 signaling.

In addition, we examined expression levels of the other teneurin members, Ten-1, Ten-2, and Ten-3, during the chondrogenic differentiation of the human synovial mesenchymal cell culture. The expression level of Ten-1 was increased on Day 7, 14, and 21, compared with that on Day 0, before induction of differentiation. Ten-2 expression was high on Day 0, but it was reduced on Day 7. After Day 7, the expression level of Ten-2 was elevated on Day 14 and 21. The Ten-3 expression level was gradually decreased during the differentiation (Fig. 7). These data showed that all the teneurins are expressed in the human synovial mesenchymal cell culture.

DISCUSSION

In this study, we analyzed the expression pattern of Ten-4 in vivo mouse cartilage tissues and in vitro cultures of the human mesenchymal cell culture and of ATDC5 cells. These results showed that the Ten-4 expression is high at the early stages, before the expression of the chondrocyte differentiation markers becomes intense (Figs. 1–3). Our in situ hybridization data was similar to the previous result by Ben-Zur et al.[9] although they analyzed its expression in the joint between humerus and radius/ulna at E12.5, which looks like the cartilage tissue, and in the growth plate of the E18.5 tibia in mice. These in vivo and in vitro expression patterns of Ten-4 are consistent with the result of our knockdown experiment that showed the suppressive function of Ten-4 in chondrogenic differentiation of ATDC5 cells (Fig. 4). Although the Ten-4 expression was obviously attenuated during chondrogenesis in vivo (Fig. 1), its expression persisted and was not largely diminished in the in vitro cultures of the human synovial mesenchymal cells and ATDC5 cells after the peak of the Ten-4 expression (Figs. 2 and 3). This is probably due to analyzing the heterogenous cell populations from the whole cultures, which contain both differentiating and proliferating cells, by RT-PCR. Also, in Ten-4-knockdown ATDC5 cells, the elevated mRNA levels of the differentiation markers, particularly aggrecan, was striking compared with the control, while the alcian blue staining was moderately increased (Fig. 4). One of possible mechanisms to explain this phenomenon is that the abnormally overexpressed mRNA of aggrecan by the Ten-4 knockdown

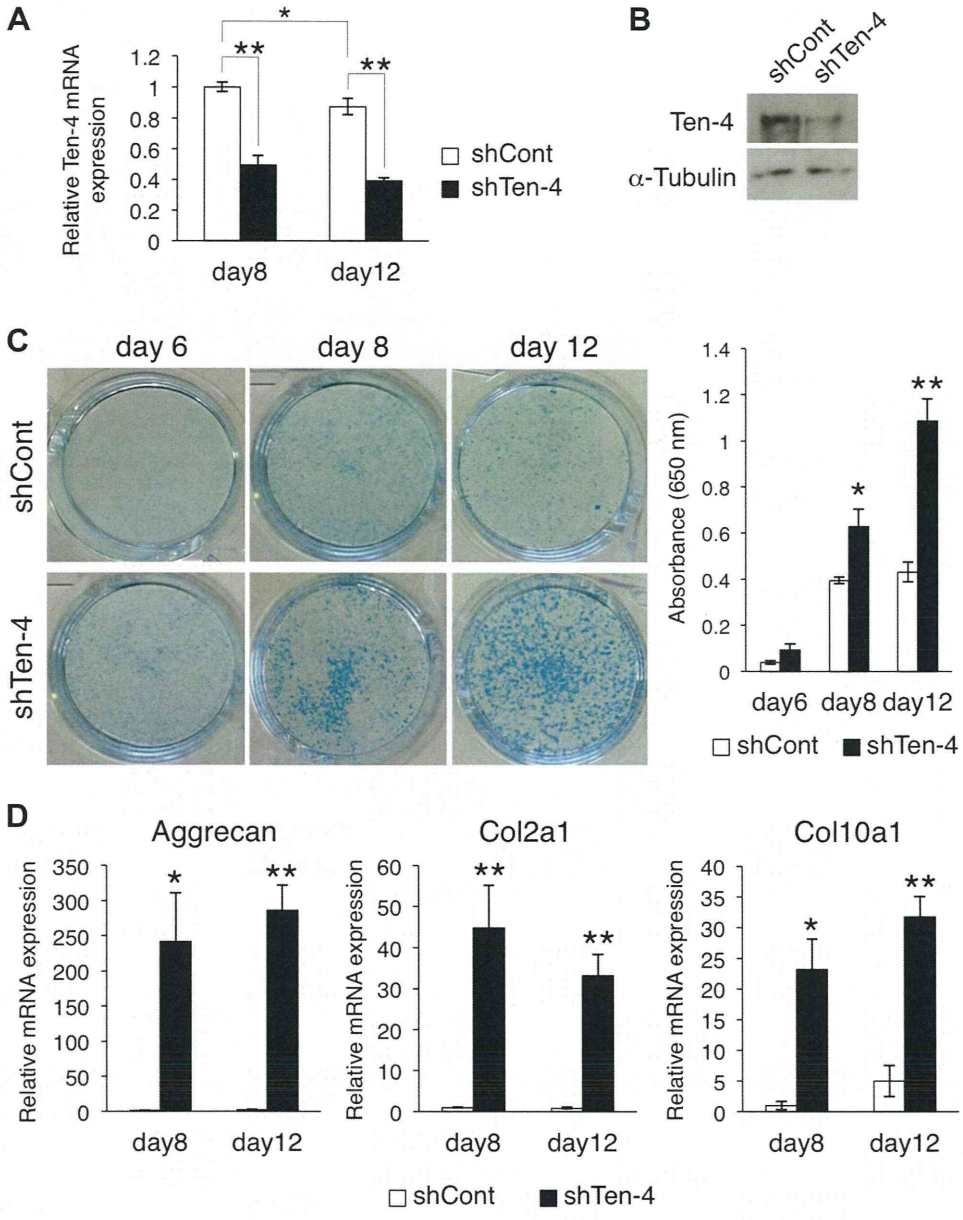

Figure 4. Promoted cell differentiation of ATDC5 by knockdown of Ten-4. (A) Quantitative RT-PCR of Ten-4 in shCont and shTen-4 cells, 8 and 12 days after induction of differentiation. The mRNA expression of each gene was normalized using HPRT mRNA expression. The normalized expression of each gene in shCont cells on Day 8 was set as 1.0. Error bars, s.e.m.; $^{*}p < 0.05$; $^{**}p < 0.01$. (B) Western blotting of Ten-4 in shCont and shTen-4 cells on Day 8. The expression of α-tubulin was used as the control. (C) Alcian blue staining in shCont and shTen-4 cell cultures during differentiation. For quantification, the values of the optical density (650 nm) were measured. Error bars, s.e.m.; $^{*}p < 0.05$; $^{**}p < 0.01$. (D) Quantitative RT-PCR of aggrecan, type II collagen (Col2a1), and type X collagen (Col10a1) on Day 8 and 12. The mRNA expression of each gene was normalized using HPRT mRNA expression. The normalized expression of each gene in shCont cells on Day 8 was set as 1.0. Error bars, s.e.m.; $^{*}p < 0.05$; $^{**}p < 0.01$.

might not be efficiently processed to the mature proteoglycan form in the extracellular matrix, which is stainable with alcian blue.

The transcription factor Sox9 is a master regulator of chondrogenesis and is expressed in both chondrocyte progenitor cells and differentiating chondrocytes, except for terminally differentiated hypertrophic chondrocytes. Sox9 is necessary for the mesenchymal condensation and chondrocyte differentiation, but prevents terminal differentiation of chondrocytes to post-mitotic hypertrophic chondrocytes.[2] The high expression of Ten-4 is observed in the mesenchymal condensation area (Fig. 1). Knocking down Ten-4 expression in ATDC5 differentiation significantly increased the expression level of Sox9 (Fig. 5). These evidences led us to propose a hypothesis that Ten-4 may be involved in controlling an initial switch of chondrogenesis, which is regulated by Sox9. The other Sox family members Sox5 and Sox6 are required for differentiation of proliferative chondrocytes, however, they block