Placenta to cartilage: direct conversion of human placenta to chondrocytes with transformation by defined factors

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ABSTRACT Cellular differentiation and lineage commitment are considered to be robust and irreversible processes during development. Recent work has shown that mouse and human fibroblasts can be reprogrammed to a pluripotent state with a combination of four transcription factors. We hypothesized that combinatorial expression of chondrocyte-specific transcription factors could directly convert human placental cells into chondrocytes. Starting from a pool of candidate genes, we identified a combination of only five genes (5F pool)—BCL6, T (also called BRACHYURY), c-MYC, MITF, and BAF60C (also called SMARCD3)—that rapidly and efficiently convert postnatal human chorion and decidual cells into chondrocytes. The cells generated expressed multiple cartilage-specific genes, such as Collagen type II α1, LINK PROTEIN-1, and AGGRECAN, and exhibited characteristics of cartilage both in vivo and in vitro. Expression of the endogenous genes for T and MITF was initiated, implying that the cell conversion is due to not only the forced expression of the transgenes, but also to cellular reprogramming by the transgenes. This direct conversion system from noncartilage tissue to cartilaginous tissue is a substantial advance toward understanding cartilage development, cell-based therapy, and oncogenesis of chondrocytes.

INTRODUCTION The possibility of redirecting cell differentiation by overexpression of genes was suggested by H. Weintraub with the identification of the “master gene,” MyoD (Davis et al., 1987). The process was believed to involve reversion to a less differentiated state, a kind of dedifferentiation, before the new cell type is formed. Another process has since been introduced—the concept of direct conversion or direct reprogramming without dedifferentiation. This process is believed to be direct lineage switching rather than lineage switching back to a branch point and out again in a different direction (Hochedlinger and Jaenisch, 2006; Orkin and Zon, 2008). Direct conversion has been shown in β cells, cardiomyocytes, and neurons. A specific combination of three transcription factors (Ngn3, Pdx1, and MafA) reprograms differentiated pancreatic exocrine cells in adult mice into cells that closely resemble β cells (Zhou et al., 2008); a combination of three factors (Gata4, Tbx5, and Baf60c) induces noncardiac mesoderm to differentiate directly into contractile cardiomyocytes (Ieda et al., 2010); and a combination of three factors (Ascl1, Brn2, and Myt1l) converts mouse fibroblasts into functional neurons (Vierbuchen et al., 2010). In this study, we used the strategy of direct conversion to generate chondrocytes from human somatic cells.

During skeletal development, chondrogenesis starts from condensed mesenchyme tissue, which differentiates into chondrocytes and begins secreting the molecules that form the extracellular matrix and leads to endochondral ossification. Cartilage is a stiff yet flexible connective tissue found in many areas in the bodies of humans and other animals. It is composed of chondrocytes, which...
produce a large amount of collagen fiber, an abundant ground substance rich in proteoglycans, and elastin fibers. Developmentally, the undifferentiated mesenchymal cells migrate into the limb field and condense to form the cartilage anlage. Bone morphogenetic proteins and transforming growth factor-β initiate the chondrogenic program and have significant effects on chondrogenesis through distinct mechanisms in a stage-specific manner. In addition to soluble factors, the high mobility group–domain transcription factors such as Sox5, Sox6, and Sox9 control chondrogenic differentiation, maintain the chondrocyte phenotype, and regulate expression of extracellular matrix molecules, such as cartilage-specific collagen type II (Lefebvre et al., 1997).

Murine chondrocytes can be converted from fetal fibroblasts by the direct reprogramming method using the cartilage-specific transcription factors Sox9, c-Myc, and Klf4 (Hiramatsu et al., 2011), but human chondrocytes converted from different types of cells have not yet been reported. In the present study, we generated chondrosarcoma cell lines derived from human placenta by the direct reprogramming method, using a different set of genes. Placental membrane can be obtained at every delivery and is usually discarded. Therefore it is an easily accessible cellular source without ethical problems.

RESULTS

Isolation of cells from smooth chorion and decidua

We used smooth chorion and decidua for a cell source by removing the amnion from the placental membrane and used the explant culture method in which the cells are outgrown from pieces of smooth chorion and decidua attached to dishes (Figure 1A and Supplemental Figure S1). The adherent chorion- and decidua-derived cells were passaged when the cells reached ∼80% confluence. These placenta-derived cells continued to grow for 30 d, which was five population doublings (PDs), before reaching senescence (Figure 1B). The cells at four PDs were used as “parental cells” for conversion analysis.

FIGURE 1: Characterization of infected cells. (A) Cell source for infection. Smooth chorion- and decidua-derived cells were used to investigate chondrogenesis by direct reprogramming. Bars, 1 mm. (B) Cell cultivation. (a) Phase contrast micrograph of parental cells. Bars indicate 200 μm. (b) Growth curve of parental cells. (C) Cells infected with five genes. (a) Phase contrast micrograph of infected cells. Bars, 200 μm. (b) Growth curve of infected cells. Orange, clone 01; red, clone 02; blue, clone 03; green, clone 05. Vertical axis indicates population doublings (PDs), and horizontal axis indicates days after infection. (D) Quantitative RT-PCR of TERT expression in the infected cell lines (clones 01, 02, 03, and 05). Individual RNA expression levels were normalized to respective GAPDH expression levels. HeLa cells were used for reference. Error bars, SD (n = 3). (E) Genomic DNA PCR analysis of uninfected and infected cells. To investigate chromosomal integration of the genes by retroviral infection, we performed genomic DNA PCR analysis, using transgene-specific primers of each gene. Five transgenes (BCL6, T, c-MYC, MITF, and BAF60C) were detected in all of the infected cell lines. (F) Southern blot analyses of the infected cells (clone 01). Genomic DNA was digested with SpeI, MfeI, BglII, and BamHI and then probed for probes of the genes for BCL6, T, c-MYC, MITF, respectively. The transgenes (BCL6, T, c-MYC, MITF, and BAF60C) were detected in all of the infected cell lines. Arrows indicate bands corresponding to the endogenous genes. (G) STR analysis of parental cells and the infected cells. All of the infected cells exhibited the same STR patterns as parental cells. (H) G-band chromosome analysis for parental cells with XX chromosomes and infected cells 01. (I) G-band chromosome analysis for parental cells with XY chromosomes and infected cells 05.
Infection of transcription factors into placenta-derived cells
To select candidates for transcription factors that would be required to reprogram fibroblasts to a cartilage fate, we used microarray analyses to identify transcription factors and chromatin remodeling factors with greater expression in mouse embryonic stem cell that are differentiated into mesoderm. We started with a 14-gene set, that is, genes for mesoderm-specific transcription factors (T, MITF, TBX5, TBX20, CSX/NKX2.5, GATA4, MEF2C, MESP1, ISL1, BCL6, and PRDM16) and chromatin-remodeling/reprogramming factors (BAF60C, c-MYC, and KLF4). We generated individual retroviruses to efficiently express each gene. Viral infections were preceded by transfection of small interfering RNA (siRNA) to the p53 gene (Supplemental Figure S2). Parallel experiments using retrovirus carrying the EGFP gene indicated that infection efficiency was nearly 100%. We investigated expression of cartilage-associated genes such as Collagen Type II α1 (COL2A1), Collagen Type X α1 (COL10A1), LINK PROTEIN-1 (CRTL1), and AGGRECAN (ACAN) by reverse transcriptase (RT)-PCR and identified five genes (BCL6, T, c-MYC, MITF, and BAF60C) that induced chondrocyte gene expression. The induction levels of the cartilage-associated genes were greatly reduced by elimination of any one gene from the five-gene set. We thus decided to use the five-gene set for chondrogenic induction for subsequent experiments. After we seeded infected cells on mouse embryonic fibroblasts (MEFs), we detected a very large number of mouse embryonic stem cell-like colonies on MEFs 15 d after infection of the 5F pool (Figure 1C, a). Efficiency of colony formation (colony number per the number of cells infected) was 5.76 ± 0.21 × 10^{-4}. We randomly picked four clones and analyzed cell growth rates. The cells replicated at a rate of once every 2 d and continued to grow for >150 d without reaching senescence (Figure 1C, b). All four clones expressed the TERT gene after establishment as a cell line (Figure 1D). The cells infected with the five genes exhibited a chondrogenic phenotype with malignant transformation, as shown by following results, and were thus designated induced chondrosarcoma (iCS) cells.

To determine chromosomal insertion of the genes, we performed genomic DNA PCR analysis (Figure 1E). The genes encoding BCL6, T, c-MYC, MITF, and BAF60C were detected in chromosomal genome of the four clones. Southern blot analysis with cDNA probes of each of the five genes (BCL6, T, c-MYC, MITF, and BAF60C) confirmed that each clone had chromosomal integration of the exogenously infected genes (Figure 1F and Supplemental Figure S3). The analysis of the 16 short tandem repeats (STRs) revealed that the infected clones were derived from parental cells: clones 1, 2 and 3 were derived from parental cells of the same donor with XX chromosomes, and clone 5 was derived from different parental cells with XY chromosome (Figure 1G). The STR patterns of the infected cells differed from those of any cell lines deposited on National Institutes of Health website (http://stemcells.nih.gov/research/nihresearch/scunicligenotyping.htm), implying that the cells generated are not a contamination of previously established cell lines. To determine the karyotypes of the iCS cell lines, karyotypic analysis was performed at different passages (P6– P23). Chromosomal G-band analyses showed that each clone had a normal karyotype with 46XX and 46XY (Figure 1, H and I, respectively). We then performed karyotypic analysis on iCS clones after prolonged passages (P15 and P23 for iCS-01; P13 and P21 for iCS-02; P12 and P21 for iCS-03; P7 and P23 for iCS-05, and did not detect any significant karyotypic change (Supplemental Figure S4).

In vitro chondrogenic phenotypes of the cells infected with the 5F pool
To investigate whether the infected cells exhibit a chondrogenic phenotype in vitro, we performed RT-PCR analysis using primers of the cartilage-specific genes (Figure 2A and Supplemental Table S1; Sekiya et al., 2002; Shirasawa et al., 2006). All the cell lines expressed the chondrocyte-specific/associated transcription factors (SOX5, SOX6, and SOX9), structural genes (COL1A1, COL2A1, CRTL1, and ACAN), and immortalizing gene (TERT). To see whether the endogenous genes for BCL6, T, c-MYC, MITF, and BAF60C were expressed by reprogramming, we performed RT-PCR analysis by the primers specific to the endogenous gene but not the transgenes (Supplemental Figure S5). Endogenous genes such as T, MITF, and BAF60C were induced (Figure 2B). To determine the surface markers of the cells, we performed flow cytometric analysis. All clones were positive for CD44, CD49c, CD151, and CD166 but not CD117 and CD133, suggesting that the cell marker pattern of iCS cells is compatible with that of chondrocytes (Figure 2C; Grogan et al., 2007). Western blot analysis revealed that all the infected cells expressed COL2A1 and COL1A1 at the protein level (Figure 2D and Supplemental Figure S6). Comprehensive gene expression analysis showed that the expression pattern of the infected cells is similar to that of human adult chondrocytes and human fetal chondrocytes (Figure 2E).

Expression of cartilage-specific genes such as Sox9, Aggrecan, and Matrix Gla-protein was detected in the infected cells and chondrocytes but not in the parent human smooth chorion and decidua cells (Figure 2F). Conversely, expression of placenta-associated genes such as GATA3, CD200, PDCD1LG2, OLR1, TEK, HSD17B2, and FOXF1 was lost in the infected cells. Hierarchical clustering analysis revealed that the infected cells were grouped into the same category that includes chondrocytes obtained from human fetuses and adults (Figure 2G). In addition, principal component analysis (PCA) revealed that the infected cells and chondrocytes showed similar scores in the PC2 axis (Figure 2H). The representative genes (principal components) of the PC2 axis in Table 1 include cartilage-specific genes such as Aggrecan, Fibromodulin, and Matrix Gla-protein (Plass and Wong-Palms, 1993; Yagami et al., 1999; Sekiya et al., 2002; Hjorten et al., 2007; Surmann-Schmitt et al., 2009).

Inhibition of five factors by small interfering RNA
To investigate the involvement of the five factors in chondrogenesis, we suppressed their expression by siRNA (Supplemental Table S2). The mRNAs for the five factors (BCL6, T, c-MYC, MITF, and BAF60C) were significantly decreased by siRNAs compared with control cells transfected with control siRNAs (Figure 3, A and B, and Supplemental Figures S7–S9). Morphological changes in the siRNA-treated cells were too variable to interpret. Gene expression of the chondrogenic-specific/associated transcription factors (SOX5, SOX6, and SOX9) and structural genes (COL1A1, COL2A1, CRTL1, and ACAN) decreased significantly in siT (siRNA to the T gene)-transfected cells compared with cells treated with control siRNA (Figure 3C and Supplemental Figure S10), suggesting that T is necessary for chondrogenic conversion (Hoffmann et al., 2002). In addition, expression of the genes for SOX5, SOX6, COL1A1, and COL2A1 decreased significantly in siMitF-transfected cells compared with cells transfected with control siRNA, suggesting that MITF is also necessary for chondrogenic conversion. In contrast, treatment of siRNA to BCL6, c-MYC, and BAF60C did not alter cartilage-related genes (Zelzer and Olsen, 2003; Levy and Fisher, 2006). siMitF diminished the cobblestone appearance of iCS colonies and the cell lining at the periphery of iCS colonies and altered the appearance of the iCS cells to a fibroblast-like morphology, which may be related to decreased expression of the cartilage-associated genes.
FIGURE 2: Chondrogenic phenotypes of infected cells. (A) RT-PCR analysis of the genes encoding cartilage-specific proteins (SOX5, SOX6, SOX9, COL1A1, COL2A1, COL10A1, CRTL1, and ACAN), immortalizing gene (TERT), and the infected genes (BCL6, T, c-MYC, MITF, and BAF60C). Primers that detect both the transgenes and endogenous genes for BCL6, T, c-MYC, MITF, and BAF60C were used (Supplemental Figure S5C). RNAs from the following sources were used for positive controls: heart for BCL6, MITF, BAF60C, and GAPDH; iPS cells for T, c-MYC, and TERT; and cartilage for COL1A1, COL2A1, COL10A1, CRTL1, ACAN, SOX5, SOX6, and SOX9. H2O (water without RNA) served as a
negative control. (B) RT-PCR analysis of the endogenous genes encoding T, MITF, and BAF60C. The primers were prepared to amplify the endogenous genes but not the transgenes. RNAs from the following sources were used for positive controls: heart for BCL6, MITF, BAF60C, and GAPDH; and iPS cells for T and c-MYC. H2O (water without RNA) served as a negative control. (C) Flow cytometric analysis of cell surface markers on the parental cells and infected cells. All of the results were compared with each isotype control. The X- and Y-axes indicate the intensity and the cell number, respectively. (D) Western blot analysis of COL2A1 protein in the infected cells and parental cells. (E, F) The heat map in the infected cells and parental cells. Each row represents a gene; each column represents a cell population. Expression levels of representative genes are shown in F. (G) Hierarchical clustering analysis (TIGR MeV; see Materials and Methods), based on expression levels of the cartilage-associated genes. (H) Principal component analysis of gene expression levels.

**TABLE 1:** Representative genes in PC2 axis of the PCA.

| Gene symbol | Description | Gene symbol | Description |
|-------------|-------------|-------------|-------------|
| ACAN        | Aggrecan    | CLEC4D      | C-type lectin domain family 4, member D |
| FMOD        | Fibromodulin| NRAP        | Nebulin-related anchoring protein |
| MGP         | Matrix Gla protein | OR2V2 | Olfactory receptor, family 2, subfamily V, member 2 |
| LRRC48      | Leucine-rich repeat containing 48 | KCNH7 | Potassium voltage-gated channel, subfamily H (eag-related), member 7 |
| SLPI        | Secretory leukocyte peptidase inhibitor | KCNK17 | Potassium channel, subfamily K, member 17 |
| RAB11FIP4   | RAB11 family interacting protein 4 (class II) | DRD1 | Dopamine receptor D1 |
| TLR5        | Toll-like receptor 5 | CTNN2 | Catenin (cadherin-associated protein), α2 |
| NEBL        | Nebulette    | FMR1NB      | Fragile X mental retardation 1 neighbor |
| RAB11FIP4   | RAB11 family interacting protein 4 (class II) | ABCC12 | ATP-binding cassette, subfamily C (CFTR/MRP), member 12 |
| CAPG        | Capping protein (actin filament), gelsolin-like | SLITRK3 | SLIT and NTRK-like family, member 3 |
| SLC26A4     | Solute carrier family 26, member 4 | CIITA | Class II, major histocompatibility complex, transactivator |
| MIF         | Macrophage migration inhibitory factor (glycosylation-inhibiting factor) | GP2 | Glycoprotein 2 (zymogen granule membrane) |
| CALR3       | Calreticulin 3 | OR12D3 | Olfactory receptor, family 12, subfamily D, member 3 |
| ESPN        | Espin        | GALNTL4     | UDP-N-acetyl-α-D-galactosamine |
| SLC7A2      | Solute carrier family 7 (cationic amino acid transporter, γ+ system), member 2 | BRSK2 | BR serine/threonine kinase 2 |
| ZBTB10      | Zinc finger and BTB–domain containing 10 | L08961 | Transmembrane tyrosine kinase mRNA |
| ND3         | NADH-ubiquinone oxidoreductase chain 3(NADH dehydrogenase subunit 3) | RAB33B | RAB33B, member RAS oncogene family |
| EFNA1       | Ephrin-A1    | ELA1        | Elastase 1, pancreatic |
| RGMA        | RGM domain family, member A | ASPA | Asparoacylase (Canavan disease) |
| ENST00000390243 | Immunoglobulin κ-light-chain V gene segment | IL18RAP | Interleukin 18 receptor accessory protein |
| GPA33       | Glycoprotein A33 (transmembrane) | EPHA8 | EPH receptor A8 |
| CLMN        | Calmin (calponin-like, transmembrane) | CXCR6 | Chemokine (C-X-C motif) receptor 6 |
| RAB11FIP4   | RAB11 family interacting protein 4 (class II) | BAGE | B melanoma antigen |
| KRT26       | Keratin 26   | SIRPG       | Signal-regulatory protein γ |
| YBX2        | Y box–binding protein 2 | AF083118 | CATX-2 mRNA |
| EEF1G       | Eukaryotic translation elongation factor 1 γ | TSPAN16 | Tetraspanin 16 |
| NAG18       | NAG18 protein on chromosome 19 | AF028840 | Kruppel-associated box protein mRNA |
| CX62        | Connexin 62  | WIF1        | WNT inhibitory factor 1 |
| KCNC2       | Potassium voltage-gated channel, Shaw-related subfamily, member 2 | TTY9A | Testis-specific transcript, Y-linked 9A (TTY9A) on chromosome Y |
| TSPAN33     | Tetraspanin 33 | LRRCS5 | Leucine-rich repeat containing 50 |
| PTCH1       | Patched homologue 1 (Drosophila) | ENST0000037416 | Collagen, type XXVII, α1 |
| DEFB126     | Defensin, β126 | WFDC12 | WAP four-disulfide c ore domain 12 |

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injected cells generated cartilage that exhibited metachromasia by toluidine blue staining and were light blue when stained with Alcian blue (Figure 4A). In contrast, implantation of parental cells produced neither tumor nor cartilage (Figure 4E). RT-PCR analysis showed that iCS cartilage expressed genes for COL1A1, COL2A1, COL10A1, CRTL1, ACAN, CD44, CD49c, CD151, and CD166 (Figure 4B). Western blot analysis showed that iCS cartilage produced collagen type II at the protein level (Figure 4C). We also performed immunohistochemical analysis using antibodies to vimentin, collagen type II, and Ki-67. The antibody for vimentin that we used specifically reacts with human protein but not murine protein. The antibody for Ki-67 reacts with a human nuclear cell proliferation–associated antigen, and thus it does not react with differentiated chondrocytes. iCS cells stained positive for human vimentin, and extracellular matrix was positive for collagen type II, implying that the injected human cells generate cartilage (Figure 4D). Nearly 30% of iCS cells stained positive for Ki-67, indicating that iCS cells continued to replicate in cartilage at 7 wk after injection. iCS cells in the tumor had large nuclei with coarse chromatin structure and one or two nucleoli, and the ratio of nucleus/cytoplasm was large. The tumors generated by iCS cells had large nuclei with coarse chromatin structure and one or two nucleoli, and the ratio of nucleus/cytoplasm was large. The tumors generated by iCS cells were histopathologically diagnosed as chondrosarcoma by a certified pathologist (A.U.). Anchorage-independent colony formation is a hallmark of transformation and an in vitro correlate of tumorigenicity in vivo (Cremona and Lloyd, 2009). After cultivation in MethoCult H4034 medium, colony formation was evaluated (Figure 5). The colony-forming assay clearly revealed that iCS cells formed colonies but parental cells did not, indicating that iCS cells are transformed cells with chondrogenic potential.

Generation of chondrocytes from other human somatic cells

In addition to human smooth chorion, we used primary cultured cells from human menstrual blood and placental artery. We obtained 10 and 9 clones, respectively, from menstrual blood-derived cells and placental arterial endothelium. All of them proliferated as a chondrogenic cells with transformation and exhibited the same morphology with iCS in vitro (Figure 6). The growth rates of the clones generated from menstrual blood and placental artery were essentially the same as those of iCS cells. After implantation into the dermal tissue of nude mice, they generated chondrogenic tissue that showed metachromasia with the toluidine blue stain.
DISCUSSION

In mammals, cartilage does not regenerate in limb tissue, but cells that derive cartilage retain a strong memory of their embryonic origin in the axolotl (Kragl et al., 2009). Cells are undergoing reprogramming that allows them to reenter embryonic programs of tissue formation, even if they do not revert back to the pluripotent state. Here we show that expression of five transcription factors can rapidly and efficiently convert nonchondrocytes (chorion- and decidua-derived cells) into chondrocytes. iCS cells displayed functional chondrogenic properties such as the generation of extracellular matrices. The possibility of redirecting cell differentiation by overexpression of genes was suggested by Weintraub with the identification of the MyoD “master” gene (Davis et al., 1987). The process was believed to involve reversion to a less differentiated state, a kind of dedifferentiation, before the new cell type is formed. Another process has since been suggested, the concept of direct conversion or direct reprogramming without dedifferentiation. This process is believed to be direct lineage switching rather than lineage switching back to a branch point and out again in a different direction. Direct conversion has been shown in β cells, cardiomyocytes, and neurons. A specific combination of three transcription factors (Ngn3, Pdx1, and MafA) reprograms differentiated pancreatic exocrine cells in adult mice into cells that closely resemble β cells (Zhou et al., 2008); a combination of three factors (Gata4, Tbx5, and Baf60c) induces noncardiac mesoderm to differentiate directly into contractile cardiomyocytes (Ieda et al., 2010); and a combination of three factors (Ascl1, Brn2, and Myt1l) converts mouse fibroblasts into functional neurons (Vierbuchen et al., 2010).

In this study, we used the strategy of direct conversion to generate chondrocytes from human extraembryonic somatic cells. Based on the same method, murine chondrocytes were generated from skin fibroblasts (Hiramatsu et al., 2011) using the three transcription factors Sox9, c-Myc, and Klf4. Sox9 is a determinant of chondrogenic lineage (Lefebvre et al., 1997), c-Myc is a cell cycle driver (Schmidt, 1999), and Klf4 is involved in the down-regulation of p53 (Rowland et al., 2000).

We performed histological analysis and immunohistochemical analysis using the human vimentin-specific antibody. The parental cells did not exhibit cartilage formation at the injected site. Left, HE stain. Right, immunohistochemistry using human-specific antibody to vimentin. Bars, 100 μm.

FIGURE 4: In vivo chondrogenic phenotypes of iCS cells. (A) Cartilage at 7 wk after injection of iCS cells. (a) HE stain, (b) Alcian blue, (c) toluidine blue. iCS cells at passage 3 were injected subcutaneously to the dorsal flank of athymic nude mice. Areas of extracellular matrix accumulation stain light to dark blue with Alcian blue (b) or light- to dark-red/purple with toluidine blue (c). Bars, 50 μm. These results are representative of five independent experiments. (B) RT-PCR analysis of the genes encoding SOX5, SOX6, SOX9, COL1A1, COL2A1, COL10A1, CRTL1, and ACAN in cartilage generated by iCS cells. Human cartilage and H2O (water without RNA) served as positive and negative controls, respectively. Parental cells in culture serve for comparison. (C) Western blot analysis of COL2A1 protein in iCS cartilage at 7 wk after subcutaneous injection of iCS cells into athymic nude mice. Human cartilage and H2O (water without RNA) served as positive and negative controls, respectively. Parental cells in culture serve for comparison. (D) Immunohistochemical analysis of iCS cartilage. (a) Vimentin, (b) collagen type II (COL II), (c) Ki-67. (E) Implantation of the parental cells. We injected parental cells into athymic nude mice but did not detect any tumor formation.
et al., 2005). In this study, T and MITF may act as inducers of chondrogenic fate determinant, and BA6F0C may act as epigenetic modifier. Klf4 was unnecessary for conversion from placental cells to chondrocytes, unlike the reprogramming described for iPSC cells (Takahashi and Yamanaka, 2006) and chondrocytes (Hiramatsu et al., 2011). Ba6F0C permits binding of Gata4 to cardiac genes for reprogramming toward cardiomyocytes (Takeuchi and Bruneau, 2009). Likewise, BA6F0C may initiate expression of chondrogenic gene sets in combination with T and MITF in iCS cells.

We showed that the transgene set was sufficient for chondrogenesis without exogenous SOX9 gene. The T gene, one of the transcription factors used in this study, is a member of the T-box family of transcription factors (Papaioannou and Silver, 1998), all of which play key roles during early development, mostly in the formation and differentiation of normal mesoderm (Showell et al., 2004). T is also transiently induced in vivo in rhesus monkey embryonic stem cells and mouse embryonal carcinoma cells undergoing mesodermal differentiation (Vidricaire et al., 1994; Behr et al., 2005). Both T and Sox9 are downstream of mitogen-activated protein kinase signaling via fibroblast growth factor receptors in chondrogenesis (Hoffmann et al., 2002). Chondrogenic conversion of the human extraembryonic cells was accompanied by induction of the endogenous Sox9 gene; however, the induced Sox9 did not complement reduction of chondrogenesis by siRNA to the T gene. MITF was also shown to be involved in chondrogenic conversion, although involvement of MITF in chondrogenesis has not been reported. MITF belongs to an evolutionarily ancient family of the bHLH/LZ proteins (Atchley and Fitch, 1997) and is known to regulate a number of genes of importance in differentiation and maintenance of the melanocytic lineage (Tachibana et al., 1996); conversely, loss-of-function mutations in MITF produce depigmentation (Newton et al., 2001). Disruption of MITF does not affect chondrocytic differentiation during development, and alternative factors may therefore be present in cells of chondrogenic lineage. The chondrogenesis by T and MITF may not reflect developmental or physiological pathway, but these two factors are requisite for in vitro chondrogenic conversion of nonchondrocytes.

Stability of the chondrogenic phenotype of iCS cells after long-term cultivation is probably due to lack of transgene silencing—in other words, continuity of transgene expression during cultivation and expression of endogenous chondrogenic genes in some cases. Chondrogenic induction is mediated at least in part by reprogramming, because expression of the endogenous T gene was initiated by the five factors. It remains unclear whether the process of the conversion is via dedifferentiation into multipotent, oligopotent stem cells or undifferentiated progenitors. Placenta is developmentally distinct from cartilage, and conversion from placenta to chondrocytes is considered lineage switching or transdifferentiation rather than differentiation. In β cells (Zhou et al., 2008) and retinal cells (Osakada et al., 2009), direct reprogramming is achieved with hepatocytes and iris cells, respectively, which are developmentally close to the generated cells. Successful reprogramming with other somatic cells for parental cells (Figure 6) indicates that the conversion is indeed reprogramming. Autoregulatory feedback and feedforward activation of downstream transcriptional regulators reinforces the expression of important cell fate–determining genes and helps to further stabilize the induced transcriptional program. Robust changes in transcriptional activity can be explained by genome-wide adjustments of repressive and active epigenetic features, such as DNA methylation, histone modifications, and changes of chromatin-remodeling complexes that further stabilize the new transcriptional network (Zhou et al., 2008). It is possible that certain subpopulations of cells are “primed” to respond to these factors, depending on their preexisting transcriptional or epigenetic states (Yamanaka, 2009).

Our study opens an avenue to generate human chondrocytes. Even with the presence of retroviral integration, human iCS cells can possibly be used for tissue engineering experiments such as screening of suitable scaffold of cartilage and can be an alternative to murine ATDC5 teratocarcinoma cells, an ideal cell line for development of tissue engineering strategies aimed at cartilage generation. Once the safety issue, that is, cell transformation, is overcome, iCS cells should also be applicable for repair of defective cartilage in regenerative medicine. We should, however, exercise caution because human iCS cells are not identical to human chondrocytes from the viewpoint of global gene expression. Further studies are essential to determine whether a nontransformed counterpart of iCS can replace chondrocytes in medical applications.

**MATERIALS AND METHODS**

**Preparation of tissue and procedure for cell culture**

A human placenta was collected after delivery of a male neonate with the approval of the Ethics Committee of the National Research Institute for Child Health and Development, Tokyo, Japan. Signed informed consent was obtained from the donors, and the specimens were irreversibly deidentified. All experiments handling human cells and tissues were performed in line with the Tenets of the Declaration of Helsinki. This study was wholly carried out at the National Research Institute for Child Health and Development, Tokyo, Japan.
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ered saline with 0.1% trypsin and 0.25 mM EDTA for 5 min at 37°C and counted. The harvested cells were reseeded at a density of \(3 \times 10^5\) cells in a 10-cm dish. Confluent monolayers of cells were subcultured at a 1:8 split ratio onto new 10-cm dishes. The culture medium was replaced with fresh culture medium every 3 or 4 d.

**Plasmid construction**

Full-length of transcription factors BCL6, T, c-MYC, MITF, and BAF60C were amplified from cDNAs prepared from total RNA of adult human heart cells (Clontech, Mountain View, CA) and embryonic body-formed iPS cells (day 3–4) by reverse transcription-PCR with the primers listed in Supplementary Table 1, and by digestion with HindIII sites of pMXs and T4 DNA polymerase accessory they were used for DNA synthesis. The cDNA plasmid was subcloned into pMXs vector using HindIII restriction sites. pMXs was a gift from T. Kitamura (Tokyo University, Tokyo, Japan).

**Retroviral infection**

293FT cells (5 \(\times\) \(10^6\)) were plated in a 10-cm dish and incubated overnight. The next day, the cells were cotransfected with pMXs-BCL6, pMXs-T, pMXs-c-MYC, pMXs-MITF, pMXs-BAF60C, pCL-GagPol, and pHCMV-VSV-G vectors with TransIT-293 reagent (Mirus, Madison, WI). The medium was replaced with fresh medium 24 h after transfection. The medium was collected after 48 h as the virus-containing supernatant. Placenta-derived cells in primary culture were seeded at 1 \(\times\) \(10^5\) cells per six-well plate 1 d before infection. The virus-containing supernatants were filtered through a 0.45-μm pore-size filter, ultracentrifuged at 8000 \(\times\) g for 24 h, and then resuspended in Knockout-DMEM (Invitrogen) supplemented with 2 mg/ml Polybrene (Nacalai Tesque, Kyoto, Japan). Cells in six-well plates were transfected with siRNA to the p53 gene (siTP53) using RNAiMAX (Invitrogen, Carlsbad, CA) according to the protocols recommended by the manufacturer (Hong et al., 2009). Cells were transfected overnight, washed, and resuspended in Opti-MEM (Life Technologies/Invitrogen) and were used for virus infection. Cells were trypsinized and plated in six-well plates at 1 \(\times\) \(10^5\) cells per well for 24 h before transfection. The medium was changed every 2 d with fresh Poweredby10 (GP Biosciences, Yokohama, Japan). Colonies were picked up and transferred into 2-ml Poweredby10 medium at \(\sim\)10 d after infection. The colonies were mechanically dissociated to small clumps by pipetting up and down. The cell suspension was transferred on irradiated MEF feeder in 60-mm dish (Iwaki; Asahi Techno Glass, Tokyo, Japan). We defined this stage as passage 1.

To isolate placenta-derived cells, we used the explant culture method, in which the cells were outgrown from pieces of chorion and decidual cells attached to dishes (Supplemental Figure S1). Briefly, the smooth chorion and decidua were cut into pieces ~2 mm³ in size. The pieces were washed in DMEM (high glucose; Sigma-Aldrich, St. Louis, MO) supplemented with 100 U/ml penicillin–streptomycin (Life Technologies, Carlsbad, CA), 1 μg/ml amphotericin B (Life Technologies), and 4 U/ml Novo-Heparin Injection 1000 (Mochida Seiyaku, Tokyo, Japan) until the supernatant was free of erythrocytes. Some pieces were attached to the substratum in a 10-cm dish. The cells migrated out from the cut ends after \(\sim\)20 d of incubation at 37°C. The migrated cells were harvested with Dulbecco’s phosphate-buff-

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**FIGURE 6:** Generation of iCS cells from other human somatic cells. (A) iCS generation from human menstrual blood. (a) Phase contrast micrograph of menstrual blood–derived cells. (b) Phase contrast micrograph of iCS cells from menstrual blood. (c–e) Histological analysis of iCS cartilage generated from menstrual blood. (c) HE stain, (d) Alcian blue (Al-B), (e) toluidine blue (ToI-B). Bars, 200 μm (a, b) and 50 μm (c–e). (B) iCS generation from human placental artery (hPAE). (a) Phase contrast micrograph of hPAE cells. (b) Phase contrast micrograph of iCS cells from hPAE cells. (c–e) Histological analysis of iCS cartilage generated from hPAE cells. (c) HE stain, (d) Alcian Blue (Al-B), (e) toluidine blue (ToI-B). Bars, 200 μm (a, b) and 50 μm (c–e).
RT-PCR
Total cellular RNA was isolated from cells using an Isogen extraction kit (Nippon Gene, Tokyo, Japan) according to the manufacturer’s protocol. Total RNA (1.0 μg each) for RT-PCR was converted to cDNA with Superscript III RNase H– reverse transcriptase (Invitrogen), according to the manufacturer’s manual. PCR conditions were optimized, and the linear amplification range was determined for each primer by varying annealing temperature and cycle number. PCR products were identified by positive control primer. Sequences are provided in Supplemental Table S1 and Supplemental Figure S5. RT-PCR was performed by using the primers for the genes BCL6, T, c-MYC, MITF, Baf60C, COL1A1, COL2A1, COL10A1, CRT1L, ACAN, SOX5, SOX6, SOX9, TERT, and GAPDH. Adult chondrocyte RNA (Cell Applications (San Diego, CA), human heart RNA (Clontech), and human iPSC cell RNA were used as positive controls for RT-PCR analysis. PCR was performed with KOD FX DNA polymerase and PCR buffer (Toyobo, Osaka, Japan), 35 cycles, with each cycle consisting of 95°C for 30 s, 60°C for 45 s, and 72°C for 45 s, with additional 5-min incubation at 72°C after completion of the final cycle. The PCR products were size fractionated by 2% agarose gel electrophoresis.

Quantitative RT-PCR
RNA was extracted from cells using an Isogen extraction kit according to the manufacturer’s protocol. An aliquot of total RNA was reverse transcribed by using an oligo(dT) primer. For the thermal cycle reaction, the cDNA template was amplified (PRISM 7900HT Sequence Detection System; Applied Biosystems, Foster City, CA) using the Platinum Quantitative PCR SuperMix-UDG with ROX (11743-100; Invitrogen) under the following reaction conditions: 40 cycles of PCR (95°C for 15 s, and 60°C for 1 min) after an initial denaturation (95°C for 2 min). Fluorescence was monitored during every PCR cycle at the annealing step. The authenticity and size of the PCR products were confirmed using a melting curve analysis (using software provided by Applied Biosystems) and gel analysis. mRNA levels were normalized using GAPDH as a housekeeping gene.

Flow cytometric analysis
Cells were stained for 1 h at 4°C with primary antibodies and immunofluorescent secondary antibodies. The cells were then analyzed on a Cytomics FC 500 (Beckman Coulter, Brea, CA), and the data were analyzed with FlowJo, version 7 (Tree Star, Ashland, OR). Antibodies against human CD44, CD49c, CD151, and CD166 (all from BD Biosciences Pharmingen, San Diego, CA) were adopted as primary antibodies.

Histological analysis
Infected cells were harvested by trypsin/EDTA treatment, collected into tubes, and centrifuged at 300 × g for 5 min, and the pellets were suspended in the DMEM medium. The same volume of Base- ment Membrane Matrix (BD Biosciences Pharmingen) was added to the cell suspension. The cells were implanted subcutaneously to a BALB/c-nu/nu mouse (CREA, Tokyo, Japan) for 7 wk. Tumors were dissected and fixed with phosphate-buffered saline containing 4% formaldehyde. Paraffin-embedded tissue was sliced and stained with hematoxylin and eosin.

Western blotting
Semiconfluent cells were lysed with Celllytic M cell Lysis Reagent (Sigma-Aldrich) supplemented with a protease inhibitor cocktail (Sigma-Aldrich). Cell lysates (20 μg each) were separated by electrophoresis on NuPAGE Novex Tris-Acetate gel (Invitrogen), and transferred to Immobilon-P transfer membrane (Millipore, Billerica, MA). The membrane was soaked in protein blocking solution (Blocking One solution; Nacalai Tesque) for 30 min at room temperature before an overnight incubation at 4°C with primary antibody for COL2A1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) and GAPDH (1:1000; Cell Signaling, Beverly, MA) also diluted in blocking solution. The membrane was then washed three times with TBST (20 mM Tris-HCl, pH 7.6, 136 mM NaCl, and 0.1% Tween-20), incubated with a horseradish peroxidase–conjugated secondary antibody (0.04 μg/ml) directed against the primary antibody for 45 min, and washed three times with TBST. The signal was detected by an enhanced ECL Plus Western Blotting Detection System (GE Healthcare, Pisccataway, NJ) and an LAS3000 imaging system (Fujifilm, Tokyo, Japan), following the manufacturers’ recommendations.

siRNA transfection
The infected cells in six-well plates were transfected with siRNA using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the protocols recommended by the manufacturer (Cui et al., 2011). The cells were harvested 48 h after transfection and analyzed by real-time PCR and RT-PCR.

Gene expression microarray
Total RNA was prepared from duplicate biological samples, and human adult chondrocyte RNA was purchased from Cell Applications. With the use of Low RNA Input Fluorescent Linear Amplification Kits (Agilent Technologies, Santa Clara, CA), cDNA was reverse transcribed from each RNA sample, as well as from a pooled reference control, and cRNA was then transcribed and fluorescently labeled with Cy3. cRNA was purified using an Agilent One Color Spike Mix Kit (Agilent Technologies). We hybridized 1650 ng of Cy3-labeled and amplified cRNA to Agilent 4 × 44 K whole human genome microarrays and processed it according to the manufacturer’s instructions. The array was scanned using a G2505B DNA microarray scanner (Agilent Technologies). The image files were extracted using Feature Extraction software, version 10.7.3.1 (Agilent Technologies), with background subtraction and dye normalization. The data were analyzed using GeneSpring GX 10.0 (Agilent Technologies).

Hierarchical clustering analysis and principal component analysis
To analyze the microarray data, we used agglomerative hierarchical clustering and PCA. The hierarchical clustering techniques classify data by similarity using NIA Array Analysis (http://lgsun.grc.nia.nih.gov/ANOVA), and their results are represented by dendrograms. PCA is a multivariate analysis technique that finds major patterns in data variability using TIGR MeV (www.tm4.org/mev.html; Toyoda et al., 2011).

Southern blot analysis
For Southern blot analysis, genomic DNA was isolated using the DNeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol, digested with SpeI and BamHI for BCL6, SpeI and NcoI for BAF60C, SpeI and BglII for MITF and T, and SpeI and MfeI or BamHI and MfeI for c-MYC, and separated via 0.8% agarose gel electrophoresis. Transfer was to Hybond-N membranes (GE Healthcare). The membrane was fixed under UV irradiation. The probe was hybridized to the blot and detected using CDP-Star detection reagent (GE Healthcare). Signals from the labeled DNA were quantified using a Hyper film ECL (GE Healthcare).

Short-tandem repeat analysis
Genomic DNA was isolated from cultured cell samples using DNeasy columns (Qiagen). This was used as template for STR analysis using
the PowerPlex 16 System (Promega, Madison, WI) and PRISM instrumentation (Applied Biosystems). Numbers shown denote base pair lengths of the 15 autosomal fragments. The analysis was carried out at Nihon Gene Research Laboratories (Sendai, Japan).

Karyotypic analysis

The cells were fixed with methanol/glacial acetic acid (2:5) three times and dropped onto glass slides (Nihon Gene Research Laboratories). Chromosome spreads were Giemsa banded and photographed. A minimum of 10 metaphase spreads were analyzed for each sample, and they were karyotyped using a chromosome imaging analyzer system (Applied Spectral Imaging, Carlsbad, CA).

Colonies formation assay

A total of 50,000 cells were resuspended in MethoCult H4034 medium and plated into a six-well plate. Colonies were counted 14 d after plating.

Gene Expression Omnibus accession numbers

National Center for Biotechnology Information Gene Expression Omnibus gene expression microarray data were submitted under accession number GSE29745.

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