Membrane-bound transferrin-like protein (MTf) is expressed in parallel with the expression of cartilage-characteristic genes during differentiation of chondrocytes, and the MTf level is much higher in cartilage than in other tissues. To investigate the role of MTf in cartilage, we examined the effects of growth factors on MTf expression in mouse prechondrogenic ATDC5 cells and the effect of MTf overexpression on differentiation of ATDC5 and mouse pluripotent mesenchymal C3H10T1/2 cells. In ATDC5 cultures, bone morphogenetic protein-2 and transforming growth factor-β as well as insulin induced MTf mRNA expression when these peptides induced chondrogenic differentiation. Forced expression of rabbit MTf in ATDC5 cells induced aggrecan, type II collagen, matrilin-1, type X collagen mRNAs, and cell-shape changes from fibroblastic cells to spherical chondrocytes. Accordingly, the synthesis and accumulation of proteoglycans were higher in MTf-expressing cultures than in control cultures. These effects of MTf overexpression correlated with the MTf protein level on the cell surface and decreased in the presence of anti-MTf antibody. However, the aggrecan mRNA level in the ATDC5 cells overexpressing MTf was lower than that in wild type ATDC5 cells exposed to 10 μg/ml insulin. MTf overexpression in C3H10T1/2 cells also induced aggrecan and/or type II collagen mRNA but not the spherical phenotype. These findings suggest that the expression of MTf on the cell surface facilitates the differentiation of prechondrogenic cells, although MTf overexpression alone seems to be insufficient to commit pluripotent mesenchymal cells to the chondrocyte lineage.

Membrane-bound transferrin-like protein (MTf) was identified originally as a human tumor-associated antigen of 97 kDa (1–3). MTf is linked to the outer surface of the plasma membrane via glycosylphosphatidylinositol (GPI) anchor (4). MTf is present at high or moderate levels in melanomas and some other tumors (5, 6). In addition, it is expressed in many fetal tissues including the intestine and several adult tissues including salivary glands, sweat glands, eosinophils, brain capillaries, and cartilage (7–11). MTf may be involved in iron metabolism, proliferation, or differentiation in these tissues or pathological responses in certain tissues such as plaques of Alzheimer disease (12). However, the role of MTf remains unknown.

Recently we purified MTf from the plasma membrane of rabbit chondrocytes as a 76-kDa concanavalin A-binding “chondrocyte-specific” protein (7). We found that the MTf level is much higher (100-fold) in the cartilage of young rabbits and adult mice than in the other tested tissues including the intestine, liver, and brain (7, 13). Furthermore, in cultures of ATDC5 cells, MTf is expressed developmentally in parallel with the expression of type II collagen and aggrecan, in a pattern commensurate with the onset of chondrogenesis to form cartilage nodules. In rabbit chondrocyte cultures, MTf expression is down-regulated by retinoic acid or passage of the culture, when these treatments suppressed chondrocyte phenotypic expression. These observations suggested that MTf is involved in chondrogenic differentiation.

ATDC5 cells maintain an undifferentiated state or the properties of chondroprogenitor cells in the presence of 5% serum in the absence of added growth factors. However, insulin (10 μg/ml) or insulin growth factor I (IGF-I) (300 ng/ml) induces chondrogenic differentiation of the cells through a cellular condensation process, resulting in the formation of cartilage nodules and the expression of type II collagen and aggrecan mRNAs (14, 15). Bone morphogenic protein (BMP)-2 or transforming growth factor-β (TGF-β) also up-regulates the expression of type II collagen mRNA in these cells (16, 17). ATDC5 cells synthesize fibroblast growth factor, parathyroid hormone-related protein, BMP, TGF-β, and other signaling molecules, each with a unique temporal pattern (15, 16). The differentiation process in ATDC5 cultures is similar to that in cartilage in vivo, and thus ATDC5 cells have been used extensively for studies on chondrogenic differentiation. On the other hand, a mouse mesenchymal cell line C3H10T1/2 has been used for studies on differentiation of mesenchymal cells to adipocytes, osteoblasts, or chondrocytes. In C3H10T1/2 cells, BMP-2 or TGF-β induces osteogenic, adipogenic, or chondrogenic differentiation (18, 19).

We show here that BMP-2 and TGF-β, as well as insulin, induce MTf expression in ATDC5 cells. Furthermore, forced expression of MTf enhanced the expression of aggrecan and/or type II collagen in ATDC5 and C3H10T1/2 cells. MTf seems to be an important target for these growth factors and
MTf Induces Chondrogenic Differentiation

EXPERIMENTAL PROCEDURES

Cell Cultures—ATDC5 cells were obtained from RIKEN cell bank (Tsukuba, Japan). Cells were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium (DMEM) (Nissui Pharmaceutical, Japan) and Ham’s F-12 medium (Nissui Pharmaceutical, Japan) supplemented with 5% fetal bovine serum (Invitrogen), 10 μg/ml human transferrin (Roche Molecular Biochemicals), and 3 × 10−6 m sodium selenite (Sigma) in the presence or absence (Medium A) of 10 μg/ml bovine insulin (Sigma). Insulin density of the medium was 4 × 106 cells/16-mm dish, 8 × 106 cells/35-mm dish, or 20 × 106 cells/10-cm dish. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO2 in air. The medium was replaced every other day.

ChS0/1T1/2 cells were obtained from RIKEN cell bank and maintained in α-modified essential medium (Sanko Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO2 in air. The medium was replaced every other day.

Chondrocytes were isolated from the rib rest cartilage or growth plates of 4-week-old male Japanese white rabbits, as described previously (20). Cells were seeded at 105 cells/10-cm dish and maintained in α-modified essential medium supplemented with 10% fetal bovine serum, 50 μg/ml ascorbic acid, 32 unit/ml penicillin, 60 μg/ml kanamycin (Meiji Seika Co., Tokyo, Japan), and 250 ng/ml amphotericin B (Dainippon Pharmaceutical Co., Osaka, Japan) at 37 °C in a humidified atmosphere of 5% CO2 in air.

Transfection—For construction of a rabbit MTf expression vector, rabbit MTf cDNA (full-length) was subcloned into pC3DNA3.1Zeot (+) (Invitrogen). MTf was expressed under the control of cytomegalovirus (C. Meiji Seika Co., Tokyo, Japan). Cells were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 μg/ml human transferrin, 50 μM sodium selenite (Sigma), and 250 ng/ml amphotericin B (Dainippon Pharmaceutical Co.) and washed several times with TTBS buffer, as described previously (14). Inoculum density of the cells was 106 cells/10-cm dish and maintained in a humidified atmosphere of 5% CO2 in air. The growth rate was estimated using CellTiter 96 Aqueous One Solution cell proliferation assay (Promega, Madison, WI) for 18, 23, 22, and 22 cycles for aggrecan, type II collagen, type X collagen, and Sox9, respectively. The cycles were optimal for the comparison between the amplified products. For normalization of the RNA loading, RT-PCR of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also performed in each RT-PCR reaction as an internal control (18 cycles). The pairs of oligonucleotides, 5′-TG-CTACTTCATGCAGCCCAT-3′ and 5′-AAAGACCTCACCTCCTCATCT-3′ for aggrecan; 5′-TACGCTTGTCAAGGCCAGATCTG-3′ and 5′-GGTCACCAGCAGTACCTCTG-3′ for type II collagen; 5′-CGTGGATT-ACGTGAGGAGCTCAATG-3′ and 5′-GGCTCTCTCCTCCACACAGA-T-3′ for matrilin-1; 5′-CAGGAAAAAAGTCGACGAG-3′ and 5′-ACCC-TGAGGACATGGAC-3′ for type X collagen; and 5′-ACCTCCAAACCGCGCTT-3′ and 5′-CGTGGATTACGTGAGGAGCTCAATG-3′ for GAPDH were used as primers for the PCR. The PCR products were separated on 1% agarose gels and transferred to Nytran membranes (Schleicher & Schuell). The membranes were hybridized with 32P-labeled mouse aggrecan cDNA, 32P-labeled mouse type II collagen cDNA, 32P-labeled matrilin-1 cDNA, 32P-labeled mouse type X collagen cDNA, 32P-labeled mouse Sox5 cDNA, and 32P-labeled mouse GAPDH cDNA as described previously (13). The membranes were exposed to BioMax x-ray film at −80 °C with an intensifying screen.

Quantitative Real Time PCR Analysis—The quantitative real time PCR analysis was performed using an ABI PRISM 7700 sequence detection system instrument and software (PerkinElmer Life Sciences). The first-strand cDNA prepared by RT-PCR reaction was amplified using 5′-GGAGCTTGCATTGCTCTT-3′ and 5′-GTCTTCTTGAGTACGACA-3′ for mouse MTf. The amplified cDNA was quantified using 6FAM-TCTGGTGTCCAGGGCAACTCCG-TAMRA for mouse MTf. For an internal control, mouse β-actin cDNA was amplified using 5′-AAGGATGCTCCTGCTCAGG-3′ and 5′-CAAGAAGAGGACTTGG-3′ for GAPDH. The amplified cDNA was quantified using VIC-CAACAGCGGTCTCGAT-GCC-TAMRA for mouse β-actin.

[S]Sulfate Incorporation and DNA Content—Cells were maintained in 0.5 ml of Medium A in 16-mm dishes for 16 days. The cultures were exposed for 6 h to 5 μM [35S]sulfate in Medium A. Proteoglycan synthesis was monitored by measuring the incorporation of [35S]sulfate into material precipitated with cetylpyridinium chloride after treatment with Pronase E (protease type XIV; Sigma) as described previously (23). DNA content was determined using a fluorescent DNA quantitation kit (Bio-Rad).

Toluidine Blue Staining—Cell layers were fixed with ethanol for 20 min and washed twice with PBS and stained with 0.5% toluidine blue solution for 20 min and then washed five times with PBS. The cell layers were washed with 50% ethanol and then with 30% ethanol and were washed five times with PBS and dried up.

Determination of Uronic Acid—Cells were maintained in Medium A in the 16-mm dishes for 25 days. After the further rinsing, the cell layers were washed with PBS and then incubated at 37 °C for 1 h with 2 mg/ml Pronase E (protease type XIV; Sigma) in 0.2 ml Tris-HCl buffer, pH 7.8, containing 5 mM CaCl2. Uronic acid was determined by the method of Bitter and Muir (24).

Proliferation Assay—The growth rate was estimated using CellTiter 96 Aqueous One Solution cell proliferation assay (Promega, Madison, WI).

Statistics—Experiments were performed at least in triplicate and expressed as mean ± S.D. The student’s t test was used for statistical analysis.

RESULTS

Effects of BMP-2, TGF-β, and Insulin/IGF-I on MTf mRNA Levels in ATDC5 Cells—To examine a relationship between the MTf level and the degree of chondrogenic differentiation, ATDC5 cells were exposed to BMP-2 or TGF-β in the medium.
IGF-I receptors. The incubation with BMP-2 or TGF-β/H9252
stimulated MTf expression in repeated studies. Similar results were obtained in repeated
mRNA expression of Sox9 was determined by real time PCR. D, wild type ATDC5 cells were exposed to BMP-2 (1 μg/ml) for 0, 6, 12, or 24 h. The MTf mRNA expression was examined by RT-PCR-Southern blot analysis. E, the mRNA expression of Sox9 was determined by RT-PCR-Southern blot analysis. Similar results were obtained in repeated studies.

FIG. 1. Effects of BMP-2, TGF-β, and insulin on MTf mRNA levels in ATDC5 cells. Wild type ATDC5 cells were seeded in 35-mm dishes and maintained for 5 days. The confluent cultures were incubated in DMEM/Ham’s F12 (1:1) medium containing 5% fetal bovine serum (A–E) or 0.5% fetal bovine serum (E) in the absence or presence of 10 μg/ml insulin (ins) and in the presence of BMP-2 (1 μg/ml) or TGF-β1 (5 ng/ml) for 4 days. A, phase-contrast microphotographs were taken on day 9. B, the mRNA expression of aggrecan and type II collagen was examined by RT-PCR-Southern blot analysis. C, the fold increase of MTf mRNA was determined by real time PCR. D, wild type ATDC5 cells were exposed to BMP-2 (1 μg/ml) for 0, 6, 12, or 24 h. The MTf mRNA expression was examined by RT-PCR-Southern blot analysis. E, the mRNA expression of Sox9 was determined by RT-PCR-Southern blot analysis. Similar results were obtained in repeated studies.

containing 5% fetal bovine serum in the presence or absence of insulin at 10 μg/ml. At a high concentration, insulin activates IGF-I receptors. The incubation with BMP-2 or TGF-β for 4 days increased the number of spherical or polygonal cells, and this effect was increased further in the presence of insulin (Fig. 1A). BMP-2 also increased the expression of aggrecan and type II collagen, and this effect was increased in the presence of insulin (Fig. 1B). TGF-β alone had little effect on the expression of aggrecan or type II collagen. However, TGF-β enhanced the expression of aggrecan and type II collagen in the presence of insulin.

In these cells, BMP-2 and TGF-β increased the MTf mRNA level within 4 days 25- to 30-fold and 3- to 5-fold, respectively, and insulin increased the effect of TGF-β (12-fold) (Fig. 1C). Insulin alone increased the MTf mRNA level to a lesser extent. Insulin increased the MTf mRNA level markedly only 6 days after adding the hormone (13). BMP-2 had the greatest effect on MTf expression. Insulin partly suppressed the BMP-2-dependent MTf expression in repeated studies (Fig. 1C) (data not shown). The high MTf level in BMP plus insulin-exposed cells may be sufficient for chondrogenic differentiation. The BMP-2 stimulation of MTf mRNA expression could be observed within 24 h (Fig. 1D). The degree of MTf expression correlated roughly with that of chondrogenic differentiation, except that insulin suppressed the BMP-induced MTf expression.

In the medium containing 5% serum, insulin, BMP-2, or TGF-β had a marginal effect on the Sox9 mRNA level because of a high basal Sox9 level (Fig. 1E). However, at a low serum concentration (0.5%), insulin, BMP-2, or TGF-β increased this level 2- to 10-fold. The 5% serum-enhanced expression of Sox9 alone was insufficient to induce chondrogenic differentiation in ATDC5 cultures; no differentiation was observed in the presence of 5% serum in the absence of added growth factors.

Effects of Forced Expression of MTf on Chondrogenic Differentiation of ATDC5 Cells—If the growth factor-induced MTf is involved functionally in chondrogenic differentiation, forced expression of rabbit MTf in mouse ATDC5 cells should modulate chondrogenic differentiation in the absence of growth factors. To examine this hypothesis, we constructed pcDNA3.1/Zeo(+) expression vectors containing the entire coding region of rabbit MTf cDNA under the control of cytomegalovirus promoter. We transfected the MTf expression vector into mouse ATDC5 cells. Individual colonies were isolated, and the expression of MTf was examined by Northern blotting and immunoblotting. All clones transfected with the MTf expression vector showed the expression of MTf mRNA at high levels (data not shown). Five clones (M1, M4, M5, M6, and M8) had a high protein level of MTf, and three clones (m2, m3, and m7) had a low level (see Fig. 2A and Table I). In all clones, the protein level of MTf was lower (< 10%) than that in chondrocytes isolated from resting cartilage or the growth plate of adult rabbits (Fig. 2A).

To determine whether MTf is expressed on the surface of transfected cells, we examined the cell surface MTf level using a flow cytometer. As we expected, ATDC5 cells transfected with a rabbit MTf expression vector were stained with anti-rabbit MTf mAb2 but not with control IgG (Fig. 2B). This demonstrated that rabbit MTf could be expressed stably on the cell surface of mouse ATDC5 cells. In contrast, MTf was not detectable on the cell surface of the cells transfected with the empty vector (Fig. 2B). Most of M1, M4, M5, and M8 cells (83–96%) stained with mAb2, whereas only 58% of M6 cells showed positive staining (Fig. 2B). The pattern of MTf expression in M6 cells differed from that in the other M cells (Fig. 2B).

Parental ATDC5 cells, as well as cells transfected with the empty vector (Pc1, Pc2, and Pc3) showed the fibroblastic appearance throughout the whole culture period (see Fig. 3 and Table I). However, M1, M4, M5, and M8 cells underwent cell-
shape change from fibroblastic to spherical/polygonal cells by day 23. Such cell-shape change was less prominent in m2, m3, M6, and m7 cultures. The level of [35S]sulfate incorporation into glycosaminoglycans was significantly higher in five MTf-expressing clones (M1, m2, M4, M5, and m7) than in wild type ATDC5 cultures on day 16 (see Fig. 4A and Table I). Furthermore, all MTf-expressing cultures except for M6 were stained more intensely with toluidine blue than control cultures (wild type, Pc1, and Pc2) on day 16 (Fig. 4B). Accordingly, the content of uronic acid-containing glycosaminoglycans (proteoglycans) was higher in MTf-expressing cultures than in control cultures on day 23 (Fig. 4C).

In addition, forced expression of MTf induced aggrecan mRNA expression in all clones except for M6, and induced type II collagen mRNA in all clones except for m7. Matrilin-1, another chondrocyte marker, was expressed in Pc1, Pe2, and Pc3 at low levels, whereas it was expressed at high levels in most MTf-expressing clones (Fig. 5). These findings suggest that forced expression of MTf enhanced chondrogenic differentiation of ATDC5 cells depending upon the MTf level (Table I). M6 cells showed type II collagen and matrilin-1 syntheses but not aggrecan synthesis. Most M6 cells did not show the spherical phenotype. The reason by which M6 cells did not show aggrecan synthesis or the spherical phenotype is unknown. We did re-sequence rabbit MTf expressing in M6 cells and found no mutation. However, flow cytometric analysis showed that the percentage of M6 cells (58%) that stained with anti-rabbit MTf mAb2 was lower than that of M1, M4, M5, or M8 cells (83–96%) (Fig. 2B). The MTf expression pattern was different between M6 and the other M cells. The presence of M6 cells without MTf on the cell surface (~42%) may suppress aggrecan synthesis and the expression of the spherical phenotype.

Changes in Cell Shape and Gene Expression in MTf-expressing ATDC5 Cells—ATDC5 cells expressing MTf at a high level (M5) and empty-vector integrated cells (Pc1) became confluent on day 4. The MTf-expressing cells started to form cartilage-like nodules on day 15 (Fig. 6A). The cells in the nodules exhibited the spherical phenotype. Most cells in M5 cultures became spherical on day 25. In M5 cultures, aggrecan, and type II collagen mRNA levels were low on day 5 and then increased with time, reaching a plateau on day 15 (Fig. 6B). The type X collagen mRNA level became detectable on day 15 and then increased with time until day 25. In contrast, aggrecan, type II collagen, and type X collagen mRNAs or spherical chondrocytes were hardly detected in control cultures during the whole culture period. In both M5 and Pc1 cultures, Sox9 mRNA was expressed at high and low levels on days 5–15 and days 20–25, respectively (Fig. 6B).

Effects of Anti-MTf Monoclonal Antibody on Chondrogenesis of the MTf-expressing ATDC5 Cells—To examine whether MTf expression is necessary for chondrogenic differentiation, the antisense-mouse MTf cDNA was expressed in ATDC5 cells under the control of cytomegalovirus promoter. In three independent studies, we isolated more than 20 Zeocin-resistant clones. However, Northern blot analysis revealed that no clones except for A5 showed the expression of antisense-mouse MTf RNA (data not shown). The expression of antisense MTf RNA may inhibit clonal growth or survival of ATDC5 cells. Interestingly, the antisense MTf RNA-expressing cells (A5) hardly underwent chondrogenic differentiation even in the presence of insulin, whereas other clones, as well as parental ATDC5 cells, became chondrocytes in the presence of insulin. In the presence of insulin, A5 cells did not exhibit the expression of chondrocyte-related genes.
of insulin, the aggrecan mRNA level was far lower (<10%) in A5 cells than in parental ATDC5 cells (data not shown).

Because we could not obtain other clones expressing the antisense-MTf RNA, we used anti-rabbit MTf mAb2 to modulate the effect of rabbit MTf on the cell surface. When M4 or M5 cells were exposed to mAb2 in confluent cultures, the expression of the spherical phenotype was suppressed markedly on day 15 (Fig. 7A). This antibody also decreased [35S]sulfate incorporation into glycosaminoglycans (Fig. 7B) and aggrecan and type X collagen mRNA levels on day 15 (Fig. 7C). The antibody treatment was not toxic. The inhibition by mAb2 became less prominent with increase in the culture period even in the continuous presence of mAb2, perhaps because of the accumulation of the extracellular matrix, which may inhibit the access of the antibody to the cell surface. To demonstrate that the effect of mAb2 is reversible, M4 cells were exposed to mAb2 for 8 days (days 4–12) and then incubated for 8 days without antibody until day 20. These cells re-expressed the spherical phenotype and type X collagen mRNA on day 20 (Fig. 7D). Anti-rabbit MTf polyclonal Ab1 also suppressed the cell-shape change in M4 and M5 cultures (data not shown). These findings suggest that MTf expressed on the cell surface is involved in the enhancement of chondrocyte differentiation.

Comparison between the Effect of MTf Expression and Insulin on the mRNA Expression of Aggrecan and Type II Collagen and Cell Growth—The effect of MTf-forced expression on aggrecan expression was less than that of insulin on day 15 (the matrix-forming stage) (Fig. 8A). The effect of MTf-forced expression on type II collagen expression was also less than that of insulin (Fig. 8A). On day 5, insulin decreased aggrecan mRNA expression in M4 and M5 cultures (Fig. 8B). The degree of inhibition varied from 20 to 70% in repeated studies (data not shown). However, insulin increased aggrecan mRNA expression in M4 and M5 cultures on days 10, 15, 20, and 25 (Fig. 8, A and B). The forced expression of MTf had little effect on the proliferation of ATDC5 cells, irrespective of the presence or absence of insulin (Fig. 8C).

Effects of Forced Expression of MTf on the Expression of Aggrecan or Type II Collagen mRNA in C3H10T1/2 Cells—Mouse C3H10T1/2 cells have multilineage differentiation potential and show the expression of type II collagen only in pellet or micromass cultures exposed to TGF-β. They do not undergo chondrogenic differentiation in monolayer cultures even in the presence of TGF-β. MTf mRNA expression in C3H10T1/2 cells was very low (data not shown). To examine the role of MTf in chondrogenic differentiation, we isolated C3H10T1/2 clones (T1–T4) transfected with the rabbit MTf expression vector (Fig. 9A). The rabbit MTf level in these clones was similar to that in M5-ATDC5 cells. The forced expression of MTf induced aggrecan mRNA expression in all
C3H10T1/2 clones and type II collagen mRNA in T1 and T3 clones in monolayer cultures exposed to TGF-/H9252 (Fig. 9B), whereas these expressions were undetectable in parental and empty vector-integrated C3H10T1/2 cells. The forced expression did not induce the expression of the spherical phenotype in T2, T3, and T4 cells. T1 cells alone showed the polygonal/spherical phenotype (data not shown).

**DISCUSSION**

The marked stimulation of MTf expression by insulin/IGF-I, BMP-2, and TGF-β, which are essential for chondrogenic differentiation, suggests the involvement of MTf in the differentiation program. BMP-2 elicited the greatest stimulation of MTf expression in ATDC5 cells, whereas TGF-β had less effect. Insulin also enhanced MTf expression. The high MTf expression in chondrocytes may depend upon the insulin/IGF-I and BMP/TGF-β-signaling pathways.

To test a hypothesis that MTf is involved in chondrogenic differentiation, we used ATDC5 cells expressing rabbit MTf and anti-rabbit MTf antibodies. In the absence of added growth factors, ATDC5 cells expressing rabbit MTf underwent chondrogenic differentiation as indicated by the synthesis and accumulation of cartilage-matrix macromolecules and the spherical appearance, whereas wild type and empty vector-integrated ATDC5 cells did not show any traits of chondrogenic differentiation except for the expression of Sox9. The degree of chondrogenic differentiation in the MTf-expressing cells depended roughly upon the expression level of rabbit MTf (Table I).

The protein level of MTf was more than 10-fold higher in chondrocytes isolated from adult rabbits than in the rabbit MTf-expressing ATDC5 cells. The MTf mRNA level in chondrocytes was comparable with that in SK-MEL melanoma cells (data not shown). SK-MEL cells express 10^6-10^7 MTf molecules.
per cell (26), whereas fibroblasts express $10^3$ MTf molecules per cells (27). This high MTf level in chondrocytes may modulate growth factor signaling pathways. The effect of the forced expression of MTf in ATDC5 cells on chondrogenic differentiation was less than that of insulin. This may be because of the lower level of rabbit MTf in the MTf-overexpressing ATDC5 cells than in adult chondrocytes. It is also likely that MTf expression mediates only part of the growth factor actions in chondrogenic cells.

The forced expression of MTf in ATDC5 cells enhanced type II collagen, aggrecan, matrilin-1, and type X collagen. Type II collagen is expressed in chondrocytes and a few non-cartilaginous tissues during embryogenesis and development of the skeleton. Type II procollagen is synthesized in the perichondrium as two different forms (type IIA and type IIB) generated by alternative splicing of exon 2 in the precursor mRNA transcript. These two forms are expressed spatially during development with the type IIB mRNA expressed primarily by chondrocytes, whereas type IIA form is expressed in chondroprogenitor cells (28). The forced expression of MTf increased both type IIA and IIB collagen mRNA expressions, although it had much greater effects on type IIB collagen mRNA expression (data not shown). Aggrecan is also the major component in the cartilage matrix. Matrilin-1 is synthesized in a cartilage-characteristic manner and binds to aggrecan and type II collagen (29–31). Matrilin-1 may be an adhesion factor for chondrocytes (32). Aggrecan and matrilin-1 mRNAs were also expressed at higher levels in the MTf-expressing cells. The expression of type X collagen, a marker for hypertrophic chondrocytes, was also induced in the MTf-expressing clones at a late stage. Furthermore, anti-MTf antibodies suppressed the expressions of aggrecan and type X collagen in MTf-expressing ATDC5 cells. These findings suggest that MTf facilitates the differentiation of prechondrogenic cells from early to terminal stages.

C3H10T1/2 cells had far less chondrogenic potential than ATDC5 cells, whereas C3H10T1/2 cells had much greater osteogenic and adipogenic potential than ATDC5 cells. In C3H10T1/2 cells exposed to TGF-β1 (5 ng/ml), the mRNA expressions of aggrecan and type II collagen were determined by RT-PCR-Southern blot analysis, and phase-contrast microphotographs were taken on day 18. Similar results were obtained in repeated studies.
The mechanism by which MTf enhances chondrogenic differentiation is not known. Because chondrocytes secrete transferrin at high levels, MTf is unlikely to play a role in iron uptake. In the absence of transferrin, MTf enhances iron uptake via a transferrin receptor-independent mechanism (33). However, MTf cannot receive iron from transferrin, and thus MTf does not play a role in iron uptake in physiological conditions (12). Furthermore, the addition of transferrin had no effect on chondrogenic differentiation of wild type and MTf-overexpressing ATDC5 cells (data not shown). Thus, MTf seems to work independently of transferrin and iron uptake. It is hypothesized that MTf may have matrix metalloproteinase-like activity and inhibit lipid peroxidation (12). However, these activities of MTf cannot account for the enhancement of chondrogenic differentiation.

The anti-MTf antibodies including mAb2 and polyclonal Ab1 suppressed chondrogenic differentiation of ATDC5 cells. The antibodies may compete with an unknown MTf ligand secreted by chondrocytes or suppress the interaction between MTf and other signaling molecules on the cell surface. In contrast to these antibodies, some antibodies (polyclonal Ab4) against rabbit MTf enhanced aggrecan synthesis and the expression of the spherical phenotype by poorly differentiated rabbit chondrocytes (3). These findings suggest that cross-linking of MTf modulates chondrogenic differentiation. MTf is linked to the outer surface of the plasma membrane via GPI anchor (4). Most GPI-anchor proteins are concentrated in lipid raft, which plays an important role in signal transduction (34, 35). Several GPI-anchor proteins have been shown to modulate the growth factor or cytokine- or antigen-induced proliferation/differentiation of various cells including lymphocytes (36–38). In MTF-overexpressing ATDC5 cultures, insulin suppressed aggrecan expression in the early stage but enhanced it in the later stages. MTf may modulate the growth factor signaling pathways depending on stages.

In conclusion, MTf is a target for insulin/IGF-I, BMP-2, and TGF-β and mediates the effect of the growth factor on chondrogenic differentiation at least partly. This is the first observation that a GPI-anchor protein is involved functionally in the differentiation program of chondrogenic cells.

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