penicillin (50 units/ml), streptomycin (50 μg/ml), and FBS (10%).

HuH-7 cells (RCB1366) were obtained from the RIKEN Cell Bank (Tsukuba, Japan). HeLa cells (JCRB9004) were obtained from the JCRB Cell Bank (Osaka, Japan). HEK 293 cells were purchased from Clontech. All cell lines were cultured at 37°C under 5% CO_2 in high-glucose DMEM supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml), and FBS (10%).

In vitro cartilage formation by *Sox* gene transfer. Embryoid bodies were formed by 3-dimensional (3-D) suspension culture for 5 days and subsequent 2-D adhesive culture on gelatin-coated plates for 3 days. Then, the embryoid bodies were transduced with adenoviruses expressing the various genes listed above, including the SOX trio at 100 multiplicities of infection (MOI). Chondrogenic differentiation was detected as fluorescence by confocal fluorescent microscopy.

For spheroid culture, human MSCs and adult human DFs were cultured in 100-mm dishes until confluency, and adenoviruses expressing the *SOX* genes were transduced at 50 MOI. Two days after transduction, cells were trypsinized and 500,000 cells per tube were gently centrifuged to form spheroids. Spheroids were cultured in serum-free high-glucose DMEM or in chondrogenic medium, which consisted of 300 ng/ml of BMP-2 (Yamanouchi, Tokyo, Japan) and 10 ng/ml of TGFβ3 (Techne, Princeton, NJ) in addition to high-glucose DMEM supplemented with $10^{-7}M$ dexamethasone, 50 μg/ml of ascorbate, 40 μg/ml of proline, 100 μg/ml of pyruvate, and 1× insulin–transferrin–selenium+1 (Sigma). Cells were collected at 3, 7, 14, and 21 days after spheroid formation for histochemical analyses and real-time PCR.

For analysis of monolayer-cultured human MSCs and adult human DFs, *SOX* genes were transduced at 50 MOI. Cells were collected at 5, 9, 16, and 23 days after transduction for real-time PCR. Three-dimensional culture on collagen gel was performed with 3-D Collagen Cell Culture system (Koken, Tokyo, Japan), according to the manufacturer's instructions. The transduced human MSCs and adult human DFs were trypsinized 2 days after transduction and seeded onto a DMEM-containing collagen gel at a density of 250,000 cells/cm^2 in 24-well plates and then cultured in serum-free DMEM. Cells were collected at 7, 14, and 21 days of 3-D culture. In each culture system, the medium was replaced every 3–4 days.

Transfections of HuH-7, HeLa, and HEK 293 cell lines with GFP-SOX expression vectors were performed with FuGENE 6 transfection reagent (Roche, Mannheim, Germany). In cotransfection, the same amount of total DNA was used, and all plasmids were added in an equal ratio.

Real-time PCR analysis. Total RNAs from cells were isolated with an RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. All total RNA samples were treated with DNase I. Total RNAs (50 ng to 1 μg) were reverse-transcribed with MultiScribe reverse transcriptase (ABI, Foster City, CA) and random hexamers in a 50-μl reaction volume, according to the manufacturer's instructions, and 1 μl of each reverse transcriptase reaction was used as a template for the second-step SYBR Green real-time PCR. The full-length or partial-length cDNA of target genes, including PCR amplicon sequences, were amplified by PCR, cloned into pCR-TOPO Zero II or pCR-TOPO II vectors (Invitrogen, Carlsbad, CA), and used as standard templates after linearization. QuantiTect SYBR Green PCR Master Mix (Qiagen) was used for the second-step SYBR Green real-time PCR according to the manufacturer's instructions. SYBR Green PCR amplification and real-time fluorescence detection were performed with an ABI 7700 Sequence Detection system. All reactions were run in quadruplicate. Copy numbers of target gene messenger RNA (mRNA) in each total RNA were calculated by reference to standard curves and were adjusted to the human or mouse standard total RNA (ABI) with the human GAPDH or rodent Gapdh as an internal control.

Each primer position in the coding sequences of target genes is described below. SOX5 and SOX6 primer sets were designed on the N-terminal domain of their long isoforms. The human set was as follows: for aggrecan, 6497–6796; for chondromodulin 1, 175–431; for COL2A1, 3856–4123; for COL9A1, 338–635; for COL10A1, 1641–1843; for COL11A2, 2543–2836; for matrilin 3, 232–422; for SOX5, 354–854; for SOX6, 315–593; for SOX9, 651–762; for RUNX2, 1270–1447; for COL1A1, 1184–1411; and for osteopontin (OPN), 251–446.

The mouse set was as follows: for aggrecan, 6013–6177; for chondromodulin 1, 192–474; for Col2a1, 3713–3951; for Col9a1, 1969–2196; for Col11a2, 910–1120; for Sox5, 1775–2010; and for Sox6, 2114–2271.

Western blot analysis. Western blot analysis was performed with cell extracts from SOX-overexpressing cell lines, human MSCs, and adult human DFs. Whole cell lysates or nuclear extracts (5 μg) were separated by 5–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride filters. The filters were incubated with an anti-GFP antibody (1:200; Clontech), anti-SOX antibody mixture (1:200–1:1,000 each; Santa Cruz Biotechnology, Santa Cruz, CA, and a generous gift from Dr. Yoshihiko Yamada, National Institutes of Health, Bethesda, MD, and Dr. Tomoatsu Kimura, Toyama Medical and Pharmaceutical University, Toyama, Japan). Antigen–antibody complexes were detected with horseradish peroxidase–conjugated secondary antibodies and visualized with the use of an ECL-Plus system (Amersham, Piscataway, NJ).

Histologic analysis. Spheroids and mouse tibias were fixed overnight at 4°C in 4% paraformaldehyde/phosphate buffered saline, transferred to 70% ethyl alcohol, and stored at 4°C until they were used. Subsequently, the samples were either frozen in OCT compound and then sectioned at 10 μm or embedded in paraffin and sectioned at 5 μm. Sections were stained with Alcian blue, toluidine blue, or Safranin O to evaluate the cartilaginous matrix, and with hematoxylin and eosin to evaluate the morphology, as previously described (32). Immunohistochemistry for Col2 and LacZ was performed as previously described (32).

In vivo *SOX* gene transfer. Ten 8-week-old C57BL/6J mice were divided into 2 groups and anesthetized with an intraperitoneal injection of pentobarbiturate (5 mg/100 gm of body weight). Then, 10 μl of a suspension of adenovirus vector expressing LacZ or the SOX trio (10^8 MOI) was injected into the subcutaneous tissue in front of the anteromedial diaphysis of the tibia. The mice were killed 1 week after surgery, and the entire tibia and surrounding tissue were harvested for histologic and immunohistochemical analyses. Whole tibias were dissected and fixed for 2 hours in 4% paraformaldehyde/phosphate buffered saline, pH 7.4, and decalcified for 2 weeks

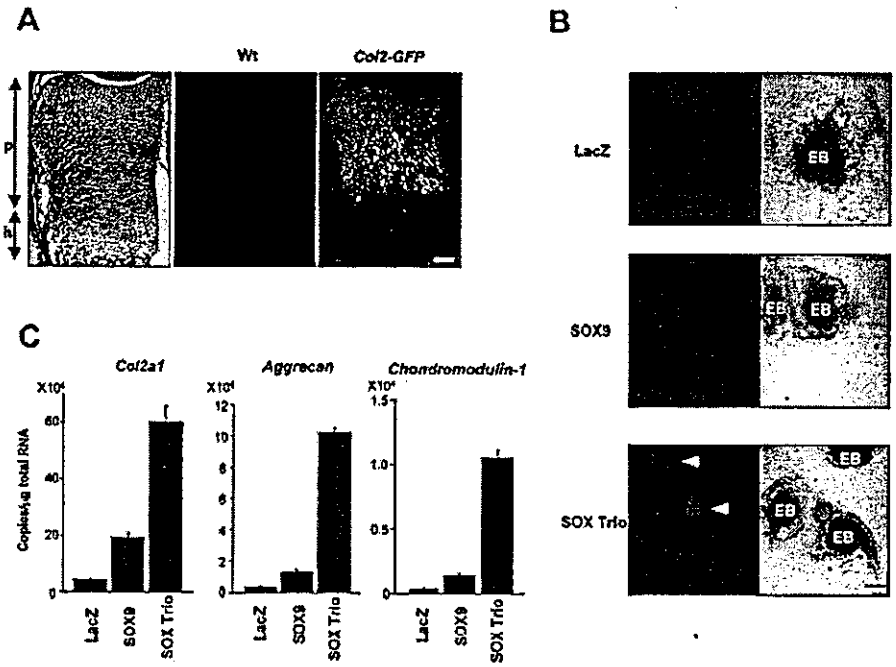

Figure 1. Induction of chondrocytic phenotypes in embryonic stem (ES) cells by the SOX trio. **A,** Fluorescence of growth plate chondrocytes from the *Col2-GFP*–transgenic mouse at embryonic day 18.5. The tibias from wild-type (Wt) and *Col2-GFP* neonate mice were sectioned, and the distal portions were examined by fluorescence microscopy. The morphology of the growth plate is shown at the left with hematoxylin and eosin staining. p = proliferating layer of growth plate chondrocytes; h = hypertrophic layer of growth plate chondrocytes. Bar = 100 μm. **B,** Fluorescence of *Col2-GFP* ES cells treated with the combination of SOX5, SOX6, and SOX9 (the SOX trio). LacZ, SOX9, or the SOX trio was adenovirally expressed in embryoid bodies (EB) of ES cells established from the *Col2-GFP*–transgenic mouse, and fluorescence was evaluated on day 3 after transduction (arrowheads). The left half of each panel shows green fluorescence protein (GFP) fluorescence; the right half shows a merging of the GFP fluorescence image and the transmitted image. Bar = 200 μm. **C,** Expression of the cartilage marker genes *Col2a1*, *Aggrecan*, and *Chondromodulin 1* by ES cells treated with LacZ, SOX9, or the SOX trio for 7 days. Levels of mRNA expression were analyzed by real-time polymerase chain reaction.

in 10% EDTA, pH 7.4. After processing and embedding in paraffin, 3-μm sagittal sections were cut and stained with Safranin O and fast green. Immunohistochemistry for type II collagen was performed as previously described (32).

Animal care was in accordance with the policies of the University of Tokyo School of Medicine.

GenBank sequences. Human gene sequences were obtained from GenBank (accession nos. M55172 for *AGGRECAN*, AB006000 for *CHONDROMODULIN 1*, X16468 for *COL2A1*, X54412 *COL9A1*, X60382 for *COL10A1*, NM_080679 for *COL11A2*, AJ224741 for *MATRILIN 3*, AB081589 for *SOX5*, AF309034 for *SOX6*, Z46629 for *SOX9*, NM_004348 for *RUNX2*, Z74615 for *COL1A1*, and AF052124 for *OPN*).

Mouse gene sequences were also obtained from GenBank (accession nos. L07049 for *Aggrecan*, NM_010701.1 for *Chondromodulin 1*, NM_031163 for *Col2a1*, D17511 for *Col9a1*, NM_009926 for *Col11a2*, AB006330 for *Sox5*, and U32614 for *Sox6*).

Image acquisition. An Axioskop 2 Plus (Carl Zeiss, Oberkochen, Germany) microscope was used for microscopic observation (bright and fluorescence fields at ×100, ×200, and ×400 magnifications). Photographs were taken with an AxioCam HRc (Carl Zeiss) camera, and images were acquired with AxioVision 3.0 software (Carl Zeiss).

RESULTS

Induction of cartilage marker gene expression in ES cells by the SOX trio. To screen for sufficient conditions for chondrogenesis, we needed a monitoring system that could detect chondrocyte differentiation in an easy, precise, and noninvasive manner. For this

purpose, we established transgenic mice expressing the chondrocyte-specific *Col2a1* promoter–*GFP* reporter gene and isolated totipotent, undifferentiated ES cells from them. Since GFP expression was specifically localized to the cartilage in these mice (Figure 1A), ES cells from these mice were expected to fluoresce solely upon chondrocyte differentiation. Using this system, we examined the effects of gain and loss of function of representative factors that are known to be important for chondrogenesis: SOX5, SOX6, SOX9, IGF-1, FGF-2, IHH, BMP-2, TGFβ, and Wnt proteins.

Since we intended to find factors affecting chondrocyte differentiation directly rather than indirectly, the assessment of fluorescence was done within 3 days after transduction. As a result, no single factor caused fluorescence; hence, we screened for all possible combinations of these factors. It turned out that *GFP* expression was observed only upon treatment with the combination of SOX5, SOX6, and SOX9 (the SOX trio) (Figure 1B), while there was no fluorescence upon treatment with the other combinations, including each SOX alone, within this period (results not shown).

We then examined the expression levels of the cartilage marker genes, which included the cartilaginous collagens (such as *Col2a1*, *Col9a1*, and *Col11a2*), cartilaginous proteoglycans (such as *Aggrecan*), and other cartilage-specific proteins that play key roles in maintaining cartilage structures (such as *Chondromodulin 1*) (33,34). Real-time PCR analysis confirmed that the SOX trio markedly up-regulated the levels of expression of *Col2a1*, *Aggrecan*, and *Chondromodulin 1* compared with SOX9 alone or the LacZ control (Figure 1C).

Induction of chondrocytic phenotypes in human MSCs by the SOX trio. We next examined the effect of the SOX trio on the chondrocyte differentiation of human MSCs. Expression of each SOX protein by adenoviruses was confirmed by Western blot analysis with specific antibodies (Figure 2A). To characterize human MSCs treated with SOX proteins, we evaluated the levels of expression of the cartilage marker genes by real-time PCR (Figure 2B). When cultured with serum-free DMEM in spheroids, human MSCs treated with the LacZ virus did not express detectable levels of the cartilage-specific collagen genes *COL2A1*, *COL9A1*, or *COL11A2* during 3 weeks of spheroid culture. In contrast, when the SOX trio was overexpressed, expression of these genes was detected as early as 3 days after spheroid formation. The number of copies of their mRNA continued to rise during the 3 weeks of spheroid culture. After 3 weeks of spheroid culture, the copy number of *COL2A1* mRNA from human MSCs exceeded that of *COL2A1* from the tracheal cartilage and articular cartilage.

When an individual SOX gene was transduced, expression of *COL2A1*, *COL9A1*, and *COL11A2* was not detected after 1 week of spheroid culture. After 2 weeks, only human MSCs treated with SOX9 expressed low levels of their mRNA. In contrast, *AGGRECAN* was already expressed at a moderate level even in untreated human MSCs, and its expression was substantially up-regulated by treatment with SOX9 alone or with the SOX trio after 2 weeks of spheroid culture. *CHONDROMODULIN 1* and *MATRILIN 3* were also induced by treatment with the SOX trio. The induction was first observed after 3 days of spheroid culture, and the copy number of their mRNA gradually increased up to 3 weeks.

We then performed histologic examinations of human MSCs treated with LacZ or the SOX trio and cultured in spheroids with serum-free DMEM or the chondrogenic medium containing TGFβ and BMP-2 (Figure 2C). Human MSCs treated with the SOX trio and cultured in spheroids with serum-free DMEM produced a proteoglycan-rich extracellular matrix characteristic of cartilage, which showed purple staining (metachromasia) with toluidine blue as early as 1 week after spheroid formation, whereas those treated with an individual SOX failed to show any staining at this stage. After 3 weeks, induction of proteoglycan-rich matrix by the SOX trio became more prominent. At higher magnification, cells in the spheroid were found to be completely surrounded by a proteoglycan-rich matrix, resembling the lacunar structure of cartilage (Figure 2D).

When cultured in the chondrogenic medium, accumulation of proteoglycan-rich matrix was accelerated (Figure 2C). After 1 week, the SOX trio induced abundant matrix production, whereas human MSCs treated with each SOX alone showed only weak production. After 3 weeks, although all spheroids including the LacZ control produced proteoglycan-rich matrix, human MSCs treated with the SOX trio showed the most abundant production. Staining with Alcian blue and Safranin O showed similar results (results not shown).

Production of type II collagen protein was detected by immunohistochemistry (Figure 2E). Human MSCs cultured in spheroids with the chondrogenic medium and treated with the SOX trio produced the most abundant type II collagen protein. Human MSCs cultured with serum-free DMEM and treated with the SOX trio and those cultured in the chondrogenic medium and treated with LacZ produced the second most abundant type II collagen protein. No type II collagen

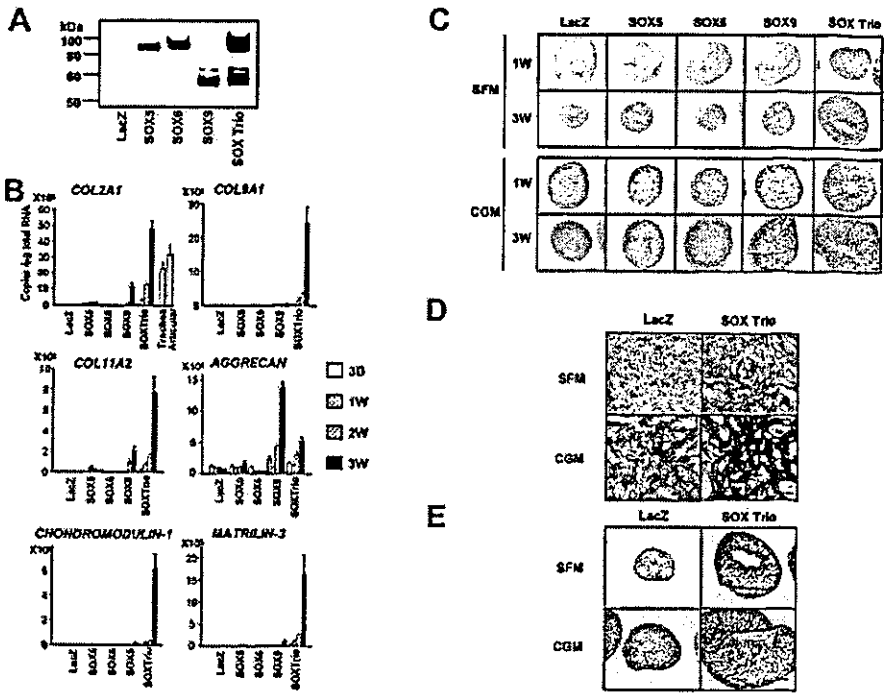

Figure 2. Induction of chondrocytic phenotypes in human mesenchymal stem cells (MSCs) by the SOX trio. **A**, Levels of adenovirally expressed SOX protein expression by human MSCs, as detected by Western blot analysis 5 days after transduction (expected sizes: 82 kd for SOX5, 87 kd for SOX6, and 56 kd for SOX9). **B**, Levels of mRNA expression of the cartilage marker genes *COL2A1*, *COL9A1*, *COL11A2*, *AGGRECAN*, *CHONDROMODULIN 1*, and *MATRILIN 3* by human MSCs. Cells were treated with LacZ, SOX5, SOX6, SOX9, or the SOX trio and cultured in spheroids with serum-free Dulbecco's modified Eagle's medium (DMEM) for 3 days, 1 week, 2 weeks, or 3 weeks, and mRNA expression was analyzed by real-time polymerase chain reaction. As positive controls, *COL2A1* mRNA levels were measured in tracheal and articular cartilage. **C**, Production of proteoglycan-rich matrix by human MSCs treated with LacZ, SOX5, SOX6, SOX9, or the SOX trio and cultured in spheroids with serum-free DMEM (SFM) or chondrogenic medium (CGM) for 1 week or 3 weeks. Spheroid sections were stained with toluidine blue. Proteoglycan-rich matrix stained purple (metachromasia). Bar = 100 μm. **D**, Higher-magnification views of proteoglycan-rich matrix produced by human MSCs treated with LacZ or the SOX trio and cultured in spheroids with SFM or CGM for 3 weeks. Spheroid sections were stained with toluidine blue. Bar = 20 μm. **E**, Expression of type II collagen protein by human MSCs treated with LacZ or the SOX trio and cultured in spheroids with SFM or CGM for 3 weeks. Type II collagen protein was detected by immunohistochemistry (brown staining). Bar = 100 μm.

production was observed in human MSCs cultured in spheroids with serum-free DMEM and treated with LacZ (Figure 2E). Interestingly, the presence of the chondrogenic medium did not cause an increase in mRNA levels of the cartilage marker genes (data not shown).

Induction of chondrocytic phenotypes in non-chondrogenic human immortalized cell lines by the SOX trio. So far, we had found that the SOX trio can induce chondrocytic phenotypes in totipotent ES cells and multipotent MSCs. If the SOX trio constitutes signals sufficient for the induction of chondrogenesis, it may induce chondrocytic phenotypes in cells already committed to other lineages. To test this possibility, we chose 3 human nonchondrogenic cell lines: HeLa cells derived from the cervix, HuH-7 cells derived from the liver (35), and HEK 293 cells derived from the embryonic kidney (36). Since these cell lines did not tolerate adenoviral transduction well, probably due to rapid proliferation of adenoviruses in these immortalized cells, we used plasmid transfection for gene delivery.

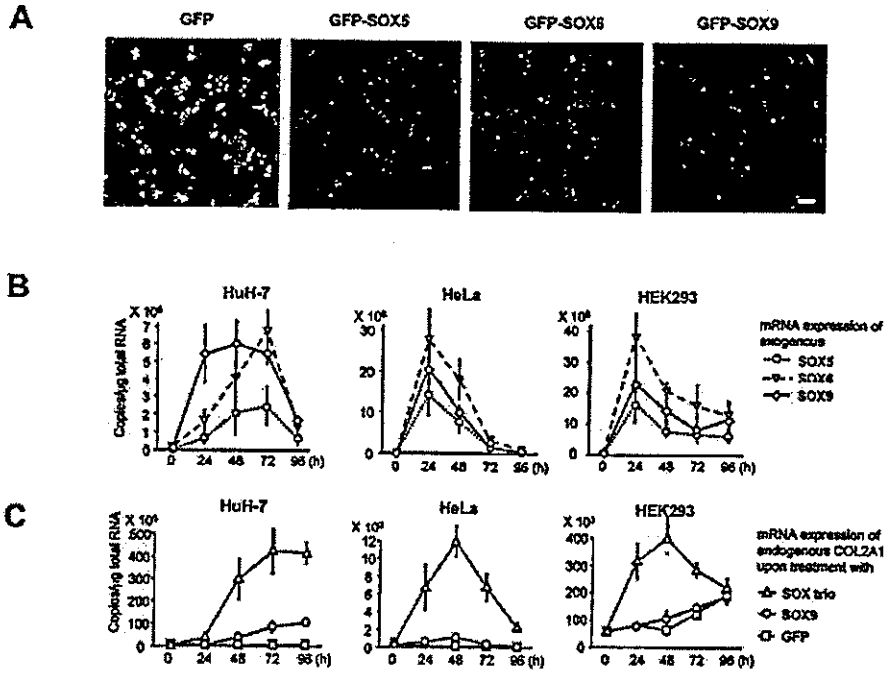

Figure 3. Induction of chondrocytic phenotypes in nonchondrogenic human cell lines by the SOX trio. A, Expression of green fluorescence protein (GFP)–tagged SOX proteins in HuH-7 cells. Each of the plasmids expressing GFP-tagged SOX genes was transiently transfected, and their expression levels and subcellular localization were detected as fluorescence using confocal fluorescence microscopy. Bar = 100 μm. B, Temporal mRNA expression profiles of exogenous SOX5, SOX6, and SOX9 in HuH-7, HEK 293, and HeLa cells transiently transfected with plasmids expressing these GFP-tagged SOX genes. Cells were cultured in monolayer with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Levels of mRNA expression were analyzed by real-time polymerase chain reaction (PCR). C, Temporal mRNA expression profiles of endogenous *COL2A1* in HuH-7, HEK 293, and HeLa cells transfected with plasmids expressing GFP, SOX9, or the SOX trio. Levels of mRNA expression were analyzed by real-time PCR.

When each of the plasmids expressing GFP-tagged *SOX* genes was transiently transfected into these cells, each GFP-tagged SOX protein was well expressed and localized in the nuclei (Figure 3A). Real-time PCR analysis revealed that the peak expression of all SOXs was achieved at 24–72 hours after transfection (Figure 3B). The SOX trio induced *COL2A1* mRNA expression within 3 days (Figure 3C). The temporal profile of *COL2A1* up-regulation correlated well with those of the exogenous *SOX* genes. Similar results were obtained with *COL9A1* and *COL11A2* (data not shown). It is noteworthy that overexpression of SOX9 alone up-regulated *COL2A1* to some extent in HuH-7 cells expressing moderate levels of endogenous *SOX5* and *SOX6* (37), but not in HeLa cells expressing no endogenous *SOX5* or *SOX6*.

Induction of chondrocytic phenotypes in adult human DFs by the SOX trio. We further examined whether the SOX trio could induce chondrocytic phenotypes in well-differentiated primary mesenchymal cells such as adult human DFs. Since adult human DFs can be easily harvested and cultured, and grow faster than human MSCs, they could be an alternative cell source for cartilage tissue engineering. Adult human DFs treated with the SOX trio were cultured in spheroids with serum-free DMEM. The SOX trio rapidly induced *COL2A1*, *COL11A2*, *AGGRECAN*, and *MATRILIN 3* within 3 days, and their levels continued to increase for up to 3 weeks (Figure 4A). *COL9A1* and *CHONDROMODULIN 1* were induced at 7 days after spheroid formation, and their expression levels continued to rise for up to 3 weeks as well. Unlike the human MSCs, adult

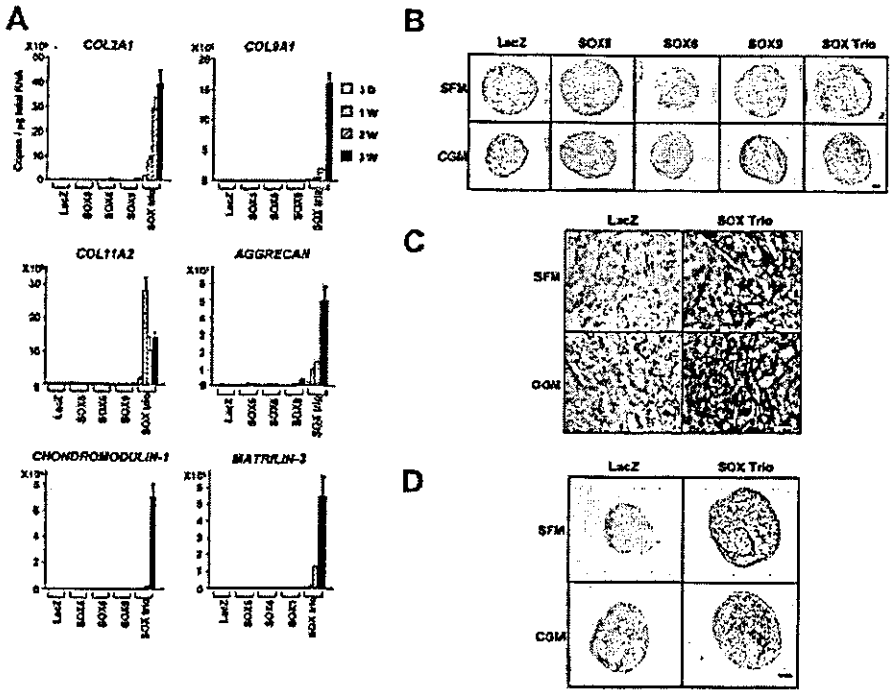

Figure 4. Induction of chondrocytic phenotypes in adult human dermal fibroblasts (DFs) by the SOX trio. A, Levels of mRNA expression of the cartilage marker genes *COL2A1*, *COL9A1*, *COL11A2*, *AGGRECAN*, *CHONDROMODULIN 1*, and *MATRILIN 3* by adult human DFs. Cells were treated with LacZ, SOX5, SOX6, SOX9, or the SOX trio and cultured in spheroids with serum-free Dulbecco's modified Eagle's medium (DMEM) for 3 days, 1 week, 2 weeks, or 3 weeks, and mRNA expression was analyzed by real-time polymerase chain reaction. B, Production of proteoglycan-rich matrix by adult human DFs treated with LacZ, SOX5, SOX6, SOX9, or the SOX trio and cultured in spheroids with serum-free DMEM (SFM) or chondrogenic medium (CGM) for 3 weeks. Proteoglycan-rich matrix stained purple (metachromasia) with toluidine blue. C, Higher-magnification views of proteoglycan-rich matrix produced by adult human DFs treated with LacZ or the SOX trio and cultured in spheroids with serum-free DMEM or chondrogenic medium for 3 weeks. Spheroid sections were stained with toluidine blue. Bar = 20 μm. D, Expression of type II collagen protein by adult human DFs treated with LacZ or the SOX trio and cultured in spheroids with serum-free DMEM or chondrogenic medium for 3 weeks. Type II collagen protein was detected with immunohistochemistry (brown staining). Bar = 100 μm.

human DFs showed low basal expression of the cartilage marker genes, and treatment with SOX9 alone resulted in very weak or no induction. We compared mRNA expression levels of the cartilage marker genes by adult human DFs and human MSCs that were treated with the SOX trio and cultured in spheroids with serum-free DMEM up to 3 weeks, and found them to be comparable (data not shown).

When cultured in spheroids with serum-free DMEM for 3 weeks, adult human DFs treated with the SOX trio exhibited an accumulation of proteoglycan-rich matrix, whereas those treated with LacZ or with each SOX alone did not (Figure 4B). When cultured with the chondrogenic medium for 3 weeks, adult human DFs treated with the SOX trio further increased the production of proteoglycan-rich matrix. At higher magnification, cells in the spheroid were found to be surrounded by proteoglycan-rich matrix, resembling the lacunar structure of cartilage (Figure 4C). Adult human DFs treated with SOX9 alone showed weak, focal production of proteoglycan-rich matrix in the presence of the chondrogenic medium, whereas those treated with LacZ, SOX5, or SOX6 did not (Figure 4B). Production of type II collagen protein by adult human DFs treated with the SOX trio and cultured with serum-free DMEM or the chondrogenic medium was confirmed by immunohistochemistry, whereas those treated with LacZ and cultured with serum-free DMEM or the chondrogenic

medium did not exhibit any immunoreactivity (Figure 4D). As with the human MSCs, the presence of the chondrogenic medium did not cause an increase in mRNA levels of the cartilage marker genes (data not shown).

Influence of different culture systems on the induction of chondrocytic phenotypes by the SOX trio. We next examined the effect of different culture systems on chondrocyte differentiation induced by the SOX trio. Three-dimensional cell–cell interactions and the extracellular matrix are known to influence the differentiation potentials of many cell types. Monolayer culture has been reported to be disadvantageous to chondrocyte differentiation, and therefore, spheroid culture and 3-D culture are preferable (38). If the SOX trio provides signals sufficient for chondrogenesis, it may obviate the need for these specific culture formats. To test this possibility, we compared the expression levels of the cartilage marker genes *COL2A1*, *AGGRECAN*, and *CHONDROMODULIN 1* by human MSCs cultured with serum-free DMEM in monolayer, in spheroids, and in 3-D collagen. Even in monolayer culture, treatment with the SOX trio induced high levels of the cartilage marker genes within 1–2 weeks, and their expression levels increased for up to 3 weeks (data not shown). Peak expression levels of the cartilage marker genes in monolayer culture were comparable to those in spheroid culture. Similar results were obtained with adult human DFs (data not shown).

Levels of expression of the cartilage marker genes by human MSCs and adult human DFs treated with the SOX trio and cultured with serum-free DMEM in 3-D collagen cultures were much higher than those cultured in spheroid or monolayer cultures (data not shown), and there was substantial accumulation of proteoglycan-rich matrix secreted into the collagen gel (data not shown).

Induction of the expression of *SOX5* and *SOX6* in vitro by SOX9. Conditional ablation of *Sox9* was shown to cause a marked down-regulation of *Sox5* and *Sox6* mRNA expression (19), strongly suggesting that *Sox9* is necessary for the expression of *Sox5* and *Sox6*. In our experiments, ES cells, human MSCs, and adult human DFs treated with SOX9 alone started to express low levels of some cartilage marker genes after 2 weeks of culture, suggesting the formation of the SOX trio at a later period (Figures 2 and 4). Taken together, it is likely that SOX9 may induce the expression of *SOX5* and *SOX6*, but the hypothesis has never been directly proven. In our experiment, human MSCs treated with SOX9 alone and cultured with serum-free DMEM in 3-D collagen for 1 week began to express *SOX5* and *SOX6* mRNA, whereas those treated with LacZ and cultured with serum-free DMEM in 3-D collagen did not (Figure 5A). This is the first direct proof that SOX9 induces *SOX5* and *SOX6*. We also demonstrated that *SOX5* and *SOX6* did not induce each other. Similar results were obtained with ES cells and adult human DFs (data not shown). This induction was also seen in monolayer or spheroid culture, but the degree of up-regulation was smaller and took 2–3 weeks (data not shown).

Suppression of hypertrophic and osteogenic markers by the SOX trio. In human MSCs, mRNA for the gene encoding the type X collagen α1 chain (*COL10A1*), a marker for hypertrophic chondrocytes, was up-regulated when it were cultured in the chondrogenic medium in spheroids (39). Levels of mRNA expression of hypertrophic and osteogenic marker genes, such as *COL10A1*, *RUNX2*, *OPN*, and *COL1A1*, were markedly increased in 3-D collagen culture with serum-free DMEM (Figure 5B). Treatment with SOX9 alone failed to suppress these genes except for *COL1A1*, whereas treatment with the SOX trio suppressed all of these genes (Figure 5B). In adult human DFs cultured in 3-D collagen with serum-free DMEM, there was no induction of hypertrophic or osteogenic marker genes, regardless of treatment with the SOX trio (data not shown).

In vivo induction of cartilage-like tissue by the SOX trio. To test whether the SOX trio could influence cartilage formation in vivo, we directly introduced the SOX trio genes in the subcutaneous tissue. Adenoviruses expressing the SOX trio were injected into the subcutaneous tissue lying above the tibia, and 1 week after treatment, the mice were killed, and the tissues were harvested and analyzed histologically and immunohistochemically. The viruses transduced subcutaneous cells efficiently, as shown by the positive staining for LacZ immunoreactivity (Figure 5C). In all 5 mice treated with the SOX trio, chondrocyte-like cells appeared in the area adjacent to the bone. These cells stained positive for Safranin O and type II collagen immunoreactivity (Figure 5D). In contrast, no such cells were seen in the 5 mice that were treated with LacZ.

DISCUSSION

In our screening combinations of factors that are known to be necessary for chondrogenesis, we found that the SOX trio induced chondrocytic phenotypes in totipotent ES cells within 3 days. Previous studies of

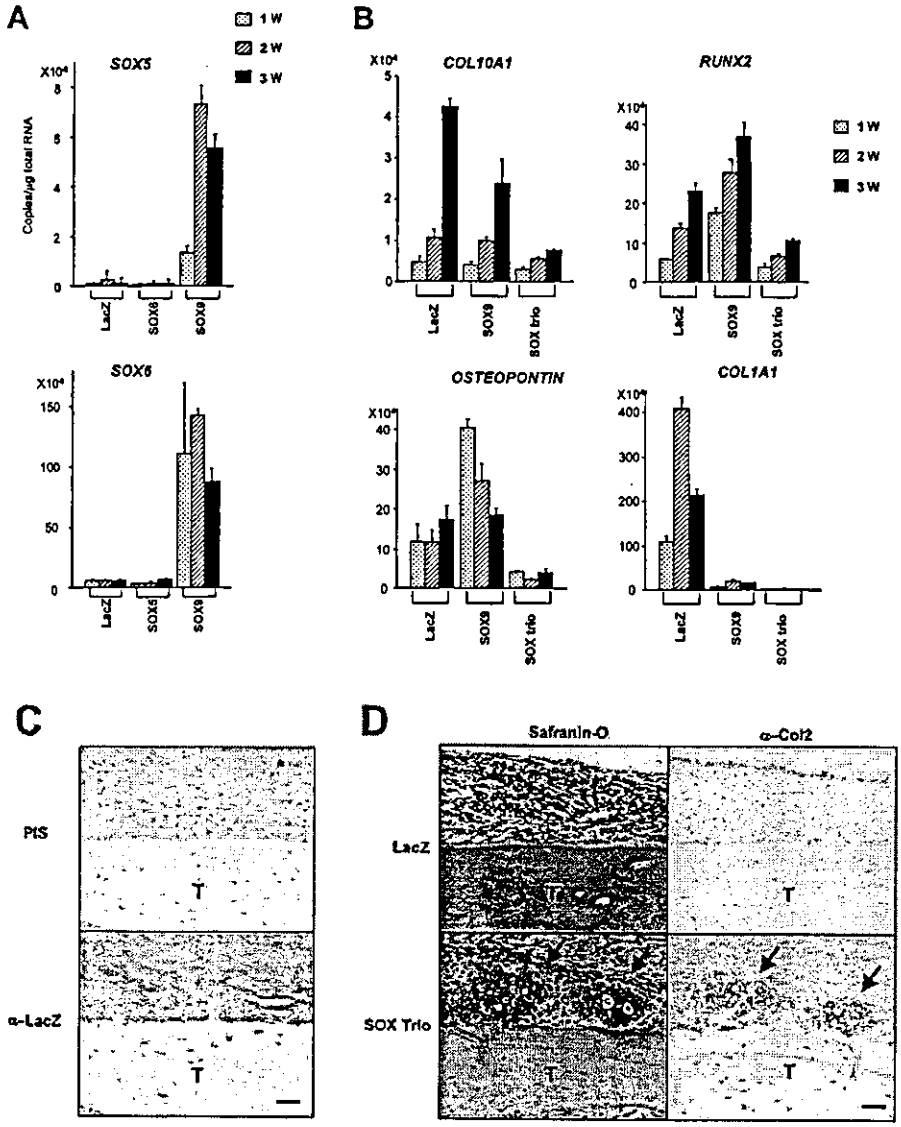

Figure 5. Induction of *Sox5* and *Sox6* expression by SOX9, suppression of hypertrophic and osteogenic differentiation by the SOX trio, and in vivo induction of cartilaginous tissue by the SOX trio. **A**, Levels of mRNA expression of *SOX5* and *SOX6* in human MSCs treated with LacZ, SOX5, SOX6, or SOX9 and cultured in 3-dimensional (3-D) collagen with serum-free Dulbecco's modified Eagle's medium (DMEM) for 1, 2, or 3 weeks, and mRNA expression levels were analyzed by real-time polymerase chain reaction (PCR). **B**, Levels of mRNA expression of the hypertrophic and osteogenic markers *COL10A1*, *RUNX2*, *OSTEOPONTIN*, and *COL1A1* by human MSCs treated with LacZ, SOX9, or the SOX trio and cultured in 3-D collagen with serum-free DMEM for 1, 2, or 3 weeks. Levels of mRNA expression were analyzed by real-time PCR. **C**, Adenoviruses expressing LacZ or the SOX trio were directly injected into the subcutaneous tissue lying above the anteromedial diaphysis of the tibia (T) and the transduction efficiency of adenoviruses was detected by immunohistochemistry for LacZ. Sections were treated with preimmune serum (PIS) or anti-LacZ antibody (α-LacZ). LacZ protein stained brown. Bar = 100 μm. **D**, Production of proteoglycan-rich matrix and induction of type II collagen protein by the SOX trio. Sections were stained with Safranin O and fast green; cartilage (arrows) stained orange. Type II collagen protein (arrows) was detected by immunohistochemistry (brown staining) with anti–type II collagen antibody (α-Col2). Bar = 100 μm.

human MSCs showed that treatment with the chondrogenic supplements TGFβ, BMP-2, or both for 2–3 weeks could induce chondrocytic phenotypes (39,40). In the present study, the SOX trio successfully induced chondrocytic phenotypes in human MSCs cultured in serum-free DMEM containing no supplements. Moreover, human MSCs treated with the SOX trio expressed the cartilage marker genes more rapidly and more potently than did those treated with the conventional chondrogenic method, and their levels of mRNA expression induced by the SOX trio were independent of the presence of TGFβ and BMP-2. These findings raised the possibility that the SOX trio may provide signals sufficient for the induction of chondrogenesis.

We found that the SOX trio induced cartilage-specific genes that did not belong to collagens or proteoglycans: *MATRILIN 3* and *CHONDROMODULIN 1*. Expression of *MATRILIN 3* is highly specific for cartilage (33). Mutations in *MATRILIN 3* cause a type of human chondrodysplasia known as multiple epiphyseal dysplasia, which is characterized by early-onset heritable osteoarthritis (33). Expression of *CHONDROMODULIN 1* is also specific for cartilage. *CHONDROMODULIN 1* stimulates chondrocyte proteoglycan synthesis and inhibits capillary network formation (34,41). The induction of these genes as well as cartilaginous collagens and proteoglycans by the SOX trio further supports the notion that the SOX trio may provide sufficient signals for the induction of chondrogenesis.

A recent study revealed that in vitro chondrogenesis of murine bone marrow–derived MSCs was enhanced by the overexpression of SOX9 (42). Our data with human MSCs partially support this, in that the cartilage marker genes (*COL2A1*, *COL11A2*, and *AGGRECAN*) were induced in human MSCs treated with SOX9 alone. However, the levels of *COL2A1* and *COL11A2* expression were much lower than those induced in human MSCs treated with the SOX trio. In addition, *COL9A1*, *MATRILIN 3*, and *CHONDROMODULIN 1* were only slightly induced by treatment with SOX9 alone. These findings suggest that SOX9 alone is not sufficient for the induction of chondrogenesis and further emphasizes the importance of the SOX trio.

Although treatment with the SOX trio successfully induced mRNA expression of the cartilage marker genes to a level comparable to that in normal cartilage and induced the production of proteoglycan-rich matrix, the addition of the chondrogenic medium containing TGFβ and BMP-2 further increased the accumulation of proteoglycan-rich matrix without increasing the mRNA expression of the cartilage marker genes in both human MSCs and adult human DFs. Thus, TGFβ and BMP-2 may induce other genes that are important for matrix accumulation, or they may be working at the posttranscriptional level. It is noteworthy that in adult human DFs, the chondrogenic medium had no effect on the production of proteoglycan-rich matrix in the absence of treatment with the SOX trio, whereas in human MSCs, the chondrogenic medium had some positive effect in the absence of treatment with the SOX trio. This difference seems to be due to some basal expression of the SOX genes in human MSCs and underscores the important role of the SOX trio in chondrogenesis. The exact mechanism(s) by which TGFβ and BMP-2 increase the accumulation of proteoglycan-rich matrix needs to be further investigated and a gene array analysis performed.

Since human MSCs consist of early mesenchymal progenitors that are already committed to some extent, there is a possibility that the SOX trio may merely be expanding the existing chondroprogenitors by increasing their proliferation or suppressing their cell death, rather than directly inducing chondrocytic phenotypes of non-committed cells. To rule out this possibility, the SOX trio was introduced into cell types other than human MSCs. The SOX trio was able to induce chondrocytic phenotypes in ES cells, which are uncommitted and undifferentiated, as well as in cells belonging to other lineages, such as immortalized cell lines derived from the kidney, liver, and cervix. The SOX trio also successfully induced chondrocytic phenotypes in adult human DFs cultured with serum-free DMEM. Expression levels of the cartilage marker genes induced by the SOX trio in adult human DFs were comparable to those in human MSCs induced by the SOX trio and were also independent of treatment with the chondrogenic medium. These findings strongly suggest that expression of the SOX trio is indeed sufficient for the induction of chondrogenesis.

The SOX trio induced chondrocytic phenotypes in cells cultured in monolayer as effectively as in cells in spheroid culture. Since the monolayer culture is usually disadvantageous for in vitro chondrogenesis and since primary chondrocytes cultured in monolayer quickly lose chondrocytic phenotypes through a process known as dedifferentiation, the conventional in vitro chondrogenic methods invariably use spheroid culture or 3-D culture. It is likely that spheroid culture and 3-D culture may provide some unknown signals that are necessary for chondrogenesis but are not present in monolayer culture. The fact that the SOX trio obviated the use of

spheroid culture further supports the importance of the SOX trio in chondrogenesis. At the same time, it shows the limitation of the SOX trio, since the results did not fully match those obtained with the 3-D culture.

We found that the SOX trio helped to maintain the phenotype of permanent cartilage by suppressing the expression of the marker genes for hypertrophic and osteogenic differentiation, which were induced with the conventional chondrogenic method. This finding may reflect in vivo reciprocal expression patterns of the SOX trio and hypertrophic/osteogenic marker genes (21) and enlargement of the hypertrophic zone in the epiphyseal growth plate of $Sox9^{+/-}$ mice (43). Although the mechanism of the down-regulation is not yet clear, the SOX trio may directly inhibit hypertrophic and osteogenic markers. Alternatively, proteins such as chondromodulin 1 induced by the SOX trio may down-regulate these markers. In either case, inhibition of hypertrophic and osteogenic markers by the SOX trio is compatible with the notion that the SOX trio directly induces chondrocyte differentiation, and this finding is advantageous for tissue engineering of articular, facial, and tracheal cartilage, which needs to remain nonhypertrophic and nonosteogenic.

This is the first study to show that SOX9 induces *SOX5* and *SOX6*. When treated with SOX9, both human MSCs and adult human DFs began to express *SOX5* and *SOX6* at 1 week after transduction. This finding fits the in vivo sequential expression patterns of *SOX5*, *SOX6*, and *SOX9* and is compatible with the previously reported data (19) that $Sox9^{flox/flox}$, *Prx1-Cre*, and *Col2a1-Cre* mice lost the expression of *Sox5* and *Sox6* in cells that lacked SOX9. This finding is also compatible with our observation that overexpression of SOX9 alone up-regulated cartilage marker genes to some extent in HuH-7 cells expressing moderate levels of endogenous *SOX5* and *SOX6*, but not in HeLa cells expressing no endogenous *SOX5* or *SOX6*. These observations further stress the importance of the SOX trio over individual SOXs in the induction of chondrocytic phenotypes. The mechanism of *SOX5* and *SOX6* induction by SOX9 should be further investigated by analyzing human MSCs and adult human DFs treated with SOX9 alone.

When the SOX trio was adenovirally expressed in the subcutaneous tissue, new cartilage formation was induced. Although the adenoviruses infected most of the cells in the injected area, the strongest induction was observed in the area adjacent to the bone, including the periosteum. This finding suggests that despite the strong chondrogenic actions of the SOX trio, there are cells in the periosteal region that are more susceptible to the signal. These cells may represent an enrichment of MSCs in the perichondrium.

In conclusion, the findings of the current study strongly suggest that the SOX trio provides signals that are sufficient for the induction of permanent cartilage in vitro. The potent in vitro chondrogenic system of the SOX trio provides a new in vitro model of chondrogenesis, which may help us to better understand the mechanism of chondrogenesis and to advance cartilage regenerative medicine.

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REFERENCES

1. De Crombrugghe B, Lefebvre V, Nakashima K. Regulatory mechanisms in the pathways of cartilage and bone formation. Curr Opin Cell Biol 2001;13:721–7.
2. Kolettas E, Muir HI, Barrett JC, Hardingham TE. Chondrocyte phenotype and cell survival are regulated by culture conditions and by specific cytokines through the expression of Sox-9 transcription factor. Rheumatology (Oxford) 2001;40:1146–56.
3. Stheneur C, Dumontier MF, Guedes C, Fulchignoni-Lataud MD, Tahiri K, Karensty G, et al. Basic fibroblast growth factor as a selective inducer of matrix Gla protein gene expression in proliferative chondrocytes. Biochem J 2003;369:63–70.
4. Church VL, Francis-West P. Wnt signalling during limb development. Int J Dev Biol 2002;46:927–36.
5. Zehentner BK, Dony C, Burtscher H. The transcription factor Sox9 is involved in BMP-2 signaling. J Bone Miner Res 1999;14:1734–41.
6. Tuli R, Tuli S, Nandi S, Wang ML, Alexander FG, Haleem-Smith H, et al. Transforming growth factor-β-mediated chondrogenesis of human mesenchymal progenitor cells involves N-cadherin and mitogen-activated protein kinase and Wnt signaling cross-talk. J Biol Chem 2003;278:41227–36.
7. Ng LJ, Wheatley S, Muscat GE, Conway-Campbell J, Bowles J, Wright E, et al. SOX9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse. Dev Biol 1997;183:108–21.
8. Zhao Q, Eberspaecher H, Lefebvre V, de Crombrugghe B. Parallel expression of Sox9 and Col2a1 in cells undergoing chondrogenesis. Dev Dyn 1997;209:377–86.
9. Foster JW, Dominguez-Steglich MA, Guioli S, Kowk G, Weller PA, Stevanovic M, et al. Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. Nature 1994;372:525–30.
10. Wagner T, Wirth J, Meyer J, Zabel B, Held M, Zimmer J, et al. Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. Cell 1994;79:1111–20.
11. Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrugghe B. Sox9 is required for cartilage formation. Nat Genet 1999;22:85–9.
12. Bell DM, Leung KK, Wheatley SC, Ng LJ, Zhou S, Ling KW, et al. SOX9 directly regulates the type-II collagen gene. Nat Genet 1997;16:174–8.
13. Bridgewater LC, Lefebvre V, de Crombrugghe B. Chondrocyte-

specific enhancer elements in the Col11a2 gene resemble the Col2a1 tissue-specific enhancer. J Biol Chem 1998;273: 14998–5006.
14. Lefebvre V, Huang W, Harley VR, Goodfellow PN, de Crombrugghe B. SOX9 is a potent activator of the chondrocyte-specific enhancer of the proα(II) collagen gene. Mol Cell Biol 1997;17: 2336–46.
15. Liu Y, Li H, Tanaka K, Tsumaki N, Yamada Y. Identification of an enhancer sequence within the first intron required for cartilage-specific transcription of the α2(XI) collagen gene. J Biol Chem 2000;275:12712–8.
16. Sekiya I, Tsuji K, Koopman P, Watanabe H, Yamada Y, Shinomiya K, et al. SOX9 enhances aggrecan gene promoter/enhancer activity and is up-regulated by retinoic acid in a cartilage-derived cell line, TC6. J Biol Chem 2000;275:10738–44.
17. Xie WF, Zhang X, Sakano S, Lefebvre V, Sandell LJ. Transactivation of the mouse cartilage-derived retinoic acid-sensitive protein gene by Sox9. J Bone Miner Res 1999;14:757–63.
18. Zhang P, Jimenez SA, Stokes DG. Regulation of human COL9A1 gene expression. Activation of the proximal promoter region by SOX9. J Biol Chem 2003;278:117–23.
19. Akiyama H, Chaboissier MC, Martin JF, Schedl A, de Crombrugghe B. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes Dev 2002;16: 2813–28.
20. Smits P, Li P, Mandel J, Deng JM, Behringer RR, de Crombrugghe B, et al. The transcription factors L-Sox5 and Sox6 are essential for cartilage formation. Dev Cell 2001;1:277–90.
21. Lefebvre V, Li P, de Crombrugghe B. A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. EMBO J 1998; 17:5718–33.
22. Miyagishi M, Taira K. RNAi expression vectors in mammalian cells. Methods Mol Biol 2004;252:483–91.
23. Matsumoto M, Ogawa W, Teshigawara K, Inoue H, Miyake K, Sakaue H, et al. Role of the insulin receptor substrate 1 and phosphatidylinositol 3-kinase signaling pathway in insulin-induced expression of sterol regulatory element binding protein 1c and glucokinase genes in rat hepatocytes. Diabetes 2002;51:1672–80.
24. Yamanaka Y, Tanaka H, Koike M, Nishimura R, Seino Y. PTHrP rescues ATDC5 cells from apoptosis induced by FGF receptor 3 mutation. J Bone Miner Res 2003;18:1395–403.
25. Long F, Zhang XM, Karp S, Yang Y, McMahon AP. Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation. Development 2001;128:5099–108.
26. Ruiz i Altaba A. Gli proteins encode context-dependent positive and negative functions: implications for development and disease. Development 1999;126:3205–16.
27. Fujii M, Takeda K, Imamura T, Aoki H, Sampath TK, Enomoto S, et al. Roles of bone morphogenetic protein type I receptors and Smad proteins in osteoblast and chondroblast differentiation. Mol Biol Cell 1999;10:3801–13.
28. Vleminckx K, Kemler R, Hecht A. The C-terminal transactivation domain of β-catenin is necessary and sufficient for signaling by the LEF-1/β-catenin complex in Xenopus laevis. Mech Dev 1999;81: 65–74.
29. Chung UI, Lanske B, Lee K, Li E, Kronenberg H. The parathyroid hormone/parathyroid hormone-related peptide receptor coordinates endochondral bone development by directly controlling chondrocyte differentiation. Proc Natl Acad Sci U S A 1998;95: 13030–5.
30. Robertson EJ. Embryoid-derived stem cell lines. In: Robertson EJ, editor. Teratocarcinomas and embryonic stem cells. 1st ed. Oxford: IRL Press; 1987. p. 71–112.
31. Rossert J, Eberspaecher H, de Crombrugghe B. Separate cis-acting DNA elements of the mouse pro-α1(I) collagen promoter direct expression of reporter genes to different type I collagen-producing cells in transgenic mice. J Cell Biol 1995;129:1421–32.
32. Hoshi K, Komori T, Ozawa H. Morphological characterization of skeletal cells in Cbfa1-deficient mice. Bone 1999;25:639–51.
33. Chapman KL, Mortier GR, Chapman K, Loughlin J, Grant ME, Briggs MD. Mutations in the region encoding the von Willebrand factor A domain of matrilin-3 are associated with multiple epiphyseal dysplasia. Nat Genet 2001;28:393–6.
34. Shukunami C, Hiraki Y. Expression of cartilage-specific functional matrix chondromodulin-I mRNA in rabbit growth plate chondrocytes and its responsiveness to growth stimuli in vitro. Biochem Biophys Res Commun 1998;249:885–90.
35. Nakabayashi H, Taketa K, Miyano K, Yamane T, Sato J. Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. Cancer Res 1982;42:3858–63.
36. Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol 1977;36:59–74.
37. Ikeda T, Zhang J, Chano T, Mabuchi A, Fukuda A, Kawaguchi H, et al. Identification and characterization of the human long form of Sox5 (L-SOX5) gene. Gene 2002;298:59–68.
38. Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 1982;30:215–24.
39. Sekiya I, Vuoristo JT, Larson BL, Prockop DJ. In vitro cartilage formation by adult human stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. Proc Natl Acad Sci U S A 2002;99:4397–402.
40. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143–7.
41. Hiraki Y, Mitsui K, Endo N, Takahashi K, Hayami T, Inoue H, et al. Molecular cloning of human chondromodulin-I, a cartilage-derived growth modulating factor, and its expression in Chinese hamster ovary cells. Eur J Biochem 1999;260:869–78.
42. Tsuchiya H, Kitoh H, Sugiura F, Ishiguro N. Chondrogenesis enhanced by overexpression of sox9 gene in mouse bone marrow-derived mesenchymal stem cells. Biochem Biophys Res Commun 2003;301:338–43.
43. Bi W, Huang W, Whitworth DJ, Deng JM, Zhang Z, Behringer RR, et al. Haploinsufficiency of Sox9 results in defective cartilage primordia and premature skeletal mineralization. Proc Natl Acad Sci U S A 2001;98:6698–703.

変形性膝関節症患者の動作解析
－日常生活動作における関節負荷の特徴－

Knee joint loads during various activities of daily living in the patients with knee osteoarthritis

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Knee loads were evaluated in 7 patients with knee osteoarthritis (OA) during walking, stairs and deep flexion activities. A motion analysis system was used to obtain knee kinematics and kinetics. Eleven healthy volunteers were also analyzed to compare the knee mechanics during the activities. The patients showed reduced the knee flexion moment during stair descending and rising from maximum flexion, while the knee adduction moments were greater than the normal knees in all activities. The different knee joint loads with OA patients should result from several clinical aspects of the patients, such as reduced function of the quadriceps muscle, pain, and the static alignment of the knee.

key words : Osteoarthritis（変形性関節症）
Joint load（関節負荷）
Deep flexion（深屈曲）

はじめに

変形性膝関節症（以下膝 OA）患者の歩行中の関節負荷の特徴として，膝外反モーメント（Knee Adduction Moment）の増大がある。この外反モーメントは下肢アライメント（荷重時 FTA）と強く相関し，膝 OA の病勢をよく反映しすることが知られている[3,4,5]。このように関節負荷の計測は膝 OA の病態を把握するうえで有用であるが，膝 OA 患者についてより負担の大きい階段降下や深屈曲動作中の関節負荷について検討している研究は少ない。膝 OA 患者ではこれらの動作で疼痛を訴えることが多く，臨床症状と関節負荷の関係が注目される。本研究では膝 OA 患者の日常生活動作，とくに 90°以上の深い屈曲動作における関節負荷について検討した。

対象および方法

X 線で腰野分類 2 度以上の変形があり日常生活動作に支障のない膝 OA 患者 7 例（年齢：67－78 歳，平均 71 歳，HSS スコア：79－94 点，平均 87 点，関節可動域：120－155 度，平均 140 度）を対象とした。OA の grade は 2 度および 3 度が 3 例，4 度が 1 例で，全例内側型 OA であった。動作解析装置（Qualysis 社製）および床反力計を用いて，下肢に貼付した 6 つのマーカーの位置と床反力を計測し，4 種類の動作中の膝関節負荷を Inverse Dynamics 法[1]をもちいて計算した。対象動作は，平地歩行，階段昇降および深屈曲からの起立とした。深屈曲からの起立では，被験者は側方に設置した台を指示として，最大屈曲位からの片脚起立を行った（Fig. 1）。対照として健常者 11 名（平均年齢 30 歳）について同様に計測を行い，膝 OA 群と比較検討した。

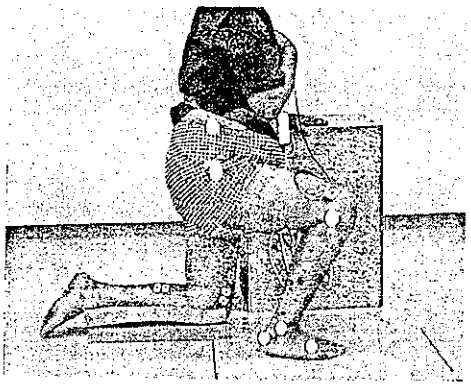

Fig. 1 最大屈曲位からの片脚起立
膝 OA 患者では側方に台を設置し，これを支持として動作を行った。

結　　果

各動作中の膝関節最大屈曲角は，階段上昇を除く 3 つの動作で膝 OA 患者において小さかった（Fig. 2）。膝屈曲モーメントは膝 OA 群で，全ての動作において小さく，階段降下，深屈曲からの起立では有意差を認めた（Fig. 3）。膝外反モーメントは，全ての動作において膝 OA 群で有意に小さかった（Fig. 4）。膝関節外力は膝 OA，コントロールとも階段降下中が最大であり，歩行中の後方，垂直外力および深屈曲からの起立中の後方外力が OA 群において小さかった（Table 1）。

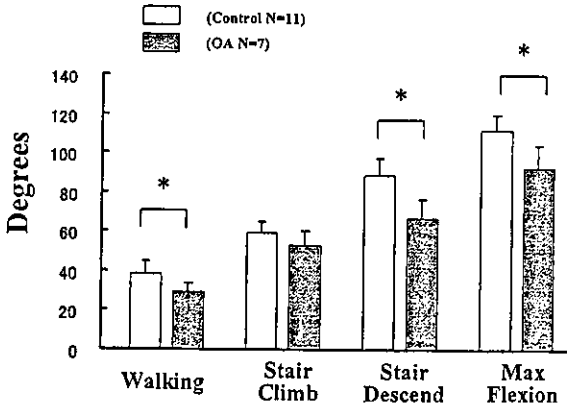

Fig. 2 各動作中の膝関節最大屈曲角度の比較
＊p<0.05

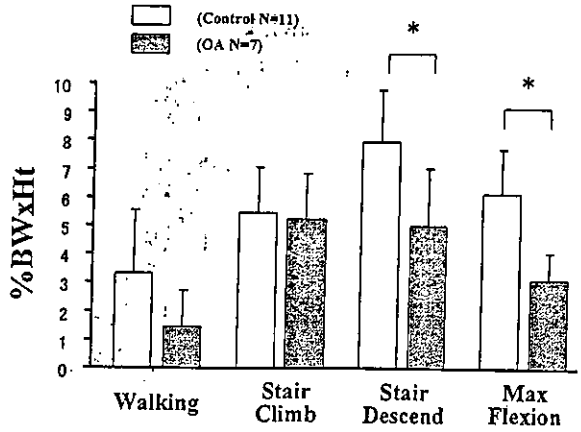

Fig. 3 各動作中の膝関節屈曲モーメントの比較
%BW × Ht ＝％体重×身長。＊p<0.05

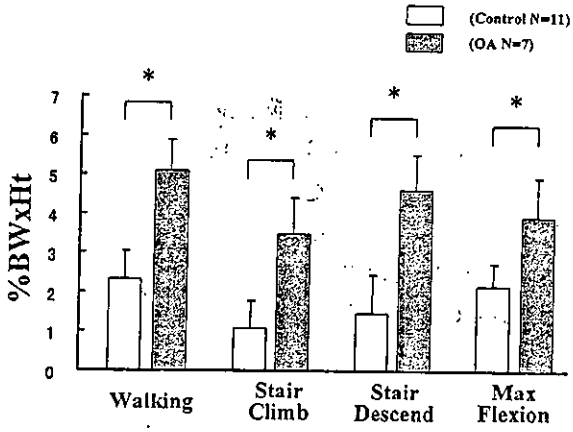

Fig. 4 各動作中の膝関節外反モーメントの比較
%BW × Ht ＝％体重×身長。＊p<0.05

Table 1 各動作中の膝関節外力の比較（単位 ％体重）

		垂直外力	後方外力
歩行	健常群	37.4 (6.2)	112.5 (14.3)
	膝OA群	25.3 (5.0)*	93.4 (5.7)*
階段上昇	健常群	41.6 (7.9)	100.3 (6.4)
	膝OA群	36.5 (5.7)	90.0 (7.8)
階段降下	健常群	60.4 (11.0)	115.6 (16.5)
	膝OA群	50.5 (5.7)	114.4 (15.9)
深屈曲から起立	健常群	47.2 (11.7)	85.5 (18.0)
	膝OA群	28.0 (5.8)*	78.3 (0.2)

＊P<0.05

考　　察

今回の検討で，膝 OA 患者ではさまざまな日常生活動作において健常者と異なる関節負荷のパターンがみられることがわかった。膝屈曲モーメントは膝伸筋（大腿四頭筋）により拮抗されるため，動作中の大腿四頭筋機能の指標と考えられる。また膝屈曲モーメントは，歩行中に疼痛のある患者では減少することが知られている[2,3]。本結果では膝 OA 患者は階段降下や深屈曲などより負荷の大きな動作で屈曲モーメントの低下が顕著であり，これらの動作で四頭筋機能の低下や疼痛の影響がより大きく反映されていたと考えられる。事実，膝スコア（HSS）

と屈曲モーメントの相関は，歩行（R＝0.06）よりも階段降下（R＝0.36）や深屈曲からの起立（R＝0.46）で高くなる傾向を示した。膝OA患者における膝外反モーメントは全ての動作で増大しており，内反変形が関節負荷に影響したものと考えられる[4,5]。立位FTAとの相関は歩行（R＝0.63）で最も高く深屈曲からの起立（R＝0.27）では相関が低かった。これは歩行では立脚期において膝が伸展位をとるため，より下肢の静的アライメントが関節負荷に反映された結果となったと考えられた。

ま と め

1）膝OA患者7名について4種類の日常生活動作中の膝関節負荷について評価した。

2）膝OA患者では，階段降下や深屈曲からの起立などより負荷の大きい動作で膝屈曲モーメントが減少していた。また膝外反モーメントが全ての動作で増大していた。

3）膝OA患者における関節負荷は，大腿四頭筋機能の低下，運動時の疼痛や下肢の内反変形を反映していると考えられた。

文　　献

1) Andriacchi T.P., Galante J.O., et al.: The influence of total knee-replacement design on walking and stair-climbing. J Bone Joint Surg Am., 64: 1328-1335, 1982.
2) Goh J.C., Bose K., et al.: Gait analysis study on patients with varus osteoarthrosis of the knee. Clin Orthop., 294: 223-231, 1993.
3) Hurwitz D.E., Ryals A.R., et al.: Knee pain and joint loading in subjects with osteoarthritis of the knee. J Orthop Res., 18: 572-579, 2000.
4) Hurwitz De., Ryals A.B., et al.: The knee adduction moment during gait in subjects with knee osteoarthritis is more closely correlated with static alignment than radiographic disease severity, toe out angle and pain. J Orthop Res., 20: 101-107, 2002.
5) Sharma L., Hurwitz D.E., et al.: Knee adduction moment, serum hyaluronan level, and disease severity in medial tibiofemoral osteoarthritis. Arthritis Rheum., 41: 1233-1240, 1998.

膝・29巻2号・123～126・2004年

変形性膝関節症患者における歩行時膝関節内反角度の変化
－動的FTA評価の試み－

Changes in varus angle during gait in patients with knee osteoarthritis
－Evaluation of dynamic femoro-tibial angle－

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Clinical evaluation of knee osteoarthritis (OA) is usually on X-ray, while the patients experience pain during the motions. And the varus deformity of the knee is also diagnosed by femoro-tibial angle (FTA) under weight bearing. This study was designed to measure the dynamic changes in varus angle of the knee during walking with knee OA patients, using skin marker besed 3D motion analysis system.

Thirty seven medial OA knees in 25 patients and 24 knees in 12 volunteers were tested at the gait laboratory. The varus angles of the knee defined by skin markers at heel strike were 180.0 ±2.4, 185.2±3.5, 188.8±4.0 degrees for normal group, moderate OA group, severe OA group, respectively. The angles increased by 0.4±1.1, 1.9±1.6, 3.1±1.6 degrees in stance phase, respectively ($p<0.05$).

This study showed that the varus angle and the changes of the varus angle increased as the grade of the knee OA advanced. An analysis of knee kinematics using skin markers was thought to be a useful tool to evaluate dynamic deformity on coronal plane in OA knees.

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key words : Knee osteoarthritis(変形性膝関節症)
Motion analysis (動作解析)
Femoro-tibial angle(大腿骨脛骨角)

はじめに

本邦における変形性膝関節症（以下OA）は内反膝を呈するものが多いが，通常その評価には単純レントゲンによる大腿骨脛骨角(以下FTA)が指標とされる。一方，OA患者は歩行・立ち上がりなどの動作時に疼痛を訴えることが多いが，その際の膝関節内反角がどの程度変化しているかは不明である。これまで膝動作時の動態解析はfluoroscopyによる報告が多い[2,3,4]が，本法は側方入射のため内外反の評価は難しい。今回われわれは三次元動作解析装置を用いて，内側型OA患者の歩行中の下肢アライメントの変化を三次元的に計測し，歩行中に生じる膝関節内反角の変化とX線による病期，臨床症状との関係を解析したので報告する。

対象および方法

X線で腰野分類2度以上の変形があり補助具

を用いずに歩行可能な内側型OA患者25例37膝（男性6膝，女性31膝，67～81歳，平均72.4歳）を対象とした。OAのgradeはgrade 2が18膝，grade 3が15膝，grade 4が4膝であった。Hospital for Special Surgery score（以下HSSスコア）はそれぞれ87.6±6.1点，79.2±6.3点，74.2±7.8点であった。対照として膝に愁訴のない健常者12例24膝（男性12膝，女性12膝，平均21歳）についても同様に計測した。

下肢に6つのマーカー（腸骨稜，大転子，膝関節外側，足関節外果，踵外側，第5中足骨骨頭）を貼付し，10mの平地歩行を4台の特殊カメラ（Qualysis社製，120Hz），床反力計（Bertec社製）を用いて計測した[6]。

得られたデータより，前額面上で大転子，膝外側，足関節外果のマーカーで成す角を‘膝関節内反角’（Fig. 1），大転子，膝外側，足関節外果の3点で成す平面と進行方向の成す角を‘下肢外旋角’（Fig. 2）と定義し，各歩行相におけるこれらの変化を分析した。ここで言う進行方向とは，歩行路の方向であり動作解析装置におけるY座標を指す。

Fig. 3に示すように膝関節内反角は膝屈曲角度の変化に影響を受けるため，立脚期において膝屈曲角度が近似する2点において膝関節内反角の計測を行った。接地後，膝が屈曲し伸展すること（double knee action）に着目し，屈曲角度の極小値を与える時点と接地時において検討した。検討項目は，(1) 立位膝関節内反角と立位FTAとの関係，(2) 接地時，立脚期（Fig. 3）の膝関節内反角の差，(3) 接地時，立脚期における膝屈曲角度及び膝外旋角度である。

上記の検討項目についてレントゲン評価でgrade 2を中等度OA群（n＝18），grade 3，4を高度OA群（n＝19）とし各群間で比較検討した。統計には分散分析一元配置を用い，$p<0.05$で有意差ありとし，相関についてはPearsonの相関係数を用いた。

結　　果

1）立位膝関節内反角と立位FTAとの関係：

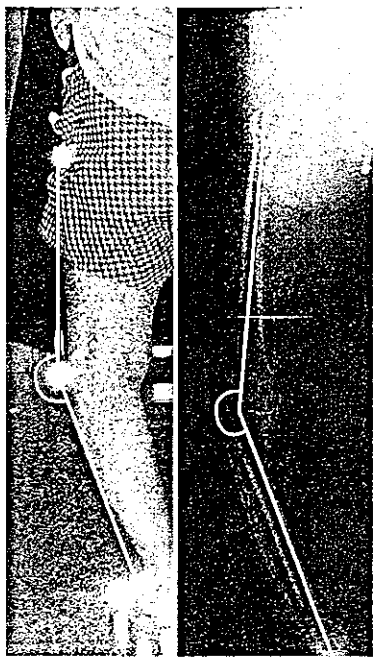

Fig. 1 立位膝関節内反角と荷重位FTA

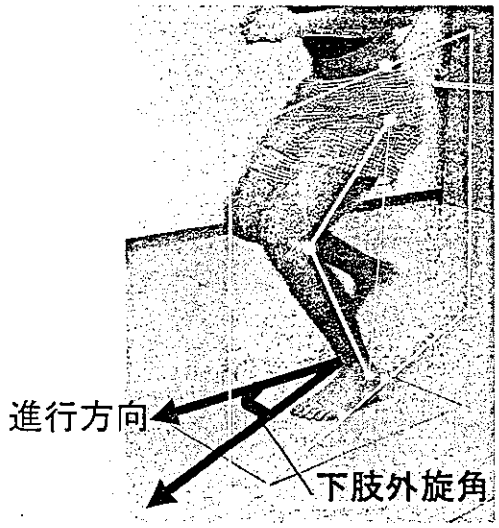

Fig. 2 “下肢外旋角”の定義
マーカー3点で成す角と進行方向の成す角を下肢外旋角と定義した。

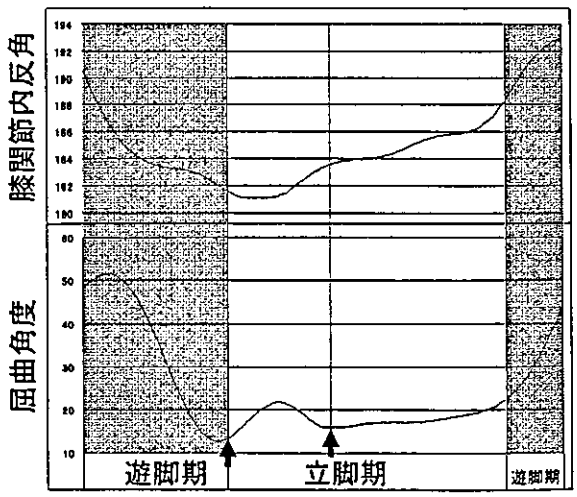

Fig. 3 計測した2点（矢印）
屈曲角度がほぼ等しくなる2点（接地時，立脚期）

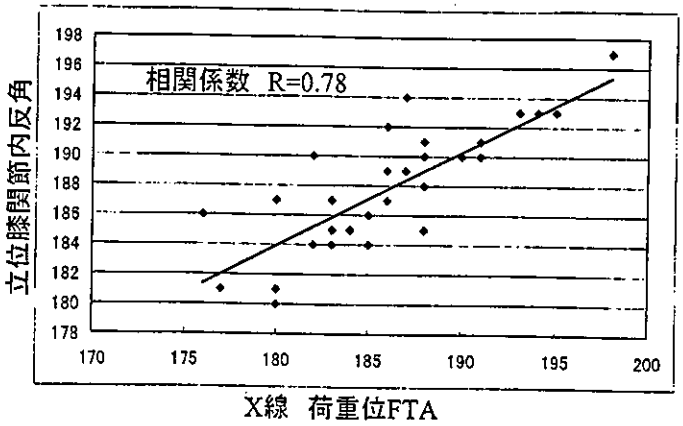

Fig. 4 荷重位 FTA と立位膝関節内反角の関係
相関係数R＝0.78と高い相関を認めた。

Table 1 歩行時膝関節内反角の変化量

膝関節内反角(°)	接地時	立脚期	変化量	
健常膝	180.0±2.4	180.4±2.3	0.4±1.1	p<0.001
中等度OA	185.2±3.5	187.1±3.3	1.9±1.6	p<0.05
高度OA	188.8±4.0	191.9±4.0	3.1±1.6	

マーカーによる立位膝関節内反角は立位 FTA と相関係数 0.78 と高い相関を認めた（Fig. 4）。

2）接地時，立脚期の膝関節内反角度の差：接地時と立脚期の膝関節内反角度の差は各群間で有意差を認めた(Table 1)。すなわち OA が高度なほど接地時の膝内反角度は大きく，立脚期には，さらにその増加量が大きかった。また膝関節内反角の変化量と HSS スコアの相関係数は－0.48であった。

3）接地時，立脚期における膝屈曲角度及び膝外旋角度：膝屈曲角度は接地時・立脚期おいて中等度 OA 群でそれぞれ 8.3 ± 5.9 度・12.7 ± 6.6 度，高度 OA 群では 10.2 ± 5.8 度・14.6 ± 8.3 度であった。また下肢外旋角は中等度 OA 群で 20.2 ± 12.5 度から 21.8 ± 10.1 度，高度 OA 群で 35.3 ± 12.5 度から 38.8 ± 9.2 度であり，いずれも高度 OA 群で大きかったが，接地時・立脚期の間に各群で有意差を認めなかった。

考　　察

膝関節の内反変形は多くの場合回旋を含む三次元的な変形である。今回検討した膝内反角はこのうち前額面における変化を評価したものである。したがって，歩行時膝関節内反角の変化については膝関節屈曲角度，脛骨の回旋角度，側方動揺性（lateral thrust）などの影響を考慮する必要がある。Koshino ら[9]は健常膝において屈曲角度が 1 度大きくなると FTA が計測上約0.1度小さくなると報告した。すなわち屈曲拘縮などにより屈曲角度が大きくなると見かけ上の内反角が変化する可能性がある。そこで，今回の検討では屈曲角度の違いによる影響をなるべく小さくなるように，接地時と荷重時のうち最も屈曲角度が小さくかつ近似した 2 点において検討した（Fig. 3）。その結果 OA の X 線上の病期が進行すると膝関節内反角度ばかりでなく，荷重による内反角の増加量も大きくなることが明らかとなった。臨床症状を反映する HSS スコアと相関係数－0.48 であるため，歩行時の下肢内反角の変化量は OA の病態を dynamic に評価する一つの指標となりえると考えられた。

本研究では大転子，膝外側，足関節外果に貼付したマーカーにより膝関節内反角を計測したに過ぎない。本システムでは skin motion による誤差を有する。しかし本法は動作を拘束することなく三次元の動態解析を行うことができる利点を有する。特に前額面における OA 膝の動的変化を捉えた研究は少ない[7]。Lateral thrust を含めた OA 膝における関節動態は依然不明な点が多く，これらの非生理的な関節動態は OA の病態，進行に深く関与していると考えられる。

近年の計測手法の発展により，多数の皮膚マーカーを用いてより詳細な計測が可能となっている[1]。今後，これらの手法の応用により OA のより詳細な動態解析を行いその有効性を検証したいと考えている。

結　　語

1）三次元動作解析装置を用いて OA 患者の歩行中の前額面での下肢アライメントを計測した。

2）OA の病期が進行すると膝関節内反角度ばかりでなく荷重による内反角の増加量も大きくなった。

3）OA の病態評価には，三次元動作解析は有用である。

文　　献

1) Andriacchi T.P., Alexander E.J., Toney M.K., et al.: A point cluster method for in vivo motion analysis.: applied to a study of knee kinematics. J Biomech Eng., 120: 743-749, 1998.

2) Banks S., Bellemans J., Nozaki H., et al.: Knee motions during maximum flexion and mobile-bearing arthroplasties. Clin Orthop., 410: 131-138, 2003.
3) Bellemans J., Banks S., Victor J., et al.: Fluoroscopic analysis of the kinamatics of deep flexion in total knee arthroplasty. J Bone Joint Surg., 84-B: 50-53, 2002.
4) Komistek D., Dennis A., Mahfouz M.: In vivo fluorpscopic analysis of the normal human knee. Clin Orthop., 410: 69-81, 2003.
5) Koshino T., Takayana M., Jiang L.S., et al.: Underestimation of varus angulation in knees with flexion deformity. Knee, 9: 275-279, 2002.
6) 名倉　武雄，松本　秀男，大谷　俊郎ほか：変形性膝関節症患者の動作解析・日常生活動作における関節負荷の特徴．膝，28: 14-16, 2003.
7) Ogata K., Yasunaga M., Nomiyama H.: The effect of insole on the thrust of osteoarthritic knees. Int Orthop., 21: 308-312, 1997.

特集II　Osteoimmunologyに関する最近の進歩と治療への応用

軟骨細胞保護におけるIL-4の役割*

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Key Words：chondrocyte, mechanical stress, interleukin-4, osteoarthritis

はじめに

関節軟骨は唯一の基質産生細胞である軟骨細胞によって維持される結合組織であり，神経，血管，リンパ管を欠くため，軟骨細胞は関節液によって栄養を受ける．軟骨細胞が正常な代謝を営むためには，関節運動による適度な力学的負荷がかかることが必須であり，これには軟骨下の骨構造の適合性，関節液を介した関節潤滑はもちろん，筋腱あるいは靱帯のバランスによって関節が安定している必要がある．これらの条件の質的・構造的破綻は，軟骨細胞の代謝異常を招来し，変形性関節症(OA)に代表される関節炎症，軟骨破壊へと進展していく．

一方，IL-4はB細胞，T細胞，肥満細胞，マクロファージといった主に免疫，アレルギーに関与する細胞に働く多面性をもったサイトカインである．近年，軟骨細胞は正常状態でIL-4を発現，産生しており[1]，膜表面のインテグリンを介したメカニカルストレスに対する反応に関与することがわかってきた．本稿では，軟骨細胞におけるメカニカルストレス，炎症性サイトカインに対するIL-4の効果について概説する．

メカニカルストレスに対する細胞の応答とIL-4の役割

われわれはエジンバラ大学との共同研究で，Dish上で単層培養したヒト軟骨細胞に，0.33Hz，16kPaの伸張負荷をかけると約20分間で細胞膜の過分極が生じること，これが細胞膜上の$\alpha_5\beta_1$インテグリンを介したものであることを報告した[2]．さらにこの膜電位の変化はneomycin, flunarizine, N-6(aminohexyl)-5-chloro-1-naphthalene sulphonamide, genistein, staurosporine, cytochalasin Dによって阻害されることから，それぞれphospholipase C，イノシトール3リン酸を介するCa^{2+}遊離，カルモデュリン，チロシンキナーゼ，protein kinase C，細胞骨格が関与する反応であることがわかった．さらに刺激後1分でpaxillin, β-cathenin, pp125FAKなどがチロシンリン酸化を受けること，このチロシンリン酸化はRGD配列を含むオリゴ蛋白で阻害されることから，$\alpha_5\beta_1$インテグリンを介した機械的刺激に細胞骨格関連蛋白を介するシグナル伝達が必須であることも判明した．興味深いことに，刺激後の培養液を無刺激の細胞に添加することで，やはり細胞膜の過分極が誘導されることから，なん

* The role of IL-4 in regulation of chondrocyte metabolism.

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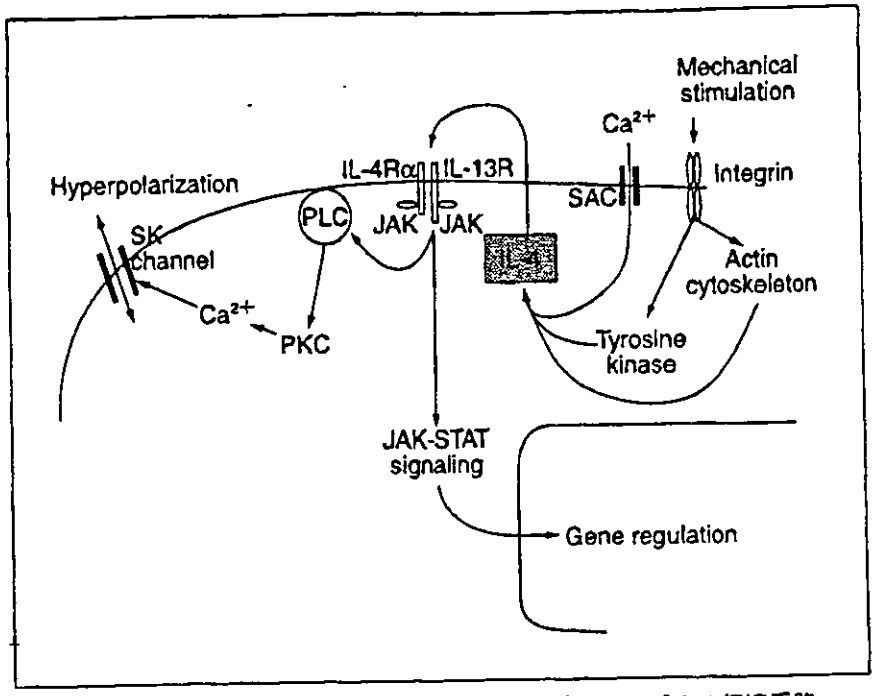

図1　IL-4を介する軟骨細胞の機械的刺激に対するシグナル伝達系路
SAC；stretch-activated ion channels, PLC；phospholipase C, PKC；protein kinase C, SK channel；small conductance calcium-activated potassium channel, IL-4Rα；IL-4 receptor α subunit, IL-13R；IL-13 receptor subunit　（文献[5]より引用）

らかの液性因子の分泌がこのカスケードに関与することが示唆され，抗IL-4抗体あるいは抗IL-4レセプター抗体が一連の細胞応答を阻害することから，メカノレセプターを介するシグナル伝達には軟骨細胞自身が分泌するIL-4が関与することが明らかとなった[3)4)]（図1）．したがって細胞内に蓄積されたIL-4の分泌，産生が負荷に追い付かない場合には，シグナル伝達機構が破綻する可能性があると思われる．

変形性関節症軟骨細胞のIL-4に対する応答

加齢，異常なメカニカルストレス，遺伝的素因は軟骨変性の重要なrisk factorである．異常なメカニカルストレスは，関節の形態，外傷，体重増加，職業，生活様式などの因子によってひき起こされ，OAの原因あるいは悪化因子となる．われわれの経験からは，ラットにおいては内側側副靱帯切離，内側半月板切除を加えると，軟骨破壊は4～6週で急速に進行することからも，加齢とは関係なく，程度によってはメカニカルストレスのみでOA病態の再現が可能であることを示す．一方，OAの進展には軟骨細胞自身からも産生される種々の炎症性サイトカインも関与し，MMPs（matrix metalloproteinases）やcathepsinをはじめとする蛋白分解酵素による基質破壊が進行する．上述のように，軟骨細胞は細胞外基質との接着によって機械的刺激を感知するため，主としてMMPsによりプロテオグリカンやII型コラーゲンをはじめとするコラーゲンの分解は細胞接着やシグナル伝達を阻害し，ひいては軟骨細胞の応答，代謝に影響を与える．したがってOA軟骨細胞は絶えず異常なメカニカルストレスにさらされることになり，細胞の分化度，応答の変化が生じている．上述の0.33Hz，16kPaの伸張ストレスにおいても，OA軟骨細胞では細胞膜は逆に脱分極を示すこと，シグナル伝達にアクチンやPKC，SKチャンネルを介さないこと，IL-4レセプターは正常軟骨細胞ではType II（IL-4Rα/IL-13R）であるのに対し，OA軟骨細胞ではType I（IL-4Rα/cγ）であること，正常軟骨細胞において生じるアグリカン発現亢進およびMMP-3発現低下が生じないことなどが判明しており[4)5)]，OA軟骨細胞では正常とは異なるシグナル伝達のカスケードが働いていると考えられる．