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In Vitro Stage-Specific Chondrogenesis of Mesenchymal Stem Cells Committed to Chondrocytes

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Objective. **Osteoarthritis is characterized by an imbalance in cartilage homeostasis, which could potentially be corrected by mesenchymal stem cell (MSC)–based therapies. However, in vivo implantation of undifferentiated MSCs has led to unexpected results. This study was undertaken to establish a model for preconditioning of MSCs toward chondrogenesis as a more effective clinical tool for cartilage regeneration.**

Methods. **A coculture preconditioning system was used to improve the chondrogenic potential of human MSCs and to study the detailed stages of chondrogenesis of MSCs, using a human MSC line, Kp-hMSC, in commitment cocultures with a human chondrocyte line, hPi (labeled with green fluorescent protein [GFP]). In addition, committed MSCs were seeded into a collagen scaffold and analyzed for their neocartilage-forming ability.**

Results. **Coculture of hPi-GFP chondrocytes with Kp-hMSCs induced chondrogenesis, as indicated by the increased expression of chondrogenic genes and accumulation of chondrogenic matrix, but with no effect on osteogenic markers. The chondrogenic process of committed MSCs was initiated with highly activated chondrogenic adhesion molecules and stimulated cartilage developmental growth factors, including members of the transforming growth factor β superfamily and their downstream regulators, the Smads, as well as endothelial growth factor, fibroblast growth factor, insulin-like growth factor, and vascular endothelial growth factor. Furthermore, committed Kp-hMSCs acquired neocartilage-forming potential within the collagen scaffold.**

Conclusion. **These findings help define the molecular markers of chondrogenesis and more accurately delineate the stages of chondrogenesis during chondrocytic differentiation of human MSCs. The results indicate that human MSCs committed to the chondroprogenitor stage of chondrocytic differentiation undergo detailed chondrogenic changes. This model of in vitro chondrogenesis of human MSCs represents an advance in cell-based transplantation for future clinical use.**

Osteoarthritis (OA), one of the most common musculoskeletal diseases, has been ascribed to an imbalance in cartilage homeostasis in the aging process (1,2). Hyaline cartilage has little capacity for self-repair. As a result, continuous mechanical stress can lead to the degradation of articular cartilage, culminating in a vicious cycle of destructive processes (3). Many therapeutic interventions directed at the restoration of the reparative capacity of chondrocytes have been explored (3–5). One of the emerging approaches is cell-based therapy, in which expanded chondrocytes are used for cartilage repair. However, the outcome of this approach has been disappointing, due to the difficulties in maintaining chondrocytic phenotypes and the decrease in proliferative capacity of autologous chondrocytes with increasing age (1,6,7).

Cell therapy based on autologous mesenchymal stem cells (MSCs), which have a vast proliferative ca-

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pacity and differentiation potential, is an attractive strategy for treating OA (8,9). MSCs with the capacity to differentiate into the mesenchymal lineage can be isolated from the bone marrow (10–12), fat tissue (11), umbilical cord blood (13), and amniotic fluid (14). It has been reported that the chondrogenic abilities of MSCs could be triggered with various growth factors, including transforming growth factor β (TGFβ) and fibroblast growth factor (FGF) (15,16).

In the case of cartilage repair in vivo, results from recent studies of cartilage defects in animal models indicate that implanted MSCs can differentiate into chondrocyte-like cells (17,18). However, unexpected results from the direct implantation of undifferentiated MSCs were also reported, such as the calcification of implanted cells, fibrogenesis, and heterotopic tissue formation in the cartilage (19–21). To avoid such complications stemming from the implantation of undifferentiated MSCs, in vitro preconditioned MSCs may be used, since they appear to exert greater chondrogenic potential in promoting cartilage regeneration, and therefore may be considered a better candidate for OA treatment (21). A therapeutic strategy using MSCs could be an approach that guides the MSCs toward chondrogenesis by exposure to selected types of target cells (22–26).

Chondrogenesis represents one of the earliest cellular events in vertebrate skeletal development, the stages of which are mesenchymal cell condensation, the rise of chondroprogenitors, chondrogenesis, terminal differentiation of progenitor cells, and ossification (27,28). During mesenchymal cell aggregation and condensation, cell–cell interactions promote cell adhesion and the release of paracrine factors (16,28,29). Mesenchymal cells subsequently transition into chondrogenesis, which is controlled by the interplay of various factors, such as hedgehog signaling pathways, transcription factors, metabolites, and stress (16,27,28). Chimal-Monroy et al and Merino et al delineated the events involved in chondrogenesis of cartilage and bone formation in vivo, using immunohistochemical analyses of whole-mount specimens (30,31). However, the exact molecular events that occur in chondrogenesis remain unclear. Thus, a direct in vitro coculture system could more accurately determine the stages of chondrogenesis through molecular characterization.

We have previously investigated the methods of purification, characterization, differentiation, and application of multipotent MSCs (10,32–35). We have also studied the expression of matrix proteins, the type I and type II collagens, which induce chondrogenesis in human MSCs under the influence of TGFβ1 (3). In this study, we more accurately delineate the stages and define the molecular markers that are involved in chondrocytic differentiation of MSCs. In addition, we demonstrate that committed MSCs have the capacity to form neocartilage, in a collagen-based 3-dimensional (3-D) scaffold. The descriptions of the coculture conditions, molecular markers, and stages of chondrogenic differentiation of MSCs reported in this study could help in the future development of MSC-based therapies for cartilage regeneration.

MATERIALS AND METHODS

Cell lines. To examine cell–cell interactions during chondrogenesis, immortalized human articular chondrocytes were obtained from the hPi cell line (4), and immortalized human MSCs (from bone marrow) were obtained from the Kp-hMSC line as previously described (35). Both cell lines were transduced with E6/E7 genes of human papillomavirus 16 and have been previously characterized for their proliferation and differentiation potentials. For routine cultures, hPi chondrocytes were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), while Kp-hMSCs were maintained in DMEM/low glucose (LG) with 10% FBS (Gibco), in a humidified atmosphere containing 5% CO_2. Cells were passaged twice a week, and the culture medium was changed every 2 days. The hPi cells were then transduced with packaged virions of a bicistronic retroviral vector containing a green fluorescent protein (GFP) reporter gene (36), which is commonly used as a cell marker in coculture systems. As previously described, GFP-expressing stable clones (designated hPi-GFP) were selected in culture medium containing G418 (Invitrogen, Carlsbad, CA), and the transduction efficiency was determined using FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Commitment of Kp-hMSCs to hPi-GFP cells. Cocultures of hPi-GFP cells and Kp-hMSCs were established in 6-well plates. The tissue culture insert (Nalge Nunc International, Naperville, IL) used in our study consisted of a polycarbonate membrane with 0.4-μm pores, which prevents the exchange of cellular components but allows nutrient diffusions. The 4 experimental groups were as follows: group A, hPi-GFP cells cultured in DMEM/F12; group B, Kp-hMSCs cultured in DMEM/LG; group C, hPi-GFP cells cultured on the upper side of the insert membrane to achieve contact with Kp-hMSCs on the opposite membrane; and group D, coculture of hPi-GFP cells and Kp-hMSCs brought in direct contact in a monolayer mixture in DMEM/LG (Figure 1). The hPi-GFP cells (group A) and Kp-hMSCs (group B) were seeded alone (10^5 cells in each) as the control groups, while 5×10^4 hPi-GFP cells and 5×10^4 Kp-hMSCs were seeded in the 2 different coculture systems as the experimental groups (groups C and D). The medium was changed every 2 days, and cells were trypsinized at different time points for analysis.

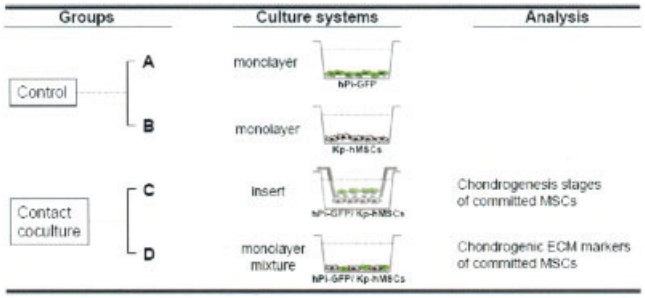

Figure 1. Experimental design. Different culture conditions were used for determining the stages of chondrogenesis of human mesenchymal stem cells (Kp-hMSCs) committed to human chondrocytes (hPi cells labeled with green fluorescent protein [hPi-GFP]). Individual cultures of hPi-GFP cells (group A) and Kp-hMSCs (group B) were used as control groups for comparisons with hPi-GFP–committed Kp-hMSCs that were brought into contact by coculture systems, involving either an insert membrane system (group C) or a direct monolayer mixture system (group D). For group C, the stages of MSC chondrogenesis were evaluated, while for group D, anti–type II collagen and Alcian blue staining were utilized to examine the synthetic chondrogenic markers of extracellular matrix (ECM) accumulation.

RNA extraction and reverse transcription–polymerase chain reaction (RT-PCR). Total RNA from cells was extracted using TRIzol reagent (Invitrogen) and subjected to RT reaction, followed by PCR amplification of the genes of interest. RT was performed with SuperScript III (Invitrogen) and an oligo$(dT)_{12–18}$ primer. Four microliters of RNA was added into a final volume of 21-μl solution containing 10 m*M* dNTP mix, 10× RT buffer, 25 m*M* $MgCl_2$, 0.1*M* dithiothreitol, RNase inhibitor, and RNase H. Six microliters of RT product was used for the amplification reaction in a final volume of 50 μl, containing 2.5 m*M* dNTP, 25 m*M* $MgCl_2$, primers specific for each gene (for *SOX9*, primer 1 [P1] GGCAGCTGTGAACTGGCCA, and P2 GCACACGGGGAACTTGTCC; for *COL2A1*, P1 CACGCAGAAGTTCACCAAGAA, and P2 CCTTGCTCCAGGGCCAGC; for aggrecan, P1 TGAGGAGGGCTGGAACAAGTACC, and P2 GGAGGTGGTAATTGCAGGGAACA; for *RUNX2*, P1 ACCATGGTGGAGATCATCGC, and P2 CATCAAGCTTCTGTCTGTGC; for *ALP*, P1 ACGTGGCTAAGAATGTCATC, and P2 CTGGTAGGCGATGTCCTTA; for osteocalcin, P1 CATGAGAGCCCTCACA, and P2 AGAGCGACACCCTAGAC; for osteopontin, P1 CAGAATCTCCTAGCCCCACA, and P2 AACTCCTCGCTTTCCATGTG; and for GAPDH, P1 GCTCTCCAGAACATCATCCCTGCC, and P2 CGTTGTCATACCAGGAAATGAGCTT), and *Taq* DNA polymerase (Invitrogen). The gene amplification reaction was carried out using a Touchgene Gradient PCR machine (Techne, Cambridge, UK), and the annealing temperatures differed depending on the genes of interest. PCR products were then run on 1% agarose gels (Agarose I; Amresco, Solon, OH) and visualized with ethidium bromide staining. Images were analyzed using FloGel-I (Fluorescent Gel Image System; Top Bio, Taipei, Taiwan). GAPDH was used as an internal control.

Microarray with real-time PCR. Real-time PCR in combination with microarray analyses was performed to quantify and identify the genes involved in skeletogenesis in our coculture system (see Figure 3A for gene classifications). Complementary DNA was used for each real-time PCR reaction (ABI Prism 7300 system; Applied BioSystems, Foster City, CA) in an RT^2 Profiler PCR Array human osteogenesis system (APHS-026A; SuperArray, Frederick, MD) with RT^2 Real-Time SYBR Green PCR master mix (PA-012; SuperArray) according to the manufacturer's instructions. Collected data were interpreted using the $\Delta\Delta C_t$ method. The C_t value for each gene from the designated groups was first standardized to its respective GAPDH value, calculated as follows: ΔC_t = gene C_t − GAPDH C_t. This normalized value was then compared between Kp-hMSC controls and cocultured Kp-hMSCs, with differences in gene expression between the 2 groups calculated using the $\Delta\Delta C_t$ method, as follows: $\Delta\Delta C_t$ = cocultured Kp-hMSC gene ΔC_t − control Kp-hMSC gene ΔC_t. To differentiate the fold change in expression of specific genes in Kp-hMSCs upon contact coculture, the fold change was calculated as $2^{-\Delta\Delta C_t}$, and these fold change values for each gene were normalized to that for GAPDH as the housekeeping gene.

Immunofluorescence staining for cell–cell interactions. Immunofluorescence staining was performed to demonstrate chondrogenesis of Kp-hMSCs upon cell–cell contact in monolayer mixture (group D, versus groups A and B as controls) (Figure 1). Cells were fixed with 80% chilled methanol and washed twice in phosphate buffered saline (PBS), followed by a blocking step using avidin/biotin blocking kit (Vector, Burlingame, CA) for 20 minutes. Immunoglobulin reactions were carried out in incubations overnight with mouse anti-human type II collagen monoclonal antibody as the primary antibody (Chemicon International, Temecula, CA) and for 30 minutes at room temperature with biotinylated anti-mouse IgG. After incubation with the antibodies, cells were then reacted with fluorescent rhodamine–avidin. Results were observed using a fluorescence microscope, and images were acquired and processed using DPC controller software (Olympus, Hamburg, Germany).

Alcian blue staining for matrix markers. The hPi-GFP cells, Kp-hMSCs, and the monolayer mixture group (group D) (Figure 1) were seeded into 6-well plates to compare their capacity for glycosaminoglycan and proteoglycan accumulation. At various time points, cells were washed twice with PBS and fixed in 10% formalin for 10 minutes at room temperature. After washing with distilled water twice, the cells were then stained in 1% Alcian blue 8GX reagent (Sigma, St. Louis, MO) in 3% glacial acetic acid (pH 2.5) for 30 minutes at room temperature, and were then dehydrated through a series of graded alcohol and cleared in xylene. Samples were mounted using aqueous mounting medium (Faramount; Dako, Carpinteria, CA) and photographed using an Olympus microscope.

Histologic examination and assessment of neocartilage formation in 3-D collagen scaffolds. The hPi-GFP cells (group A), Kp-hMSCs (group B), and committed Kp-hMSCs (group C) were prepared as described above (Figure 1). After 1-week cultures, cells in these different groups were collected and embedded at a density of 2×10^6 cells/ml in scaffold matrices suspended in DMEM/F12 containing 2 mg/ml purified type I collagen mixed with type II collagen (in a ratio of 4:1), using a protocol previously established by our laboratory (37). Cell/collagen constructs were then cultured in DMEM/F12 containing 10% FBS (for group A) or DMEM/LG con-

taining 10% FBS (for groups B and C) in an incubator at 37°C in 5% CO_2 for 4 weeks. At the end of the incubation period, samples were processed for immunoperoxidase staining (38). The primary antibody used was mouse anti-human type II collagen monoclonal antibody (100× dilution; Chemicon International). Samples were counterstained with hematoxylin and eosin (H&E). The presence of extracellular matrix (ECM) was detected with Alcian blue stain.

RESULTS

Experimental design. The goals of this study were to determine the stages of chondrogenesis of human MSCs that were committed to chondrocytes, and to define the molecular markers involved in this process. Previously, we established and characterized the phenotypes of the immortalized human articular chondrocytes (hPi cells) (4) and the immortalized human MSCs (Kp-hMSCs) (35). GFP was transduced into the hPi cells as a cell marker to distinguish the hPi chondrocytes from the Kp-hMSCs during coculture. The experimental design is illustrated in Figure 1.

Briefly, 2 separate control groups, hPi-GFP cells (group A) and Kp-hMSCs (group B), were seeded in 6-well plates in their respective media. Our observations from previous studies using direct cell–cell contact coculture have suggested that after exposure to hPi-GFP cells, committed Kp-hMSCs can be collected to investigate the stages of MSC chondrogenesis in an insert membrane system (23); this was performed in experimental group C. In addition, our studies have shown that accumulation of synthetic chondrogenic ECM can be examined in cultures of committed Kp-hMSCs using immunohistochemical analysis with anti–type II collagen and Alcian blue staining for chondrogenic ECM markers in a monolayer mixture system (23); this was performed in experimental group D.

Meanwhile, both direct-contact coculture groups (groups C and D) were tested in either DMEM/F12 (for analysis of chondrocyte growth) or DMEM/LG (for analysis of MSC growth). An unexpected pattern of gene expression was observed in the committed Kp-hMSCs in DMEM/F12 (results not shown), suggesting that this medium might contain factors favoring chondrogenesis. Therefore, DMEM/LG was chosen as the medium instead of DMEM/F12 in all further commitment experiments with groups C and D. In addition, committed Kp-hMSCs from group C were collected and reconstituted in a collagen scaffold to examine their neocartilage-forming abilities.

Proliferation of committed Kp-hMSCs. In order to evaluate the effects exerted by chondrocytic culture conditions on the proliferation of Kp-hMSCs, cell numbers were counted in the chondrocyte-committed coculture group in insert membrane experiments (group C) and in its control groups (groups A and B) (Figure 2A). After a 1-week culture period, the cocultured Kp-hMSCs showed a more pronounced rate of proliferation when compared with the control group of Kp-hMSCs ($P < 0.05$).

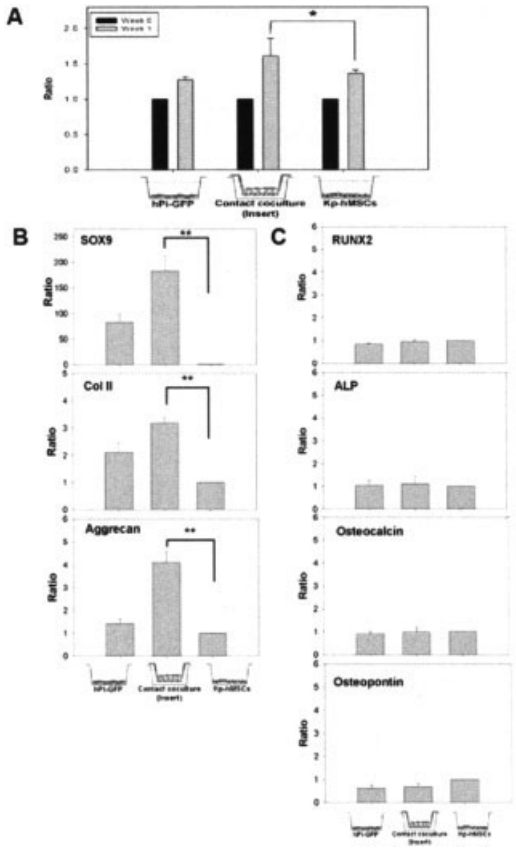

Figure 2. Chondrogenic efficacy of human mesenchymal stem cells (Kp-hMSCs) induced by human chondrocytes (hPi cells labeled with green fluorescent protein [hPi-GFP]). **A,** Rate of proliferation of Kp-hMSCs cocultured with hPi-GFP cells during 1-week cultures. **B** and **C,** Chondrogenic and osteogenic gene expression of committed Kp-hMSCs. The relative gene expression of *SOX9*, *Col2A1*, and aggrecan during chondrogenesis **(B)** and of *RUNX2*, *ALP*, osteocalcin, and osteopontin during osteogenesis **(C)** was detected using semiquantitative reverse transcription–polymerase chain reaction. Bars show the mean and SD expression ratios relative to controls, with results normalized to GAPDH. $* = P < 0.05$; $** = P < 0.01$.

Determination of the cell fate of committed Kp-hMSCs. To determine the cell fate of Kp-hMSCs committed to differentiated chondrocytes, profiles of gene expression were analyzed by RT-PCR. Genes involved in chondrogenesis were all significantly up-regulated in

committed Kp-hMSCs (mean ± SD relative fold increase in *SOX9*, 180 ± 29.16, in *Col2A1*, 3 ± 0.19, and in aggrecan, 4 ± 0.44) as compared with the gene expression profiles in the control group of parental Kp-hMSCs ($P < 0.01$) (Figure 2B). However, there was no apparent change in the levels of the osteogenic markers *ALP*, osteocalcin, and osteopontin nor were there any changes in the levels of *RUNX2* in any of these groups after 1 week of culture (Figure 2C)

Analysis of markers of chondrogenic differentiation of committed Kp-hMSCs. After establishing the coculture system, we then analyzed the molecular events involved in chondrocytic differentiation of the committed Kp-hMSCs (in comparison with their parental Kp-hMSCs) after the 7-day coculture period with chondrocytes. There were 87 genes from the skeletogenesis array kit that were available for examination of the chondrogenic process. The genes were classified into 3 groups: adhesion molecules, signaling pathways, and phenotypic determinants (Figure 3A). For determining the process of chondrogenesis, 4 stages were analyzed: mesenchymal cell proliferation and condensation, chondroprogenitor differentiation, chondrogenesis, and hypertrophic and terminal differentiation (Figure 3B).

Genes involved in regulating the cell–cell and cell–matrix interactions in the transition from the initial stage of mesenchymal cell condensation to the chondroprogenitor stage were detected in committed Kp-hMSCs after they were cocultured with hPi-GFP cells. As expected, genes for the integrins (ITGs), which are the major receptors of fibronectin, were significantly up-regulated after coculture, whereas fibronectin itself was only minimally up-regulated (1.23-fold). In particular, at the transcriptional level, ITG subunit α1 (*ITGA1*) and *ITGA3* exhibited increases in expression of 33.17-fold and 3.4-fold, respectively, relative to the levels in Kp-hMSCs alone, whereas ITG subunit β1 (*ITGB1*) exhibited a level that was almost unchanged after 7 days in coculture (1.15-fold increase). Another prominently up-regulated transcript was that for intracellular adhesion molecule 1 (*ICAM1*), which had a 36.76-fold increase in relative gene expression in the committed Kp-hMSCs.

Subsequently, activated signaling pathways and associated regulator molecules were detected during the transition from mesenchymal cell condensation to chondrogenic differentiation. Among these processes, genes from the TGF superfamily, including the TGFβs and bone morphogenetic proteins (BMPs), were elevated after coculture of committed Kp-hMSCs. In particular, the transcript levels of *TGFβ2* (7.05-fold increase) and *TGFβ3* (3.56-fold increase) showed a greater increase

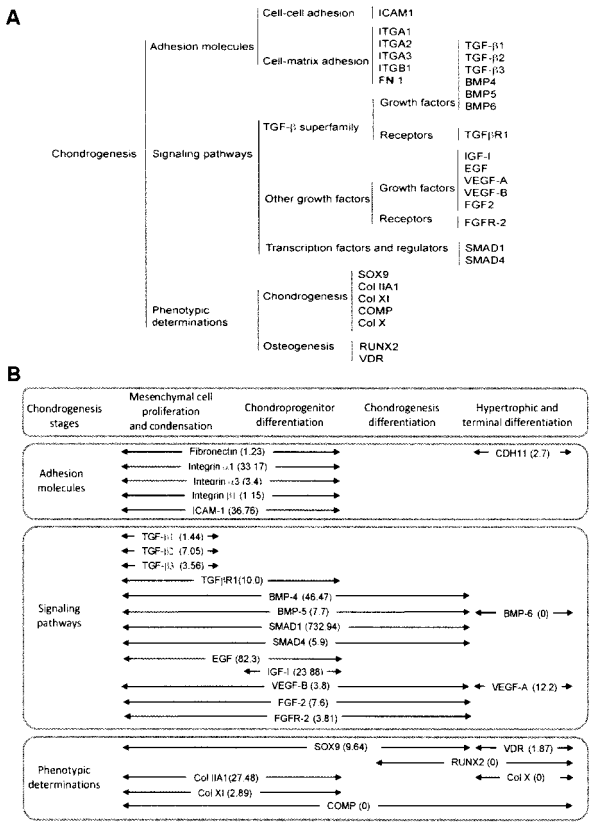

Figure 3. Analysis of the stages of chondrogenesis of human mesenchymal stem cells (Kp-hMSCs) committed to human chondrocytes (hPi cells labeled with green fluorescent protein [hPi-GFP]), using microarray with real-time polymerase chain reaction. **A,** Molecular classifications of developmental genes. **B,** Changes in adhesion molecules, changes in signaling pathways, and phenotypic determinations at different stages of chondrogenesis. Values are the expression ratios of committed Kp-hMSCs relative to Kp-hMSC controls. TGFβ = transforming growth factor β; ICAM-1 = intracellular adhesion molecule 1; ITGA1 = integrin subunit α1; ITGB1 = ITG subunit β1; FN 1 = fibronectin 1; VDR = vitamin receptor D; BMP-4 = bone morphogenetic protein 4; TGFβR1 = TGFβ receptor I; IGF-1 = insulin-like growth factor 1; EGF = endothelial growth factor; VEGF-A = vascular endothelial growth factor A; FGF-2 = fibroblast growth factor 2; FGFR-2 = FGF receptor 2.

than those of *TGFβ1* (1.44-fold increase), and there was also a large increase in the levels of their cognate receptor, *TGFβR1* (10.0-fold increase). Comparatively, levels of the *BMP4* transcript were greatly amplified (46.47-fold increase) as compared with the levels in the control group, while *BMP5* was only moderately elevated (7.7-fold increase).

Following the activation of the TGF superfamily, their major downstream molecules, the Smads, were stimulated. In particular, the levels of *Smad1* were

significantly and highly elevated (732.94-fold increase), while the levels of *Smad4* were moderately elevated (5.9-fold increase), compared with the levels of these Smads in the control group. In addition, other growth factors that regulate chondrogenic processes were all highly up-regulated in the committed Kp-hMSCs, including insulin-like growth factor 1 (*IGF1*; 23.88-fold increase), endothelial growth factor (*EGF*; 82.3-fold increase), vascular endothelial growth factors (*VEGFs*; 3.8-fold to 12.16-fold increase), and *FGF2* (7.6-fold increase) and its receptor, *FGFR2* (3.81-fold increase).

Following the analyses of the mesenchymal cell condensation and chondroprogenitor differentiation stages, the phenotypes of the committed Kp-hMSCs were analyzed. The major chondrogenic transcription factor *SOX9* exhibited a 9.6-fold increase in expression in these cocultured cells, similar to the results from our RT-PCR analyses. Chondroprogenitor cell differentiation was further indicated by the increased relative gene expression levels of *Col2A1* (27.46-fold increase) and *Col11* (2.89-fold increase). Conversely, *BMP6*, which regulates hypertrophic and terminal differentiation, was not detected. Some of the genes known to be involved in terminal chondrogenesis/osteogenesis did not seem to be affected; for example, there was no increase in the levels of *RUNX2* or *Col10* and only a minimal increase (1.87-fold) in vitamin receptor D.

Induction of chondrogenic matrix accumulation by Kp-hMSCs under chondrocytic culture conditions. To demonstrate the capacity for synthesis of major chondrogenic matrix fibril proteins, the hPi-GFP cells and Kp-hMSCs were directly mixed and cultured in a monolayer coculture system (group D) (Figure 1). Two separate control groups, hPi-GFP cells (Figure 4A, panels a, d, g, and j) and Kp-hMSCs (Figure 4A, panels c, f, i, and l), were used for comparison with the direct coculture of hPi-GFP cells and Kp-hMSCs (Figure 4A, panels b, e, h, and k). These groups were compared on the basis of their morphologic differences (Figure 4A, panels a–c) and GFP expression (Figure 4A, panels d–f). Cells were then stained for type II collagen and subjected to dual immunofluorescence microscopy using both rhodamine (Figure 4A, panels g–i) and GFP labeling (Figure 4B, merged images in panels j–l) as fluorochrome imaging methods. The hPi-GFP cells coexpressed GFP and type II collagen, thus serving as positive controls (Figure 4A, panels d, g, and j), whereas neither GFP nor type II collagen was expressed in the Kp-hMSCs, thus serving as negative controls (Figure 4A, panels f, i, and l).

After chondrocytic commitment in the monolayer coculture system, the committed Kp-hMSCs expressed type II collagen, but not GFP, and showed a higher ratio of rhodamine staining (Figure 4A, panels e, h, and k) than that in the control groups. The increased amount of type II collagen in the contact monolayer coculture of hPi-GFP cells and Kp-hMSCs was 2.5 ± 0.8–fold higher ($P < 0.05$) than that in the hPi-GFP control culture (Figure 4B).

The deposition of the chondrogenic matrix marker glycosaminoglycan in cultures of committed Kp-hMSCs was examined with Alcian blue staining (Figure 4C). Compared with the hPi-GFP control group

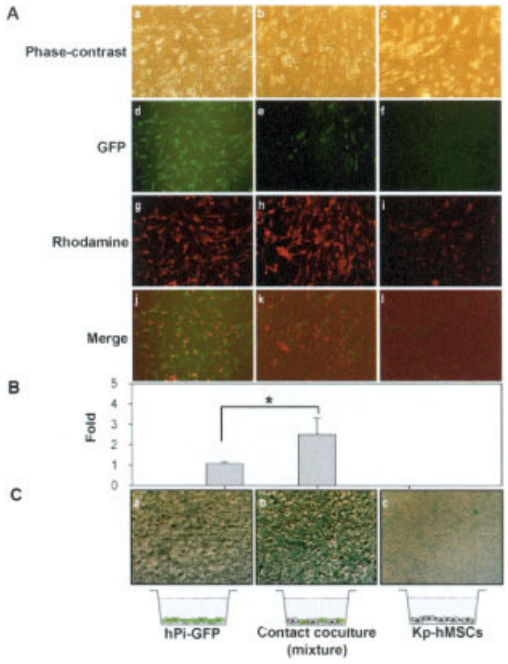

Figure 4. Determination of chondrogenic-specific matrices from human mesenchymal stem cells (Kp-hMSCs) committed to human chondrocytes (hPi cells labeled with green fluorescent protein [hPi-GFP]). The hPi-GFP cells and Kp-hMSCs were directly mixed on a monolayer culture to evaluate chondrogenic extracellular matrix accumulation in cell–cell interactions. **A,** Morphologic features (panels **a–c**) and GFP expression (panels **d–f**) were observed using phase-contrast analysis with a fluorescence microscope (original magnification × 100). In addition, cells were stained for type II collagen (rhodamine) (panels **g–i**), and the results were then merged with GFP images (panels **j–l**). **B,** To evaluate the effects of hPi-GFP cells on Kp-hMSC chondrogenesis, the ratio of fold expression of type II collagen–positive cells to GFP-positive cells was determined in the direct-contact coculture mixture of hPi-GFP cells and Kp-hMSCs compared with the hPi-GFP cells alone. * = $P < 0.05$. **C,** Accumulation of glycosaminoglycan from cell–cell interactions was detected by Alcian blue staining in the direct-contact coculture group (panel **b**) in comparison with positive controls (hPi-GFP cells; panel **a**) or negative controls (Kp-hMSCs; panel **c**).

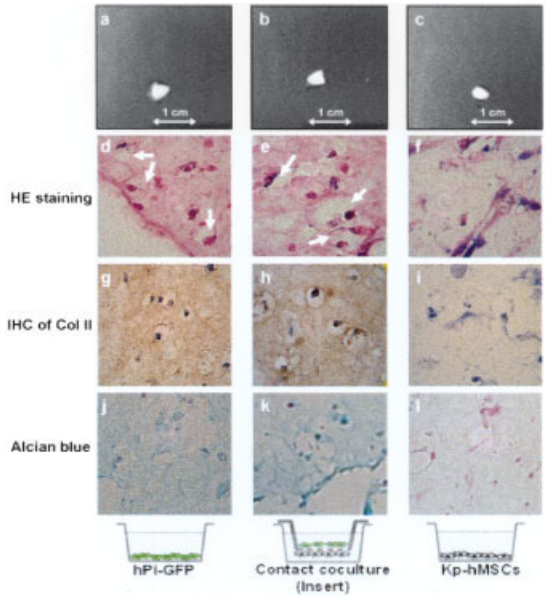

Figure 5. In vitro neocartilage formation. **a–c,** The formation of neocartilage was examined in cultures of human chondrocytes (hPi cells labeled with green fluorescent protein [hPi-GFP]) (**a**), committed human mesenchymal stem cells (Kp-hMSCs) (**b**), and Kp-hMSCs alone (**c**). Each was seeded in a collagen scaffold and cultured for 4 weeks. **d–l,** Histologic analysis with hematoxylin and eosin (H&E) staining (**d–f**), immunohistochemical (IHC) analysis of type II collagen (**g–i**), and Alcian blue staining (**j–l**) of the 3-dimensional cultures were performed. The results showed chondrogenic-specific lacunae (**arrows** on H&E staining) that surrounded the cells, and chondrogenic matrices that had accumulated between the cells and matrices. (Original magnification × 1,000 in **d–l**.)

(Figure 4C, panel a), no obvious positive staining was detected in Kp-hMSCs after 7 days in culture (Figure 4C, panel c). In contrast, a greater intensity of Alcian blue staining was expressed in the contact coculture group (Figure 4C, panel b). Similarly, it was observed that the accumulation of chondrogenic matrix–specific proteoglycan was also significantly increased in committed Kp-hMSCs when they had direct interactions with hPi-GFP cells.

Promotion of neocartilage formation by committed Kp-hMSCs. To determine the neocartilage-forming ability of the cells, 3 cell lines, hPi-GFP cells and Kp-hMSCs alone as controls and committed Kp-hMSCs isolated from the insert membrane, were separately seeded into a 3-D collagen scaffold. Following a 4-week culture period, 3-D cultures from the 3 groups were harvested, formalin-fixed, and subsequently analyzed for histologic changes (Figure 5).

The evaluation of gross morphologic features demonstrated that the size of the hPi-GFP cells alone and that of the committed Kp-hMSCs were both larger than the size of the Kp-hMSCs alone (Figures 5a–c). Both hPi-GFP cells and committed Kp-hMSCs exhibited a round chondrocytic appearance on morphology, and in both cultures, the cells were surrounded by hyaline cartilage–specific lacunae, as evident from H&E staining (Figures 5d and e, arrows). Sections of hPi-GFP cells and committed Kp-hMSCs were strongly stained with anti–type II collagen antibodies, thus indicating the deposition of chondrogenic fibril proteins between the cells and their matrices (Figures 5g and h). Moreover, similar to the hPi-GFP cells, neocartilage formation by Kp-hMSCs in the chondrocyte-commitment cocultures was evident in the detection of significantly positive signals by Alcian blue staining (Figures 5j and k).

In contrast, in the absence of chondrogenic induction, we observed fibroblastic morphologic features, resembling those of undifferentiated MSCs, in the control group of Kp-hMSCs in the collagen scaffold (Figure 5f). Moreover, no obvious chondrogenic-specific staining was detected in the Kp-hMSC control group (Figures 5i and l).

DISCUSSION

In this study, we have more accurately delineated the stages of chondrogenesis and defined the molecular markers that are involved in chondrocytic differentiation of MSCs. Chondrogenesis is a process that results in the formation of the cartilage intermediate layer and leads to endochondral ossification during vertebrate skeletal development. This process requires precise control of cellular interactions with the surrounding matrix and differentiation factors, in a temporal-spatial manner. Developmental complexity presents a great challenge for in vitro replication of chondrogenesis when devising a stem-cell–based therapeutic platform for clinical purposes. For instance, direct implantation of undifferentiated MSCs often results in heterotopic tissue formation; use of preconditioned MSCs and knowledge of their precise chondrogenic differentiation status are therefore crucial to the efficacy of MSC-based cartilage regeneration (19,20).

We have previously investigated the signaling pathways involved in chondrogenesis under the control of type I and type II collagen, TGFβ1, and platelet-rich plasma (3,4,32,33,38). Building on the observations from our previous studies, we established a coculture system in which MSCs were directed toward chondrogenesis under the influence of mature chondrocytes, thereby creating a better simulation of physiologic conditions.

In the present study, with the establishment of a coculture system for the preconditioning of MSCs, we found that direct cell–cell contact could enhance chondrocytic differentiation, thus mimicking the in vivo environment of tissue development and renewal. Use of the E6/E7 human immortalized MSCs (Kp-hMSCs) yielded cytofluorimetric profiles of CD marker expression similar to those observed in the parental primary human MSCs, and the differentiation potential was also preserved (35). Under our system, the number of Kp-hMSCs increased substantially during the first week of coculture, indicating that the cells were in transition to the first stage of chondrogenesis, i.e., mesenchymal cell proliferation (27). Concomitantly, these Kp-hMSCs showed an up-regulation in chondrogenic gene expression, including increased levels of *SOX9*, *Col2A1*, and aggrecan, but no change in osteogenic genes or *RUNX2*. Among these chondrogenic-related genes, *SOX9* expression in the cocultured Kp-hMSCs was 2-fold higher than in the hPi-GFP control group, the latter being representative of mature articular chondrocytes. These findings suggest that the Kp-hMSCs are capable of differentiating into the chondroprogenitor stage.

Our results are supported by the observations from studies by Yamamoto et al and other investigators, in which it was shown that the differentiation lineages of MSCs depend on their cocultured cell types and, possibly, their activated adhesion molecules and respective signaling pathways (22–24,39). Thus, our coculture system would provide conditions that favor chondrogenesis, rather than osteogenesis.

Because the activation of specific adhesion molecules and the induction of growth factors from progenitor or mature cells have been shown to promote differentiation of MSCs (7,25,26,40), a large number of genes that are involved in skeletogenesis were examined, in a temporal manner, using an array method. Integrins mediating cellular communication (41,42), or in this case, chondrocytic interactions, were moderately up-regulated in MSCs under our coculture system. In addition, *ICAM1* was significantly activated in committed Kp-hMSCs. ICAM-1, as well as N-cadherin, has a crucial role during mesenchymal cell condensation and tissue morphogenesis (43). These observations indicate that our coculture system successfully directed MSCs toward the completion of the early stages of chondrogenesis, namely, mesenchymal cell proliferation and condensation.

After the cellular condensation stage, followed by an increase in cell adhesion in the chondroprogenitor stage, the subsequent stages of chondrogenesis involve a complex process that includes interactions of growth factors in the earliest stages (27,44). Our results demonstrated that *TGFβ1*, *TGFβ2*, *TGFβ3*, *BMP4*, and *BMP5* were all highly induced, and this led to significant activation of both *Smad1* and *Smad4*, which served to regulate the proliferation and chondrogenic differentiation of the committed MSCs. Of particular importance, the Smads mediated by the TGFβ genes activated the receptor *TGFβR1*, which plays an essential role in promoting MSCs to chondroprogenitors and chondrogenic differentiation, especially *Smad1*. Alternatively, the *BMP* signaling molecules collectively controlled the activation of transcription factor *SOX9*, through receptors *BMPR1A* and *BMPR1B* (45,46).

In addition, activated growth factors, such as *EGF*, *IGF1*, and *VEGFs*, were significantly manifested in committed MSCs; these growth factors have been reported to modulate the process of osteochondrogenesis (15,44,47). Furthermore, *FGF2* and its receptor, *FGFR2*, were also found to be highly induced during chondrogenesis of committed Kp-hMSCs. In accordance with the findings from previous studies, *FGF2* and *FGFR*s cooperatively contribute to growth plate development, and thus are determining factors in the adult skeleton (48).

Prior to the cooperation of adhesion molecules and growth factors, the synthesis of major chondrogenic ECM proteins, including type II collagen and proteoglycans, was also observed in committed Kp-hMSCs. Chon-

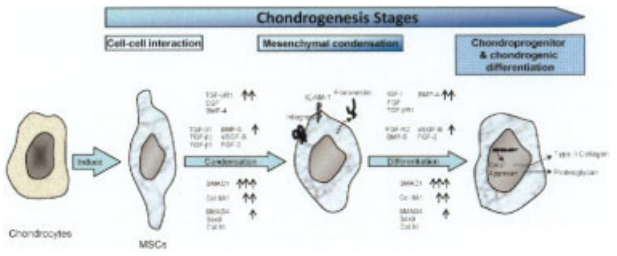

Figure 6. Schematic model of the stages of chondrogenesis of human mesenchymal stem cells (Kp-hMSCs) committed to chondrocytes. Observations of the chondrogenic process, including cell–cell interactions, mesenchymal cell condensation, and chondroprogenitor and chondrogenic differentiation, indicate that committed Kp-hMSCs were directed into the chondroprogenitor and chondrogenic differentiation stages by chondrocytes. Up arrows indicate an up-regulation in expression (single arrow = 1-fold to <10-fold increase; double arrow = 10-fold to <100-fold increase; triple arrow = at least 100-fold increase). TGFβR1 = transforming growth factor β receptor I; EGF = endothelial growth factor; BMP-4 = bone morphogenetic protein 4; VEGF-B = vascular endothelial growth factor B; FGF-2 = fibroblast growth factor 2; ICAM-1 = intracellular adhesion molecule 1; IGF-1 = insulin-like growth factor 1; FGFR-2 = FGF receptor 2.

drogenic ECM proteins such as type I and type II collagen, hyaluronic acid, and proteoglycans are produced from cells as a marker of the presence of functional articular chondrocytes (49). In contrast to the markers of chondrogenesis, molecular markers that represent stages of terminal chondrogenic differentiation, calcification, and even osteogenesis were rarely detected. Taken together, our results support the notion that a cell–cell direct-contact coculture system induces differentiation of MSCs and closely mimics the natural chondrogenic process, in particular, in the transition from the chondroprogenitor stage to partial differentiation of chondrocytes.

Thus far, we have shown that our coculture system can sustain chondrogenesis up to the chondroprogenitor stage. However, for complete chondrogenesis, the establishment of ECM is essential. Therefore, we further improved our coculture system by seeding the chondroprogenitor cells in 3-D collagen scaffolds. Previously, we have successfully used the collagen scaffold to reconstitute human tissue–engineered nucleus pulposus and human articular neocartilage (4,38). The committed Kp-hMSCs seeded in the collagen scaffold were surrounded by numerous lacunae (a characteristic of hyaline cartilage) composed of proteoglycans.

Strong type II collagen and Alcian blue staining also indicated the presence of major chondrogenic matrices that were synthesized and accumulated by the committed Kp-hMSCs. In comparison with monolayer cultures, 3-D cultures could maintain better chondrocyte phenotypes, particularly in the use of biocompatible matrices, which are critical for chondrocyte survival in tissue-engineered constructs (4,38,50). Therefore, our results show that the coculture system established in the present study is superior to others, in that chondrogenesis was represented under 3-D culture conditions that facilitate the identification of ECM formations, which can play an important role in cell–cell and cell–matrix communications.

Our results indicate that a coculture system to model the commitment of Kp-hMSCs to chondrocytes could better represent the stages of chondrogenesis, the chondrocytic phenotypes, and the physiologic conditions in musculoskeletal diseases such as OA, and the cells thus established could potentially be used for clinical joint repair. We have demonstrated that chondrogenic differentiation of human MSCs can be effectively achieved by a coculture system with chondrocytes using both the insert membrane and direct-contact coculture approaches. In addition, based on the identification of specific markers and phenotypes that pattern the process of chondrogenesis in human skeletal development, we found that MSCs could transition to the stages between chondroprogenitor and chondrogenic differentiation by observing the cooperative activation of adhesion molecules and growth factors. Finally, effective mature chondrogenesis was represented by the formation of neocartilage by committed Kp-hMSCs embedded in a collagen scaffold.

We have thus established a coculture system in which the chondrogenic differentiation stages of MSCs could be specifically manipulated (Figure 6). Our findings not only provide insights into the stage-specific molecular pathways involved in in vitro chondrogenesis of MSCs but also contribute to the advancement of cell-based transplantation techniques for future clinical use.

AUTHOR CONTRIBUTIONS

Dr. Deng had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Chen, Lai, Gelovani, Deng.
Acquisition of data. Chen, A. T. H. Wu, Hung, Deng.
Analysis and interpretation of data. Chen, C-C. Wu, Lin, Hung, Chiu.
Manuscript preparation. Chen, Lai, A. T. H. Wu, Deng.
Statistical analysis. A. T. H. Wu, Gelovani, Deng.

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