Since permanent cartilage has poor self-regenerative capacity, its regeneration from autologous human chondrocytes using a tissue engineering technique may greatly benefit the treatment of various skeletal disorders. However, the conventional autologous chondrocyte implantation is insufficient both in quantity and in quality due to two major limitations: dedifferentiation during a long term culture for multiplication and hypertrophic differentiation by stimulation for the redifferentiation. To overcome the limitations, this study attempted to determine the optimal combination in primary human chondrocyte cultures for the redifferentiation. To over-engineered cartilage from autologous chondrocytes is already available for clinical use (1–3), the size is less than 1 ml and is limited to just a filler of a small defect or an injectable plaque of the affected cartilage. To broaden its clinical indication to major disorders such as osteoarthritis joints, microtia, and cleft lip/ palate, cartilaginous matrix should be abundantly produced by a sufficient number of autologous chondrocytes. Since the number of chondrocytes that can be isolated primarily from the native cartilage is limited, it should be markedly increased in culture. However, during their multiplication in culture through repeated passaging, chondrocytes inevitably lose their ability to produce cartilaginous matrix such as glycosaminoglycan (GAG) and type II collagen (COL2) and begin producing type I collagen (COL1), which is called dedifferentiation (4).

Although biochemical factors such as hormones and growth factors are known to stimulate redifferentiation of the dedifferentiated chondrocytes to restore the chondrocytic properties (5, 6), there remains another inevitable limitation, that is, induction of hypertrophic differentiation of chondrocytes (7), which leads to endochondral ossification as seen in the growth plates.

To develop a clinically practical tissue engineering of permanently cartilage that overcomes the limitations above, the present study focused on 12 representative soluble factors (8) that are known to be potent regulators of proliferation or differentiation of chondrocytes and have already been approved for safe clinical use. We initially determined a combination of two factors among them that most efficiently induce redifferentiation of the dedifferentiated human chondrocytes under a serum-free condition using a statistical method termed “analysis of variance by fractional factorial design” (9). We then selected a third factor that prevents the redifferentiated chondrocytes from hypertrophic differentiation and investigated the underlying

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Since permanent cartilage has poor self-regenerative capacity, its regeneration using a tissue engineering technique may greatly benefit the treatment of various skeletal disorders. However, the conventional autologous chondrocyte implantation is insufficient both in quantity and quality due to two major limitations: dedifferentiation during long term culture for multiplication and hypertrophic differentiation by stimulation for the redifferentiation. To overcome the limitations, this study attempted to determine the optimal combination in primary human chondrocyte cultures for the redifferentiation. To over-engineered cartilage from autologous chondrocytes is already available for clinical use (1–3), the size is less than 1 ml and is limited to just a filler of a small defect or an injectable plaque of the affected cartilage. To broaden its clinical indication to major disorders such as osteoarthritis joints, microtia, and cleft lip/palate, cartilaginous matrix should be abundantly produced by a sufficient number of autologous chondrocytes. Since the number of chondrocytes that can be isolated primarily from the native cartilage is limited, it should be markedly increased in culture. However, during their multiplication in culture through repeated passaging, chondrocytes inevitably lose their ability to produce cartilaginous matrix such as glycosaminoglycan (GAG) and type II collagen (COL2) and begin producing type I collagen (COL1), which is called dedifferentiation (4).

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molecular mechanism. Finally, we evaluated the properties of the tissue-engineered cartilage by the selected combination to examine whether it is applicable to clinical use for the treatment of major skeletal disorders.

EXPERIMENTAL PROCEDURES

Cell Isolation and Monolayer Culture—All procedures of the present experiments were approved by the ethics committee of the University of Tokyo Hospital (ethics permission number 622). Both remnant auricular cartilage and costal from one of six microtia patients (10–15 years old) were obtained during surgery in adherence to the Helsinki Principles. Chondrocytes were isolated with digestion using 0.15% collagenase (Wako Pure Chemical Industries, Osaka, Japan). Primary articular chondrocytes were purchased from Sanko Junyaku (Tokyo, Japan). The chondrocytes were seeded in a 100-mm plastic tissue culture dish at a density of 6,400 cells/cm² and cultured in Dulbecco’s modified Eagle’s medium/F12 containing 5% human serum supplemented with fibroblast growth factor-2 (FGF-2, 100 ng/ml) and insulin (5 µg/ml), as we reported previously (8). Passing was performed by trypsin-EDTA solution (Sigma) and repeated until 1000-fold increase of the total cell number.

Generation of Cartilage Pellet and Implant—For three-dimensional atelocollagen culture, the dedifferentiated chondrocytes were suspended in 0.8% atelocollagen solution (Kawaken Fine Chemicals, Tokyo, Japan) at a density of 10⁶ cells/ml. The atelocollagen pellets were cultured in Dulbecco’s modified Eagle’s medium/F-12 (Sigma) medium with or without soluble factors for 1–3 weeks. To make the cartilage implant, a 100 µl-atelocollagen aliquot containing 10⁶ chondrocytes was added to a poly(l-lactic acid) (PLLA) mesh (kindly provided by Unitika, Tokyo, Japan) (10). Animal experiments were performed according to the protocol approved by the Animal Care and Use Committee of the University of Tokyo.

Analysis of Variance by Fractional Factorial Design—We used recombinant human bone morphogenetic protein-2 (rhBMP-2, kindly provided by Astellas Pharma, Tokyo, Japan), rh-insulin (MP Biomedicals, Irvine, CA), rh-insulin-like growth factor-1 (rhIGF-I, Former Genzyme-Technie, Minneapolis, MN), rh-testosterone (Ultraline Chemicals, Manchester, UK), rh-parathyroid hormone (rhPTH (1-34), Anaspec, San Jose, CA), rh-interleukin-1 receptor antagonist (rhIL-1RA, Strathmann Biotech, Hamburg, Germany), rh-bone growth hormone (Bio-genesis, Poole, UK), 17β-estradiol (EMD Bioscience, San Diego, CA), rh-1,3,5'-triiodothyronine (rhT3, EMD Bioscience), 1α-25-dihydroxy vitamin D3 (EMD Bioscience), rhFGF-2 (kindly provided by Kaken Pharmaceutical Corp., Tokyo, Japan), and dexamethasone (EMD Bioscience). The dosage of each factor was determined to be: BMP-2, 200 ng/ml; insulin, 5 µg/ml; IGF-I, 100 ng/ml; testosterone, 1 µM; PTH, 5 × 10⁻⁸ M; IL-1RA, 20 ng/ml; growth hormone, 100 ng/ml; 17β-estradiol, 100 nM; T3, 100 nM; 1α-25-dihydroxy vitamin D3, 100 nM; FGF-2, 10 ng/ml; dexamethasone, 100 nM, based on previous reports by us and others (8, 11–20). These doses of individual factors were within the range between effective dose 75 and effective dose 100 of their proper effects on chondrocytes, according to dose-response curves in the previous reports (11, 12, 15, 16, 21–28).

The software JMP-5.1.1J (SAS Institute, Cary, NC) was used to choose 256 randomized combinations in which the 12 factors independently appeared in an incidence of 50%, from 2¹² = 4,096 combinations (see the supplemental table). Auricular chondrocytes obtained from three different patients (5 × 10⁴ cells in 5 µl of 0.8% atelocollagen gel) were cultured in Dulbecco’s modified Eagle’s medium/F-12 containing the combinations for 2 weeks, and the GAG accumulation was measured as described below. Parameter estimates of the GAG accumulation by one factor or two were calculated from the F values that represent the effects of the individual factors and the interaction terms of two factors by the analysis of variance using the software above.

Real-time RT-PCR Analysis—Total RNA was isolated from chondrocytes using the chaotropic TRIzol method (Nippon-gene, Tokyo, Japan). Total mRNA (1 µg) was reverse-transcribed using SuperScript reverse transcriptase with random hexamer (Takara Shuzo, Shiga, Japan). The full-length or partial-length cDNA of target genes, including PCR amplicon sequences, was amplified by PCR, cloned into pCR-TOPO Zero II or pCR-TOPO II vectors (Invitrogen), and used as standard templates after linearization. QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) was used, and SYBR Green PCR amplification and real-time fluorescence detection were performed with an ABI 7700 sequence detection system (Foster City, CA). All reactions were run in quadruplicate. Sequences of primers were 5’-CTCCTGCCTTCTCCTCGT-3’ and 5’-GTGCTAAAGGTGCTGAT-3’ for type I collagen α1 chain (COL1A1); 5’-GAGTCAGGTGATCTGGTT-3’ and 5’-CACCTTGGTTCACAGAAGGA-3’ for type II collagen α1 chain (COL2A1); 5’-AGGAATGCCTGTCCTGCTTT-3’ and 5’-ACAGGCTTAACAAAAACTATGA-3’ for type X collagen α1 chain (COL10A1); 5’-CAGACACGGACGACCCAT-A-3’ and 5’-CAGGCTCAACACACCTACATC-3’ for Runx2; 5’-CAGTACGGAGGCACACTCC-3’ and 5’-TCGGTT-CAGGTGACGCCAGT3’ for Sox9; 5’-ATGCAGCGAGACTGTTCTCAG-3’ and 5’-GTTCTTGGAGGTCTCCTGCTG-3’ for PTH/PTHrP receptor; 5’-GAAGGTGAAAGGTCGAGGTCA-3’ and 5’-GAAGATGTTGATGGATTTTAC-3’ for glyceraldehyde-3-phosphate dehydrogenase.

GAG Measurement—The sulfated GAG content was measured using Alcian blue binding assay (Wieslab AB, Lund, Sweden). After digestion of the atelocollagen pellet containing chondrocytes in 0.3% collagenase for 1 h at 37 °C, cell debris and insoluble material were removed by centrifugation at 6,000 × g for 30 min. GAG in the supernatant was precipitated with Alcian blue solution, and the sediments by centrifugation at 6,000 × g for 15 min were dissolved again in 4 M GuHCl-33% propanol solution. The spectrophotometrical absorbance of the mixture was measured at a wavelength of 600 nm.

Enzyme-linked Immunosorbent Assay for Type I and Type II Collagen—The collagen proteins of the pellets, the implants, and the native human auricular cartilage were quantified by enzyme-linked immunosorbent assay using a human Type 1 and Type 2 collagen detection kit (Chondrex, Redmond, WA). The pellets, or the native human auricular carti-
nylenediamine and H2O2 were added to the mixture, and the alated counterparts and streptavidin peroxidase. O-phenyl-

The collagen proteins were captured by polyclonal antibodies and detected by biotin-
determination by the GAG accumulation. Since

| Statistical Analysis — All data are expressed as means ± S.E.

| RESULTS

**Determination of the Optimal Combination of Soluble Factors** — We attempted to determine the optimal combination of soluble factors for regeneration of permanent cartilage from cultured human chondrocytes that were isolated from remnant auricular cartilage of microtia patients during surgery. To gain a substantial number of cells, the chondrocytes were initially cultured in monolayer with repeated passaging until 1,000-fold increase of the total cell number as we previously reported (8), which took ~4 weeks. The dedifferentiated chondrocytes during the monolayer culture were further cultured in an atelocollagen gel in a three-dimensional condition under stimulation by combinations of 12 candidate factors, i.e., BMP-2, insulin, IGF-1, testosterone, PTH, IL-1RA, growth hormone, 17β-estradiol, T3, 1α-25-dihydroxy vitamin D3, FGF-2, and dexamethasone. The redifferentiation was determined by the GAG accumulation. Since exhaustive examination of 2^{12} = 4,096 combinations was impossible, we adopted the fractional factorial design (9) for the faultless reduction of the experimental units to a practical number of 256 combinations (see the supplemental table). As a single factor, the statistical analysis revealed that BMP-2, insulin, and IGF-1 were the most potent factors of the GAG accumulation (Fig. 1A). Regarding two factors, the combination of BMP-2 and insulin (BI) was shown to provide the most abundant GAG accumulation (Fig. 1A).

We then examined expressions of COL1A1, COL2A1, and COL10A1 by real-time RT-PCR in the cultured human auricular chondrocytes just after isolation (Fig. 1B, primary), after the monolayer culture (pre-rediff), and after the three-dimensional atelocollagen culture (Fig. 1B). The long term monolayer culture with repeated passaging led to an increase in COL1A1 and a decrease in COL2A1, confirming the dedifferentiation. The three-dimensional atelocollagen culture thereafter with no stimulation somewhat restored both COL1A1 and COL2A1 expressions, indicating redifferentiation, which was much more enhanced by the BI stimulation. However, BI also increased COL10A1, a marker of chondrocyte hypertrophic differentiation, which is a critical defect for the regeneration of permanent cartilage. Thus, we next searched among the other 10 factors for a third factor that not only enhances the COL1 inhibition and the COL2 stimulation by BI but also suppresses the COL10 induction and found that only T3 exhibited significant effects on all parameters. This indicates that T3 is the optimal third factor that enhances the advantage and cancels the disadvantage of BI for regeneration of permanent cartilage from human auricular chondrocytes.

When we used cultured chondrocytes from human articular cartilage (Fig. 1C) and human rib cartilage (Fig. 1D), the combination of BI and T3 (BIT) potently suppressed COL1A1 and COL10A1 and enhanced COL2A1 expressions,
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**FIGURE 1.** Determination of the optimal combination of soluble factors. A, parameter estimates of a single or two factors were statistically calculated from GAG accumulation in 256 combinations of 12 candidate factors. GH, growth hormone; E2, 17β-estradiol; VitD, 1α,25-dihydroxy vitamin D3; Dex, dexamethasone; Te, testosterone. B, COL1A1, COL2A1, and COL10A1 mRNA levels by real-time RT-PCR of the human auricular chondrocytes just after the isolation (primary), after the monolayer culture (pre-rediff), and after 1 week of the three-dimensional atelocollagen culture in the presence (+) and absence (−) of BMP-2 (200 ng/ml) + insulin (5 μg/ml) (BI) and a third factor from 10 other factors. C and D, COL1A1, COL2A1, and COL10A1 mRNA levels by real-time RT-PCR of cultured chondrocytes derived from human articular cartilage (C) and human rib cartilage (D). The dedifferentiated cells after multiplication in the monolayer culture underwent three-dimensional atelocollagen culture for 1 week in the presence and absence of BI or BI + T3 (100 nM) (BIT). For B–D, data are expressed as mean (bars) ± S.E. (error bars) of relative ratios of the control culture (−/−) for six cultures/group. #, p < 0.01, significant effects versus control; *, p < 0.01, significant effects by a third factor or T3 versus BI alone.

**FIGURE 2.** Dose response effects of T3 (1–1000 nM) on mRNA levels of Runx2, Sox9, PTH/PTHrP receptor, COL10A1, and COL2A1 determined by real-time RT-PCR in three-dimensional atelocollagen culture. The dedifferentiated chondrocytes after multiplication in the monolayer culture underwent the three-dimensional culture for 1 week under the stimulation. Data are expressed as mean (bars) ± S.E. (error bars) of relative ratios of the control culture for six cultures/group. BI, BMP-2 (200 ng/ml) + insulin (5 μg/ml). #, p < 0.01, significant effects versus control; *, p < 0.01, significant effects by T3 versus BI. Just as in the case of the human auricular cartilage, this indicates that BIT is universally efficacious for tissue engineering of permanent cartilage from human chondrocytes of various origins.

**Mechanism Underlying the Preventive Effect of T3 on BI-induced Hypertrophic Differentiation of Redifferentiated Chondrocytes**—To clarify the molecular mechanism whereby T3 prevented the BI-induced hypertrophic differentiation of redifferentiated chondrocytes, we examined the involvement of putative signalings that regulate hypertrophic differentiation in the three-dimensional atelocollagen culture of human auricular chondrocytes.

Runx2, a member of the runt family of transcription factors, is known to be required for chondrocyte hypertrophy (30). Sox9, an essential factor for chondrogenic differentiation (31), functions as a potent inhibitor of chondrocyte hypertrophy (32). PTH/PTHrP via the cAMP-dependent protein kinase is also known to be a major factor that inhibits chondrocyte hypertrophy (33). Among the three factors above, T3 dose-dependently suppressed the BI stimulation of expression of Runx2 similarly to that of COL10A1, but not that of Sox9 or PTH/PTHrP receptor, implying that Runx2 is involved in the prevention of the BI-induced hypertrophic differentiation by T3 (Fig. 2). The concentration of T3 that exhibited the maximum suppression of COL10A1 and Runx2, as well as the stimulation of COL2A1, was 100 nM. To further investigate the intracellular signaling involved, activation of Smads, p38 MAPK, and phosphatidylinositol-3 kinase/Akt, which are known to lie downstream of BMP-2 or insulin (34, 35) and also upstream of Runx2 (36, 37), was examined by Western blot analyses (Fig. 3A). The BMP receptor-specific Smad1/5/8, p38 MAPK, and Akt were phosphorylated 10, 10, and 5 min, respectively, after the treatment of BI. Although T3 (100 nM) hardly or faintly affected Smad or p38 MAPK activation by BI, it suppressed the Akt activation just like an Akt inhibitor. When we examined COL10A1 and Runx2 mRNA levels in the three-dimensional atelocollagen culture, both levels stimulated by BI were dose-dependently suppressed by the Akt inhibitor, similarly to those by T3 (Fig. 3D), suggesting that T3 prevented the BI-induced hypertrophic differentiation of redifferentiated chondrocytes at least partially via the suppression of the Akt signaling. The inhibitory effects of T3 or the Akt inhibitor on Akt activation by...
BI continued for 24 h and then disappeared (Fig. 3). Because the medium was changed twice/week throughout the experiment (1 week), T3 or the Akt inhibitor seemed to exert an inhibitory effect on the Akt activation in approximately one-third of the entire culture period and finally demonstrated the suppression of the Runx2 and Col10A1 expressions at the end of the culture (Fig. 3D). The phosphorylation of p38 MAPK seemed to be slightly increased by T3 (Fig. 3A), but the use of p38 MAPK inhibitor SB203580 instead of T3 rather down-regulated the expression of COL10A1 and RUNX2 (Fig. 3, C and D). The p38 inhibitor also suppressed that of COL2A1, but enhanced that of COL1A1, unlike T3 or the Akt inhibitor (Fig. 3C), suggesting that the slight up-regulation of the p38 MAPK activation by T3 may be associated not with chondrocyte hypertrophy but with enhancement of the early chondrocyte differentiation markers such as the collagen type II or proteoglycan.

Properties of the Tissue-engineered Cartilage from Human Chondrocytes by BIT—We next examined the properties of the tissue-engineered cartilage pellet generated by the three-dimensional atelocollagen culture (2 × 10⁵ cells/20 μl) with and without stimulation by BI or BIT from human auricular chondrocytes that were dedifferentiated after long term monolayer culture, as described above. The size of pellets with BI or BIT stimulation was much larger than that without stimulation (Fig. 4A, top). In addition, histological analyses of toluidine blue staining revealed more proteoglycan production shown by metachromasia with cartilage lacunae under the BI stimulation as compared with the control, which was further enhanced by BIT (Fig. 4A, bottom). Measurement of cartilaginous matrix protein levels also revealed that the redifferentiation markers GAG and COL2, but not the dedifferentiation marker COL1, were stimulated moderately by BI and strongly by BIT (Fig. 4B). However, the GAG level tended to be lower in all these groups as compared with that in native cartilage (Fig. 4B). The ratio of GAG/COL1 and that of COL2/COL1 were 0.77 ± 0.25 and 0.49 ± 0.64, respectively, for the control, 4.12 ± 0.94/1.47 ± 0.96 for BI and 5.16 ± 1.57/4.44 ± 1.66 for BIT, which successively approached the value of the native cartilage (9.37 ± 3.26/6.06 ± 3.15). The cell proliferation determined by proliferating cell nuclear antigen and cyclin D1 mRNA levels was significantly decreased by BI and
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Aiming at regenerating permanent cartilage that is applicable for clinical use, we created implants by adding the human auricular chondrocytes cultured in the three-dimensional atelocollagen gel to the PLLA mesh scaffold (10^6 cells/columnar implant 10 mm in diameter and 1 mm in thickness). The implants were cultured for 3 weeks with and without stimulation by BI or BIT (Fig. 5B, top) and thereafter transplanted subcutaneously into nude mice for 2 months (Fig. 5A). Although the size of the excised implants of the three groups seemed similar (Fig. 5B, bottom), depending on the original PLLA scaffold size, the metachromatic proteoglycan production was much more enhanced in the BIT-treated implant than in the other two groups (Fig. 5C). The implants of the control (Fig. 5D, Control) and BI (data not shown) groups contained not only hyaline cartilage with COL2-like fibrils but also fibrous cartilage with COL1-like fibrils even in the center of the implants, showing the mingling with the hyaline cartilage and fibrous one. However, the BIT-treated implants were filled with hyaline cartilage (Fig. 5D, BIT). Biochemical analyses confirmed the advantages of BIT: that GAG and COL2, but not COL1, were significantly stimulated by the treatment (Fig. 5E). The GAG level in the BIT reached that of the native cartilage, whereas COL2 in BIT tended to be higher than in the native one (Fig. 5E). The ratio of GAG/COL1 and that of COL2/COL1 were 0.44 ± 0.18 and 0.78 ± 0.43/0.71 ± 0.23 for BI, and 1.61 ± 0.61/1.68 ± 0.61 for BIT. The decrease in these ratios as compared with those of the tissue-engineered cartilage pellets cultured in vitro or the native cartilage may be due to the difficulty in the removal of the COL1-based fibrous tissues around the in vivo regenerated cartilage. Here again, COL10-positive matrices were visible only in the BI-treated implant but not in the control or BIT-treated one (Fig. 5F). Furthermore, mechanical parameters of the control, BI-, and BIT-treated implants were higher than those of the PLLA scaffold without cultured cells (Fig. 5G). Among them, both parameters of the BIT-treated cartilage were not only greater than the control and BI-treated cartilage but also comparable with that of the native cartilage.

DISCUSSION

The present study succeeded in overcoming the two major limitations of tissue engineering of permanent cartilage from autologous human chondrocytes: dedifferentiation during the long term culture for multiplication and hypertrophic differentiation by stimulation for redifferentiation. For the efficient and exhaustive search for the optimal combination, we utilized the analysis of variance by the fractional factorial design (9). This method was originally developed in the agricultural science
field; one could decide the optimal conditions to yield good crops out of many naturally fluctuating conditions including temperature, soil condition, rainfall, etc (39). Thereafter, this method was successfully adapted to industrial applications; e.g. it drastically reduced production errors of aircraft engine manufacturing to one-fifth through the grading of numerous parameters including cutting oil, cutting speed, groove ratio, tip-end angle, etc (40). Since this method is now widely approved as being useful for the maintenance of goods and services of high quality, the Food and Drug Administration Good Manufacturing Practices regulation and the International Organization for Standardization 9000 series now require its confirmation for process validation (9). This method, however, has not been applied for quality control in medical fields that contain tremendous parameter variations. The present study for the first time adopted the method to determine the optimal combination of putative factors for a regenerative medicine and demonstrated its usefulness.

BMP-2 was shown to have the strongest potency to produce GAG as a single factor, according to the result of analysis of variance by the fractional factorial design (Fig. 1A). BMP-2 is known to enhance not only chondrogenic differentiation but also further hypertrophic differentiation of chondrocytes in several cell cultures (7, 20), which is a critical weakness for the regeneration of permanent cartilage. Insulin is also reported to induce both chondrogenic and hypertrophic differentiation in cultures (11). The third factor, T3, as well as its prohormone 1-thyroxin (T4), are also known to play important roles in physiological skeletal growth by regulating terminal differentiation of chondrocytes. A childhood hypothyroidism, congenital hypothyroidism, is characterized by growth arrest and short stature, whereas childhood thyrotoxicosis causes accelerated growth and premature closure of the growth plate (41). Mice devoid of all isoforms produced from thyroid hormone receptor-α (TRα0/0) exhibited dwarfism due to impaired hypertrophic differentiation of chondrocytes in the growth plate (42). In addition, several in vitro studies have shown that T3 around physiological concentrations (0.1–1 nM) stimulates the terminal differentiation markers COL10, alkaline phosphatase, and osteopontin in several chondrocyte culture systems (43, 44). These seem to be inconsistent with the present finding that T3 prevented BI from inducing hypertrophic differentiation through suppression of the phosphatidylinositol-3 kinase/Akt signaling, a major pathway for chondrocyte differentiation (34). The thyroid hormones are generally believed to exert their influence through the genomic action by activating the nuclear receptors (TRα1 and -2 and TRβ1, -2, and -3), causing a conformational change that leads to dissociation from a repressor complex and interaction with an activation complex containing

FIGURE 5. Analyses of the tissue-engineered cartilage implants. A, the implants were generated by human auricular chondrocytes that underwent the long term monolayer culture followed by 3-week three-dimensional culture in the atelocollagen gel and the PLLA mesh scaffold with and without stimulations by BI or BIT, and further, by 2-month subcutaneous implantation in nude mice. B, macroscopic findings of the implants before (Pre) and after (Post) the in vivo transplantation. Scale bars, 5 mm. C, histological findings of the coronal section of the implants after the in vivo transplantation (toluidine blue-O staining). Scale bars, 0.5 mm. D, electron microscopic findings in the middle area of the implants after the in vivo transplantation. Scale bars, 10 μm and 100 nm for lower (top) and higher (bottom) magnifications, respectively. E, GAG, COL1, and COL2 protein levels in the implants after the in vivo transplantation. Data are expressed as mean (bars) ± S.E. (error bars) for six implants/group. Native, native human auricular cartilage. *, p < 0.05. **, p < 0.01, significant effects versus control. F, immunostainings with an anti-COL10 antibody in the middle area of the implants. Scale bar, 50 μm. G, mechanical properties of the implants after the in vivo transplantation, determined by compression strength and Young’s modulus, as compared with the PLLA scaffold alone and the native human auricular cartilage. Data are expressed as mean (bars) ± S.E. (error bars) for six implants/group. *, p < 0.05, **, p < 0.01, significant difference from the scaffold alone. #, p < 0.01, significant difference from the native cartilage.
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histone acetylase (41). In contrast, T3 at higher concentrations has been suggested to exert a nongenomic action; e.g. T3 (0.1–100 μM) caused a rapid activation of the Na+/H+ exchanger activity in isolated rat alveolar type II cells or rat AT II cell line RLE-6TN (45). The present study showed that T3 at more than 100 nM caused inhibition of COL10 and Runx2 expressions with a rapid suppression of Akt phosphorylation induced by BIT within 5 min. We speculate that the T3 action might be a nongenomic one that is somehow associated with the BIT and Runx2 signalings. In Fig. 2, Sox9 that is a potent inhibitor of the chondrocyte hypertrophy (32) was down-regulated in BIT, implying the induction of chondrocyte hypertrophy in BIT. However, the addition of T3 to BIT did not up-regulate the Sox9 expression, which may not follow the effect of BIT on the inhibition of the chondrocyte hypertrophy. It was reported that the thyroid hormone around the physiological concentration (T4, 30 ng/ml) inhibited the Sox9 expression and promoted chondrocyte hypertrophy in the rat chondrocyte pellet culture (46). These functions of the thyroid hormone around the physiological concentration seemed to work through a genomic action (44). The decrease in the Sox9 expression by the addition of 10–7 M T3 in the present experiment may suggest that T3 down-regulated the expression of Sox9 through the genomic action, even at the pharmacological concentration. However, we think that T3 at the pharmacological concentration prevents hypertrophic differentiation mainly through a nongenomic machinery. The nongenomic effect may dominate the genomic action in the pharmacological condition, leading to the inhibition of chondrocyte hypertrophy.

Considering that some tens to 100 ml of regenerated cartilage is necessary for its clinical application to major skeletal disorders with severe cartilage defects, we should initially obtain a sufficient number of cells from a limited amount of specimen that is no more than 0.1 ml (10⁶ cells), meaning that a 1,000-fold increase in cell number is needed. For efficient cell proliferation, many researchers have performed cell cultures with mitogenic growth factors in the presence of fetal bovine serum that is unfavorable for clinical use due to the risk of pathogen transmission and immune reaction (47). Previous trials of cultures using autologous human serum, however, could provide no more than 10⁷ cells, which corresponds to at most 1 ml of regenerative cartilage (2, 3). To overcome this problem, our preparatory study established a fetal bovine serum-free culture system of primary human chondrocytes that realize a 1,000-fold increase in cell number within 4 weeks under the stimulation of FGF-2 with insulin or IGF-1 (8), although the cell dedifferentiation was inevitable during the repeatedpassaging. Based on the previous study, the present study further established a serum-free culture system that causes redifferentiation of the dedifferentiated cells and maintains the properties of permanent cartilage. Since we succeeded in regenerating a high quality cartilage column 10 mm in diameter and 1 mm in thickness that is ~80 μL in volume, starting from at least primary chondrocytes in a surgical specimen, it is estimated that 80 ml of cartilage can be regenerated from 0.1 ml of a specimen containing 10⁶ cells by using our original two systems. We conclude that the BIT stimulation realized the tissue engineering of permanent cartilage whose quantity and quality satisfy the revolutionary treatment of major skeletal disorders. A clinical trial is now underway.

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