

Differentiation and enrichment of expandable chondrogenic cells from human embryonic stem cells *in vitro*

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Abstract

Human embryonic stem cells (hESCs) are considered as useful tools for pre-clinical studies in regenerative medicine. Although previous reports have shown direct chondrogenic differentiation of mouse and hESCs, low yield and cellular heterogeneity of the resulting cell population impairs the generation of sufficient numbers of differentiated cells for further testing and applications. Based on our previously established high-density micromass model system to study hESC chondrogenesis, we evaluated the effects of transforming growth factor (TGF)- β_1 and bone morphogenetic protein-2 on early stages of chondrogenic differentiation and commitment by hESCs. Significant chondrogenic induction of hESCs, as determined by quantitative measurements of cartilage-related gene expression and matrix protein synthesis, was achieved in the presence of TGF- β_1 . By means of selective growth factor combination (TGF- β_1 , FGF-2 and platelet-derived growth factor-bb) and plating on extracellular matrix substratum, we report here the reproducible isolation of a highly expandable, homogenous and unipotent chondrogenic cell population, TC1, from chondrogenically committed hESCs. Like primary chondrocytes, TC1 rapidly dedifferentiates upon isolation and monolayer expansion but retains the chondrogenic differentiation potential and responds to TGF- β_1 for cartilaginous tissue formation both *in vitro* and *in vivo*. In addition, TC1 displays a somatic cell cycle kinetics, a normal karyotype and does not produce teratoma *in vivo*. Thus, TC1 may provide a potential source of chondrogenic cells for drug testing, gene therapy and cell-based therapy.

Keywords: BMP-2 • TGF- β_1 • FGF-2 • PDGFbb • chondrogenesis • embryonic stem cells

Introduction

There is a need for musculoskeletal cartilage replacements due to the limited self-regenerative capacity of cartilaginous tissue [1]. Cell-based therapy and tissue engineering have been widely proposed as a means of cartilage reconstruction [2].

Human embryonic stem cells (hESCs) represent a promising cell source for regenerative medicine because of their unlimited self-renewal and ability to differentiate into various somatic cell

lineages [3]. However, one of the challenges in stem cell research is to understand, control and develop a stable and efficient culture milieu for directing differentiation of hESCs into a specific lineage. There is still limited understanding of the growth factors, signalling molecules, and even the environment necessary to drive the commitment of hESCs to the chondrogenic lineage [4]. Earlier studies of mouse embryonic stem cells (mESCs) have provided the knowledge base for studies of hESC chondrogenesis. Several reports have demonstrated direct chondrogenic differentiation of mESCs by various means including three-dimensional culture with growth factor induction using bone morphogenetic protein (BMP)-2 and transforming growth factor (TGF)- β [5–13], co-culture with limb buds [14] and genetic manipulation [15]. Similarly, our group has shown in hESCs, that a high-density microenvironment, coupled with growth factor stimulation, is critical in driving direct chondrogenic

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commitment of hESCs [16]. Recent studies have also demonstrated direct chondrogenic differentiation of hESCs by co-culture with primary chondrocytes [17, 18].

However, current protocols of directing chondrogenic differentiation of ESCs are greatly hampered by cellular heterogeneity of the resulting cultures [12, 19–20]. Potential application of hESCs for cartilage tissue engineering and regenerative medicine will depend on the ability to generate scalable and homogenous cultures of chondrogenic cells that are capable of forming cartilaginous tissue in a well-defined system.

Based on our previously established model system [16], we described here an extended multistep protocol to derive a highly expandable and homogenous chondrogenic cell population TC1. TC1 retains the chondrogenic differentiation potential for cartilaginous tissue formation both *in vitro* and *in vivo*. Furthermore, TC1 displays a normal karyotype, somatic cell cycle kinetics and does not form tumours *in vivo*.

Materials and methods

Culture of hESCs

The NIH-registered H9 cell line, isolated and established at the University of Wisconsin, was used in this study (Wicell Agreement No. 04-W094). hESCs were cultured and passaged as described [3].

In vitro derivation and differentiation of chondrogenic cells from hESCs

Differentiation was carried out in four stages, as shown in Fig. 1.

Stage 1: embryoid body (EB) formation

hESC colonies were dissociated into clumps through treatment with 1 mg/ml collagenase type IV and then transferred to non-adherent six-well culture plates (Corning, Inc., Lowell, MA, USA) in EB formation media consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F12 nutrient (Invitrogen, Grand Island, NY, USA), 10% knockout serum replacer (Invitrogen), 10% foetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 1mM L-glutamine (Invitrogen), 1% (v/v) non-essential amino acids (Invitrogen) and 0.1 mM β -mecaptoethanol (Sigma, St. Louis, MO, USA). When cultured on a non-adherent surface, the suspended hESC clumps form free-floating aggregates or EBs for a period of 5 days, during which the culture medium was changed on days 2 and 4 of culture.

Stage 2: chondrogenic induction

Chondrogenic differentiation of EB-derived cells was induced under high-density micromass culture conditions, as described previously [16, 21].

Cells were cultured in the basic serum-free chondrogenic medium consisted of high-glucose DMEM (Sigma) supplemented with 1% ITS⁺ (BD Biosciences, San Jose, CA, USA), 1% knockout serum replacer, 40 μ g/ml L-proline (Sigma), 1% sodium pyruvate (Sigma), 1% non-essential amino

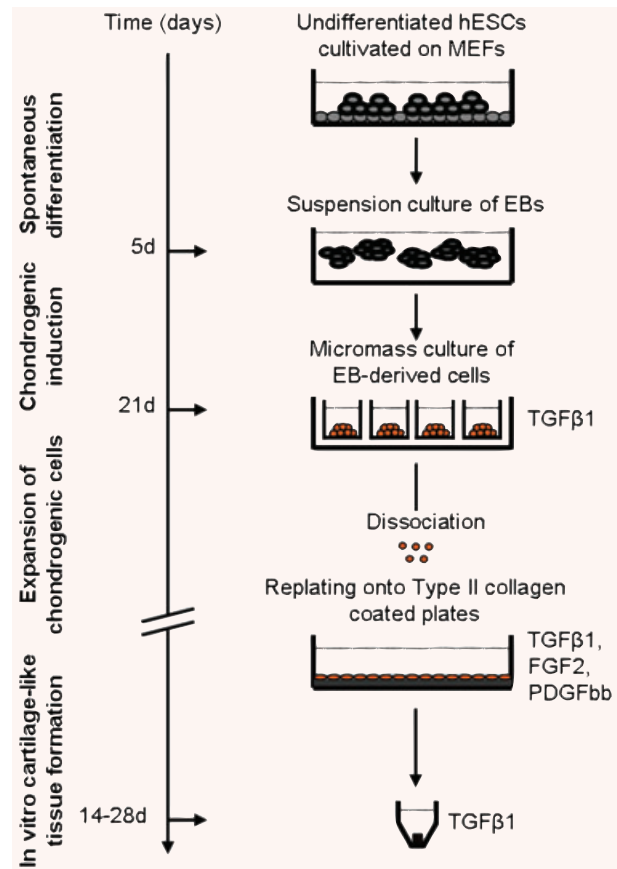


Fig. 1 Chondrogenic commitment of hESCs and isolation of expandable chondrogenic cells. Schematic representation of the differentiation protocol for hESC chondrogenesis. The hESCs were cultured on MEF feeder layer and dissociated to form EBs for a period of 5 days. 5'd EBs were then chondrogenically differentiated as high-density micromasses in serum-free chondrogenic medium in the presence of 10 ng/ml TGF- β 1 for 21 days. Chondrogenic cells are isolated by collagenase treatment and allowed to expand in monolayer in expansion medium supplemented with TFP growth factors (1 ng/ml TGF- β 1, 5 ng/ml FGF-2 and 10 ng/ml PDGF-bb) before induced to form cartilage-like pellets for up to 28 days in presence of 10 ng/ml TGF- β 1.

acids, 50 μ g/ml ascorbic acid 2-phosphate (Sigma), 10^{-7} M dexamethasone (Sigma) and 100U/100 μ g penicillin/streptomycin (Invitrogen). Following incubation for 24 hrs to allow cell attachment, the medium was replaced with fresh serum-free chondrogenic medium supplemented with 100 ng/ml recombinant human BMP-2, 10 ng/ml recombinant human TGF- β 1 or combination of both growth factors. (R&D Systems, Minneapolis, MN, USA). Differentiation was carried out for a period of 21 days with medium change every alternate day. Cultures in the basic chondrogenic medium without growth factor supplementation served as the control.

Stage 3: isolation and expansion of chondrogenic cells

Chondrogenic cells were isolated from the high-density micromass cultures by 0.15% collagenase P (Roche Diagnostics GmbH, Mannheim,

Germany) treatment overnight, and passed through the 40 μm mesh filter to obtain single cells. Isolated chondrogenic cells were plated on uncoated (UC), gelatin (GL) or type II collagen (COL2)-coated plates at a density of 2×10^4 cells/cm². After 6 hrs of incubation, cells that did not adhere to the substrates were counted. The adherent chondrogenic cells were then expanded for five passages in DMEM containing 10% FBS without (Control) or with the addition of TFP growth factors (1 ng/ml TGF- β_1 [R&D Systems], 5 ng/ml FGF-2 (Invitrogen) and 10 ng/ml PDGF-bb (Peprotech, Rocky Hill, NJ, USA).

Stage 4: *in vitro* cartilage-like tissue formation

To assess the quality of the expanded EB-derived chondrogenic cells, 3D pellet cultures were used. Aliquots of 2×10^5 cells/0.5 ml were spun down at 1100 rpm for 5 min. to form pellets and cultured in the serum-free chondrogenic medium without (Control) or with TGF- β_1 (10 ng/ml) supplementation for up to 28 days. Samples were harvested at designated time-points for histology, immunohistochemistry and biochemical analyses.

Multilineage differentiation analysis

To further assess the differentiation capacities of EB-derived chondrogenic cells, differentiation to osteogenic and adipogenic lineages were performed as previously described [22]. Human mesenchymal stem cells (MSCs) were isolated under the guidelines of the Institutional Review Board of National University Hospital of Singapore from bone marrow aspirates of donors who had given written informed consent. For osteogenic differentiation, cells were seeded as monolayer at 5×10^3 cells/cm² in high-glucose DMEM supplemented with 10% FBS (Hyclone), 10 mM β -glycerophosphate (Sigma), 10^{-8} M dexamethasone (Sigma), 50 $\mu\text{g/ml}$ ascorbic acid 2-phosphate (Sigma) and 100 U/100 μg penicillin/streptomycin (Invitrogen) and cultured for 2 weeks. For adipogenic differentiation, cells were seeded as monolayer at 2×10^4 cells/cm² in high-glucose DMEM supplemented with 10% FBS (Hyclone), 10 μM insulin (Sigma), 10^{-7} M dexamethasone (Sigma), 0.1 mM indomethacin (Sigma), 0.5 mM 3-isobutyl-methyl xanthine (Sigma) and 100 U/100 μg penicillin/streptomycin (Invitrogen) and cultured for 2 weeks. Differentiation to osteogenic and adipogenic lineages were assessed by staining with Alizarin Red S and Oil red O, respectively.

RT-PCR and real-time PCR quantitative analysis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA), and passing through the Qias shredder following the manufacturer's instructions. RNA from human palate mesenchymal cell line (HPM) (ATCC CRL-1486, American Type Culture Collection, Rockville, MD, USA) served as control to ensure primer specificity. RNA was also extracted from positive control that contained RNA extracted from EB outgrowth monolayer, EB^{OG}, cultured in control chondrogenic medium for 21 days [16]. Each sample was treated with RNase-Free DNase (Qiagen) to avoid genomic DNA contamination. Reverse transcription reaction was performed with a PCR thermal cycler, Mycycler (Bio-Rad, Hercules, CA, USA). cDNA synthesis was generated from 500 ng of total RNA per 20 μl reaction volume using the iScript cDNA synthesis kit (Bio-Rad). PCR of the cDNA samples were performed at 95°C for 5 min., followed by amplification cycles of 30-sec. denaturation at 95°C, 45-sec. annealing at 55°C to 65°C and 60-sec. elongation at 72°C, and a final extension at 72°C for 5 min. The number of

cycles varied between 23 and 38, depending on the abundance of a particular mRNA. β -actin amplification with no reverse transcriptase served as negative control to check for genomic contamination. In addition, all RNA samples were adjusted to yield equal amplification of β -actin as an internal control to normalize the PCR reactions. The amplified products were subjected to electrophoresis on 2% agarose gels and subsequently stained with ethidium bromide and photographed using the Light Imaging System (Bio-Rad). For semi-quantitative analysis, mean pixel intensity of each band was measured using the NIH public domain imaging software-*ImageJ*, and normalized to mean pixel intensity of the corresponding β -actin. Each sample was repeated at least twice for each gene of interest. PCR primers, annealing temperature, and their expected product sizes are described in Table 1. The expression of *type II collagen* isoforms (*Col2A* and *Col2B*) was also assessed by PCR and agarose gel electrophoresis using previously described parameters [23].

For quantitative analysis, *Sox9*, *type I* and *II collagen* (*Col 1* and *Col 2*) gene expression were analysed by real-time RT-PCR reaction using the Power SYBR Green PCR Master Mix System (Applied Biosystems, Foster City, CA, USA) on PCR thermocycler Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). cDNA samples (1 μl for a total volume of 20 μl per reaction) were analysed for gene of interest normalized to reference gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). The level of expression of each target gene was then calculated as $2^{-\Delta\Delta C_t}$, as previously described [24]. Each sample was analysed in duplicates for each gene of interest. Real-time RT-PCR was performed at 95°C for 10 min., followed by 40 cycles of amplifications, consisted of a denaturation step at 95°C for 15 sec., and an extension step at 60°C for 1 min. All PCR reactions were carried out with negative control with no cDNA. Primers were obtained from published literature [25]. Specificity of the PCR products was confirmed by melting curve analysis and agarose gel electrophoresis. Real-time PCR primers are listed in Table 2.

Because *Col 2* is a typical marker of differentiated chondrocytes in hyaline cartilage, as opposed to *Col 1*, which is expressed by dedifferentiated chondrocytes and other mesenchymal intermediate cells such as fibroblasts and osteoblasts, the ratio of mRNA expression levels of *Col 2* to *Col 1* (*Col2/Col1*) can be utilized as a differentiation index related to the expression of collagens during chondrogenic differentiation, and in this context of hESC chondrogenic differentiation, to better assess the efficiency of chondrogenic differentiation [25–27].

Histology and immunostaining

Cell cultures were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min., made permeable using 0.2% Triton X-100 for 15 min. at room temperature (RT) and were subsequently blocked with 10% goat serum in PBS for 45 min. at RT. The fixed cells were then incubated at RT for 2 hrs with the primary antibodies. Following three washes with PBS, the cells were incubated with secondary antibodies for 1 hr at RT. Slides were then washed and mounted with Vetashield mounting medium with DAPI for nuclear counterstaining (Vector, Burlingame, CA, USA). The sources of antibodies and dilutions used are summarized in Table 3. Pig chondrocytes and HPM cell line were used as controls for many of the antibodies against collagens and proteoglycans. Negative control was also set by using control IgG isotype or omitting the primary antibody in the immunostaining procedure. Staining was visualized with an Olympus (Tokyo, Japan) inverted fluorescence microscope and its microimage software. Cells were counted in 10–15 microscopic fields at 200 \times magnification within each chamber of a chamberslide, and two to four chambers were analysed in each experiment. For histological analysis, aggregates

Table 1 Sequence of primers used for conventional RT-PCR

Gene	Primer sequence	Product size (bp)
<i>Oct4</i>	Oct4F: 5'-CGRGAAGCTGGAGAAGGAGAAGCTG-3'	247 (55°C)
	Oct4R: 5'-AAGGGCCGCGAGCTTACACATGTTCC-3'	
<i>Rex1</i>	Rex1F: 5'-GCTGACCACCAGCACACTAGGC-3'	298 (55°C)
	Rex1R: 5'-TTTCTGGTGTCTTGTCTTTGCCCG-3'	
<i>Col 2A1</i>	Col2F: 5'-TTCAGCTATGGAGATGACAATC-3'	472
	Col2R: 5'-AGAGTCCTAGAGTGACTGAG-3'	
<i>Col2AB</i>	Col2AB-F: 5'-CTG CTC GTC GCC GCT GTC CTT-3'	432bp (splice variant A)
	Col2AB-R: 5'-AAG GGT CCC AGG TTC TCC ATC-3'	225 bp (splice variant B)
<i>Decorin</i>	DecoF:5'-CCTTTGGTGAAGTTGGAACG-3'	300 (55°C)
	DecoR:5'-AAGATGTAATTCGGTAAGGG-3'	
<i>Biglycan</i>	BiglyF:5'-TGCAGAACAACGACATCTCC-3'	475
	BiglyR: 5'-AGCTTGGAGTAGCGAAGCAG-3'	
<i>COMP</i>	COMP-F: 5'-CAACTGTCCCCAGAAGAGCAA-3'	588
	COMP-R: 5'-TGGTAGCCAAAGATGAAGCCC-3'	
<i>Col 9A1</i>	Col9-F: 5'-ACAGCAGGACTCCCTGGA-3'	410
	Col9-R: 5'-TGATCACCAGGTGCACCAG-3'	
<i>Link protein</i>	LPF: 5'-CCTATGATGAAGCGGTGC-3'	618
	LPR: 5'-TTGTGCTTGTGGAACCTG-3'	
<i>CD44s</i>	CD44F: 5'-TCCATCAAAGGCATTGGGCAG-3'	326 (56°C)
	CD44R: 5'-AACCTGCCGCTTTGCAGGTGT-3'	
<i>PDGFR</i>	PDGFR-F: 5'-ATCAATCAGCCCAGATGGAC-3'	891 (60°C)
	PDGFR-R: 5'-TTCACGGGCAGAAAGGTACT-3'	
<i>NFH</i>	NFH-F: 5'- TGAACACAGACGCTATGCGCTCAG-3'	398 (56°C)
	NFH-R: 5'- CACCTTTATGTGAGTGGACACAGAG-3'	
<i>cK14</i>	K14F: 5'-ATGATTGGCAGCGTGGAG-3'	390
	K14R: 5'-GTCCAGCTGTGAAGTGCTTG-3'	
<i>AFP</i>	AFPF: 5'-CCATGTACATGAGCACTGTTG-3'	338 (55°C)
	AFPR: 5'-CTCCAATAACTCCTGGTATCC-3'	
<i>VEGFR2</i>	VEGFR2F: 5'-AAAACCTTTTGTGCTTTTGA-3'	237 (55°C)
	VEGFR2R: 5'-GAAATGGGATTGGTAAGGATGA-3'	
<i>Cbfa1</i>	Cbfa1F: 5'-CGGAATGCCTCTGCTGTAT-3'	174
	Cbfa1R: 5'-TTCCCGAGGTCCATCTACTG-3'	
<i>Osteocalcin</i>	OC-F: 5'-ATGAGAGCCCTCACACTCCTC-3'	294
	OC-R: 5'-GCCGTAGAAGCGCCGATAGGC-3'	
<i>β-actin</i>	β-ActinF: 5'-CCAAGCCAACCGGAGAAGATGAC-3'	587
	β-ActinR: 5'-AGGGTACATGGTGGTCCGCCAGAC-3'	

All primers except *PDGFRα*, *NFH* and *β-actin* (mouse/human) are human-specific. The PCR conditions were as follows: 95°C/5 min., 95°C/30 sec., 58°C/45 sec. (except where specified otherwise), 72°C/1 min., and 72°C/5 min.

Table 2 Sequence of primers used for real-time RT-PCR

Gene	Real-time primer sequence	Product size (bp)
Sox9	Sox9F: 5'-CCTCCA CGA AGGGCCG-3'	85
	Sox9R: 5'-CGA GCT CAGCAAGACGCTG-3'	
Col 1A1	Col1F: 5'-CAGCCGCTTACCTACAGC-3'	83
	Col1R: 5'-TTTTGTATTCAATCACTGTCTTGC C-3'	
Col 2A1	Col2F: 5'-GGCAATAGCAGGTTTCAGTACA-3'	79
	Col2R: 5'-CGATAACAGTCTTGCCCACTT-3'	
GAPDH	GAPDHF: 5'-ATGGGGAAGGTGAAGGTCG-3'	119
	GAPDHR: 5'-TAAAGCAGCCCTGGTGACC-3'	

Table 3 List of antibodies

Primary antibodies	Host	Dilution	Source
Collagen II	Mouse	1:150 (IF) 1:500 (IHC)	Chemicon (Temecula, CA, USA)
Collagen I	Mouse	1:1000 (IHC)	Sigma
Collagen I	Rabbit	1:75 (IF)	Chemicon
Collagen X	Mouse	1:25 (IHC)	Quartett Immunodiagnostika (Berlin, Germany)
Ki-67	Rabbit	1:500 (IF)	Abcam (Cambridge, MA, USA)
Oct-4	Rabbit	1:100 (IF)	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
A-fetoprotein	Mouse	1:300 (IF) 1:500 (IHC)	Sigma
Secondary antibodies	Host	Dilution	Source
Alexa Flour 488	Goat	1:200	Molecular Probes (Eugene, OR, USA)
Alexa Flour 594	Goat	1:200	Molecular Probes

IF: immunofluorescence; IHC: immunohistochemistry.

were fixed in 4% paraformaldehyde in PBS overnight and processed into paraffin wax blocks. Sections were cut at 5 μ m and stained with haematoxylin and eosin, Masson's Trichrome and Alcian blue following the standard procedures as previously described [28]. Immunohistochemistry to detect collagens I, II and X was carried out following the procedures as previously described [28]. Sections of hepatocarcinoma were also included as positive control for α -fetoprotein (AFP) immunohistochemical staining.

Biochemical analysis

Samples were digested as previously described [21]. s-GAG content was measured using Biocolor Blyscan Glycosaminoglycan Assay Kit (Biocolor

Ltd, Newtownabbey, Ireland) and total collagen was assayed using Biocolor Sircol Collagen Assay Kit (Biocolor). For normalization, DNA content was measured spectrophotometrically using the Hoechst 33258 method [29]. Standard curves of s-GAG and collagen were constructed using different concentrations of bovine trachea chondroitin sulphate and collagen, respectively. Calf thymus DNA was used for construction of the standard curve for DNA quantification. Total alkaline phosphatase activities were measured as previously described [16, 21].

Growth curves

Population doubling level (PDL) was calculated following the formula $PDL = \log(N/N_0)/\log 2$, where N is the number of cells in the culture flask at the end of a period of growth and N_0 is the number of cells plated in the culture flask (2×10^4 cells/cm²). Cumulative PDL is total PDL ($\#PDL$) = $\Sigma(PDL)n$, where n is the passage number [30, 31].

Multicolour fluorescence *in situ* hybridization (mFISH)

Metaphase spreads were prepared by standard techniques and hybridized with 24Xcyte Human mFISH Painting Kit (MetaSystems GmbH, Altlußheim, Germany) as previously described [32]. Microscopic analysis was performed by a Zeiss Axioplan-2 fluorescence microscope (Carl Zeiss GmbH, Oberkochen, Germany) equipped with an HBO-103 mercury lamp, using the following filter sets: FITC, Cy3.5, Texas Red, Cy5, Aqua and DAPI. Images were captured, processed, and analysed using Isis mFISH Multicolor Chromosome Analysis Module (MetaSystems GmbH). A total of 50 metaphase spreads were analysed for each sample to check the genome integrity of the cells.

Teratoma assay and articular cartilage defect transplantation

For teratoma assay, H9 or TC1 cells (5×10^6 cells) were injected into the rear leg muscle of 4-week-old female SCID mice, as previously described [33]. Animals that developed tumours (≈ 6 weeks after injection) were killed and processed for histological analysis. For articular cartilage defect transplantation, TC1 cells (5×10^5) were pellet-cultured in the presence of TGF- β_1 for 14 days and transplanted into osteochondral defects (≈ 2 mm) created on the patellar groove of the distal femur of Sprague-Dawley rats ($n = 4$). Empty defects with no implant served as control. The rats were given daily administration of cyclosporine at (14 mg/kg body mass) to prevent immune rejection of human cells implanted. After 6 weeks, the rats were killed and distal femora were resected *en bloc* and processed for histological analysis. Animal experiments were performed in accordance with Institutional Animal Care and Use Committee (IACUC) of National University of Singapore.

Statistical analysis

All quantitative data reported here were analysed using Student's t-test or analysis of variance (one-way ANOVA) followed by Tukey's *post hoc* test as applicable. Significance was defined as $*P < 0.05$ or $**P < 0.01$. Each measurement reported here was based on duplicate analysis of at least two independent experiments.

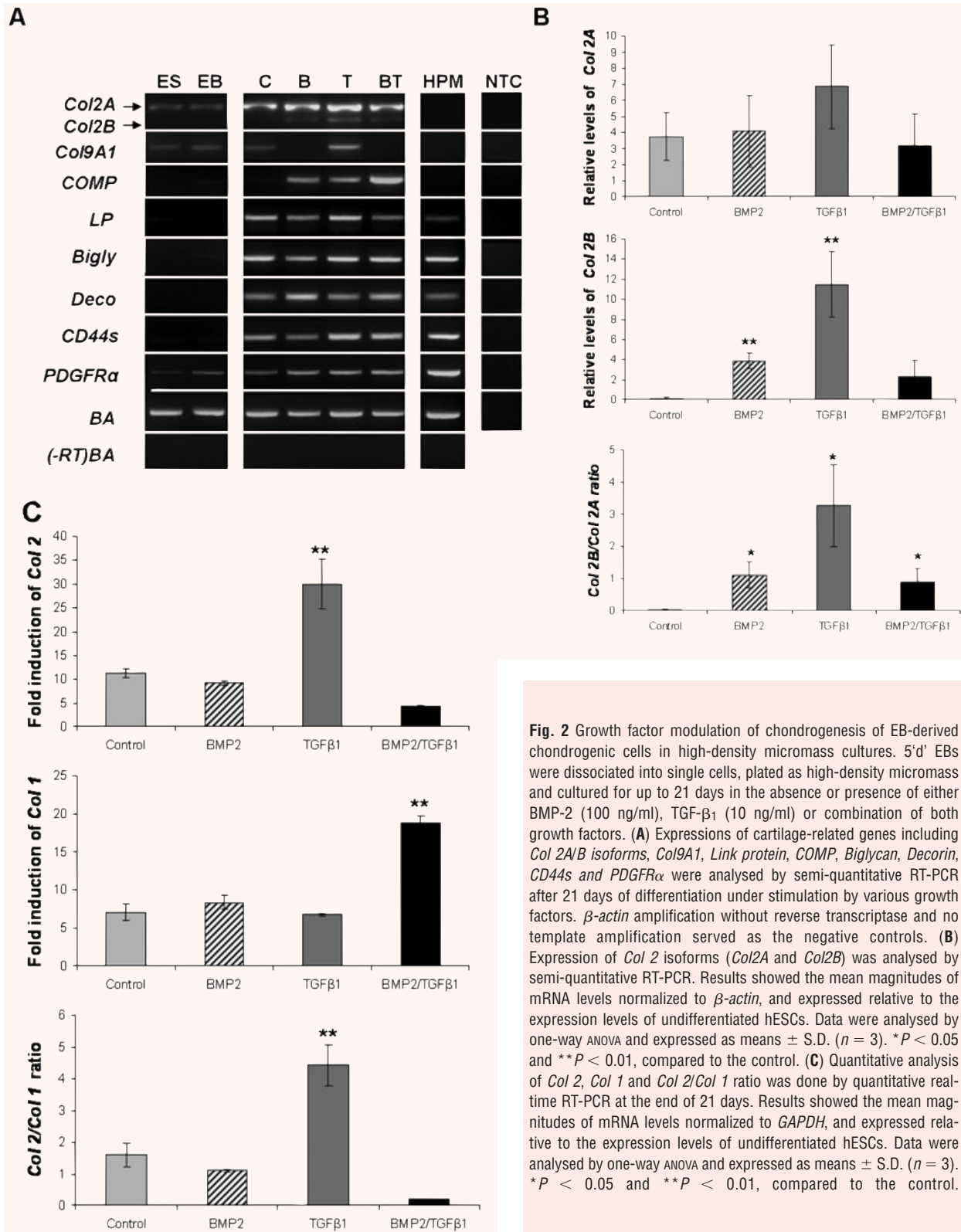
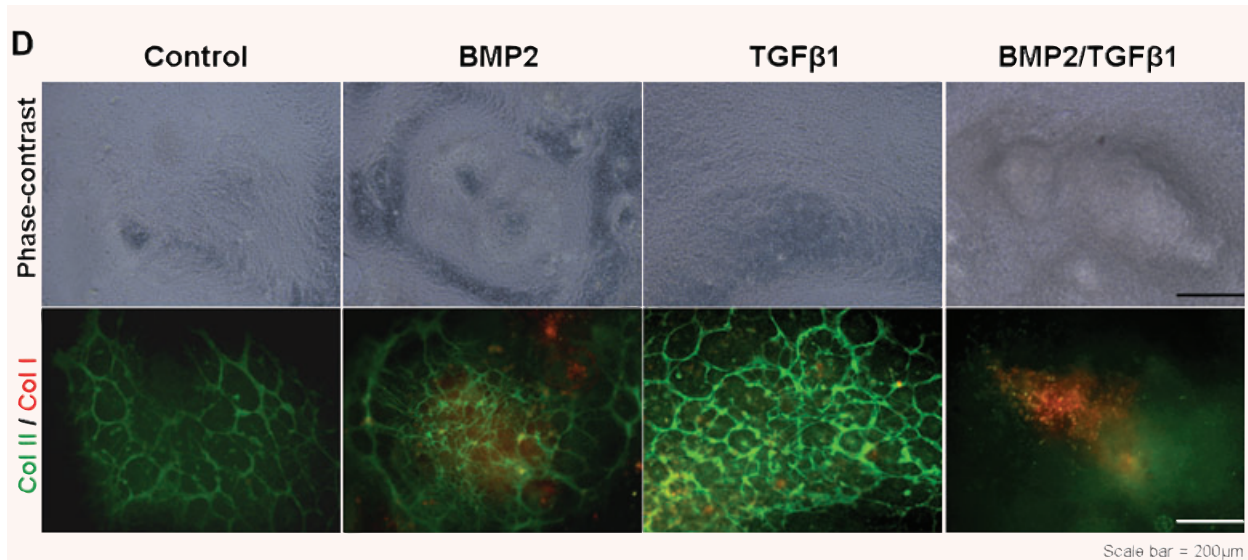
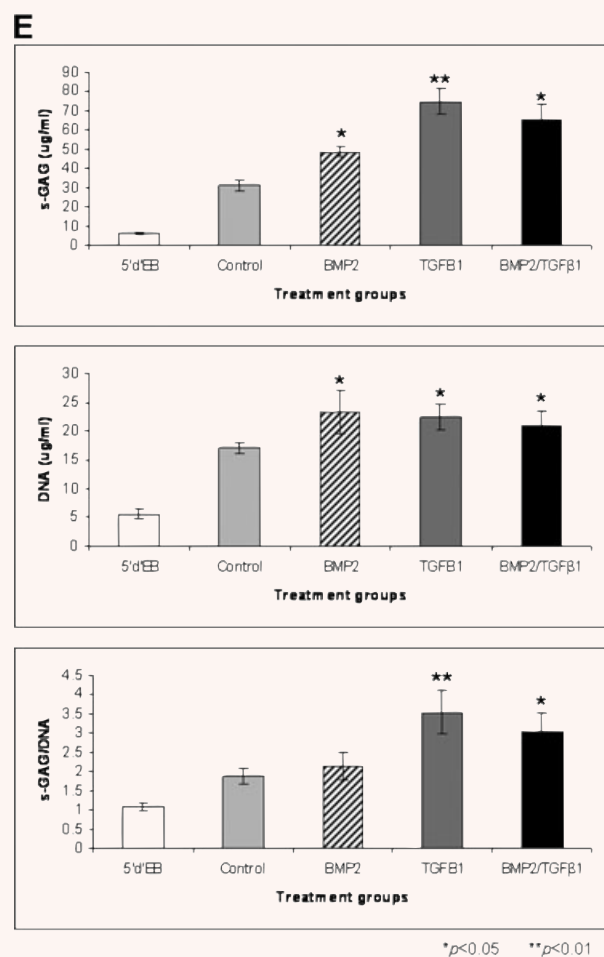


Fig. 2 Growth factor modulation of chondrogenesis of EB-derived chondrogenic cells in high-density micromass cultures. 5'd' EBs were dissociated into single cells, plated as high-density micromass and cultured for up to 21 days in the absence or presence of either BMP-2 (100 ng/ml), TGF- β 1 (10 ng/ml) or combination of both growth factors. **(A)** Expressions of cartilage-related genes including *Col 2A/B isoforms*, *Col9A1*, *Link protein*, *COMP*, *Biglycan*, *Decorin*, *CD44s* and *PDGFR α* were analysed by semi-quantitative RT-PCR after 21 days of differentiation under stimulation by various growth factors. β -actin amplification without reverse transcriptase and no template amplification served as the negative controls. **(B)** Expression of *Col 2* isoforms (*Col2A* and *Col2B*) was analysed by semi-quantitative RT-PCR. Results showed the mean magnitudes of mRNA levels normalized to β -actin, and expressed relative to the expression levels of undifferentiated hESCs. Data were analysed by one-way ANOVA and expressed as means \pm S.D. ($n = 3$). * $P < 0.05$ and ** $P < 0.01$, compared to the control. **(C)** Quantitative analysis of *Col 2*, *Col 1* and *Col 2/Col 1* ratio was done by quantitative real-time RT-PCR at the end of 21 days. Results showed the mean magnitudes of mRNA levels normalized to *GAPDH*, and expressed relative to the expression levels of undifferentiated hESCs. Data were analysed by one-way ANOVA and expressed as means \pm S.D. ($n = 3$). * $P < 0.05$ and ** $P < 0.01$, compared to the control.



(D) Representative interference contrast and immunofluorescence micrographs of micromass cultures after growth factor treatment for 21 days. Analysis of EB-derived chondrogenic cells with regard to collagen II (green) and collagen I (red). Co-localized expression of both collagen I and II results in a yellow colour. Scale bar = 200 μm. **(E)** Determination of s-GAG in micromass-papain digests by the DMMB spectrophotometric assay. The s-GAG content was normalized against the DNA content to determine the s-GAG synthesis per cell. Measurements reported were averaged from duplicates of two independent experiments, and data were analysed by one-way ANOVA and expressed as mean ± S.D. and statistical significance at * $P < 0.05$ and ** $P < 0.01$, compared to the control. Abbreviations: ES, human embryonic stem cell; EB, embryoid body; HPM, human palate mesenchymal cell line; BMP-2, bone morphogenetic protein 2; TGF-β1, transforming growth factor- β1; CD44s, CD44 standard isoform; PDGFRα, platelet-derived growth factor receptor-α; BA, β-actin; NTC, No template control; s-GAG, sulphated glycosaminoglycan; DMMB, dimethyl-methylene blue; S.D., standard deviation.



Results

Growth factor modulation of chondrogenesis

Type II collagen gene is expressed as two isoforms differentially regulated during development. The juvenile splice variant of *type II collagen (Col2A)* was readily detected in undifferentiated hESCs and 5'd' EBs, and was markedly up-regulated in micromass cultures treated with growth factors, with the highest level observed in the presence of TGF- β_1 (Fig. 2A and B). The adult cartilage splice variant of *type II collagen (Col2B)*, which is activated in mature chondrocytes, was not present under control chondrogenic condition, but was expressed after BMP-2 treatment and was more significantly elevated with TGF- β_1 treatment alone. Other cartilage-related proteins such as *Col9A1* and *link protein (LP)* also appeared to be up-regulated in the same way upon treatment with TGF- β_1 (Fig. 2A). *COMP* was not present under control chondrogenic condition, but markedly elevated with treatments of BMP-2 and/or TGF- β_1 . Co-treatment of BMP-2 and TGF- β_1 was able to induce highest expression of *COMP*. There were also up-regulation of cartilage-related receptors *CD44s* and *PDGFR α* by micromass culture of the cells. *CD44s* was not expressed in undifferentiated hESCs and EBs, but expressed specifically by micromass cultures upon chondrogenic differentiation. *PDGFR α* was readily detected in undifferentiated hESCs and 5'd' EBs, and was modestly up-regulated in micromass cultures treated with growth factors.

Quantitative real-time RT-PCR demonstrated that TGF- β_1 induced the highest level of *Col 2* expression by day 21 of differentiation, with an average threefold increase when compared to the control (Fig. 2C). No evidence of synergistic enhancement in *Col 2* expression was observed with the co-treatment of BMP-2 and TGF- β_1 , as compared to TGF- β_1 alone. *Col 1* was present at low levels in undifferentiated hESCs and 5'd' EBs, but was up-regulated by micromass cultures under chondrogenic conditions. In the presence of TGF- β_1 , the level of *Col 1* expression was comparable to the control. Therefore, TGF- β_1 was able to induce an overall highest differentiation index of *Col 2/Col 1* ratio, approximately 2.5-fold when compared to the control. On the other hand, co-treatment of BMP-2 and TGF- β_1 induced highest expression of *Col 1*, with approximately 2.5-fold increase over the control. As a result, co-treatment of BMP-2 and TGF- β_1 resulted in a lowest *Col 2/Col 1* ratio. Thus, TGF- β_1 induced an overall pattern of collagen expression showing the highest *Col 2* expression and highest ratios of *Col 2B/2A* and *Col 2/Col 1*.

Consistent with the real-time PCR results, we observed that TGF- β_1 induced enhanced chondrogenic differentiation with intense staining of collagen II-producing chondrogenic cells that form a filamentous matrix network connecting the cells (Fig. 2D). Underlying the dense matrix, small fraction of cells which co-expressed collagen II and collagen I could be observed. These cells may be mesenchymal intermediate cells which possessed high collagen I and low collagen II contents. Under the co-treatment of both BMP-2 and TGF- β_1 , these cells formed prominent clusters of cells highly enriched in collagen I. There was no sign

of osteogenesis because we did not detect any *osteocalcin (OC)* expression by RT-PCR or calcium deposition by alizarin red staining (data not shown).

Growth factor modulation of matrix synthesis

s-GAG synthesis at day 21 was measured to elucidate the influence of various growth factors on matrix accumulation during chondrogenesis (Fig. 2E). Analysing the s-GAG synthesis in a single cell level, BMP-2 treatment resulted in a ratio of s-GAG/DNA no higher than the control. On the other hand, TGF- β_1 treatment resulted in a significant enhancement (** $P < 0.01$) in the ratio of s-GAG/DNA, when compared to control and BMP-2-supplemented chondrogenic condition. However, no evidence of synergistic enhancement or inhibition exerted by BMP-2 in the ratio of s-GAG/DNA was observed at day 21, when a combination of BMP-2 and TGF- β_1 were added, as compared to TGF- β_1 alone.

TGF- β_1 induction of chondrogenic cells

Using *Col 2* as a specific marker for chondrogenesis and also the primary prominent collagen marker specific to hyaline cartilage, time-course analysis of *Col 2* expression in the TGF- β_1 -treated micromass was further carried out (Fig. 3A). Under high-density micromass culture condition, induction of *Col 2* expression by TGF- β_1 began after day 7, but was more intensified and prolonged thereafter, with an average of 1.5-fold increase by day 14 and 3-fold increase by day 21 when compared to the control. Additionally, the expression of *Sox9*, a transcription factor involved in regulation of *Col 2*, showed a similar expression pattern as *Col 2*, with an average of 1.3-fold by day 14 and 1.5-fold by day 21 when compared to the control. On the other hand, TGF- β_1 induced comparable levels of *Col 1* to the control throughout the course of differentiation. In both control and TGF- β_1 supplemented conditions, *Col 1* expression increased up to day 14 before it declined by day 21. Substantial number of cells was collagen II positive and co-localization of collagen I and II further showed that majority of the chondrogenic cells induced in the presence of TGF- β_1 are highly enriched in collagen II, and formed dense filamentous collagen II network connecting the cells in the high-density cultures (Fig. 3B). Taken together, these results suggest that TGF- β_1 , among the different growth factor treatment regimes, induced the highest *Col 2* expression and highest *Col 2/Col 1* ratio consistent with the highest number of collagen II positive chondrogenic cells observed.

Growth factor modulation of chondrogenic commitment

We also investigated how BMP-2 and TGF- β_1 may modulate the differentiation of other lineages under our chondrogenic conditions (Fig. 4). The EB micromass system showed a considerable

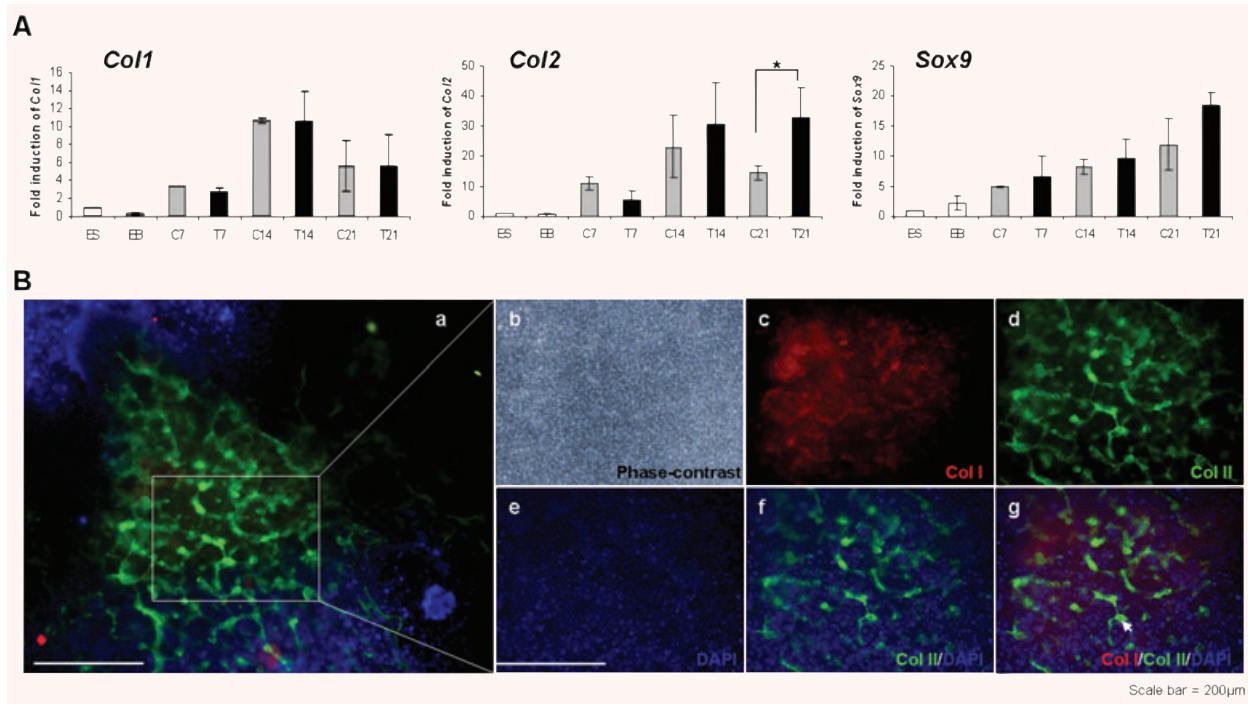


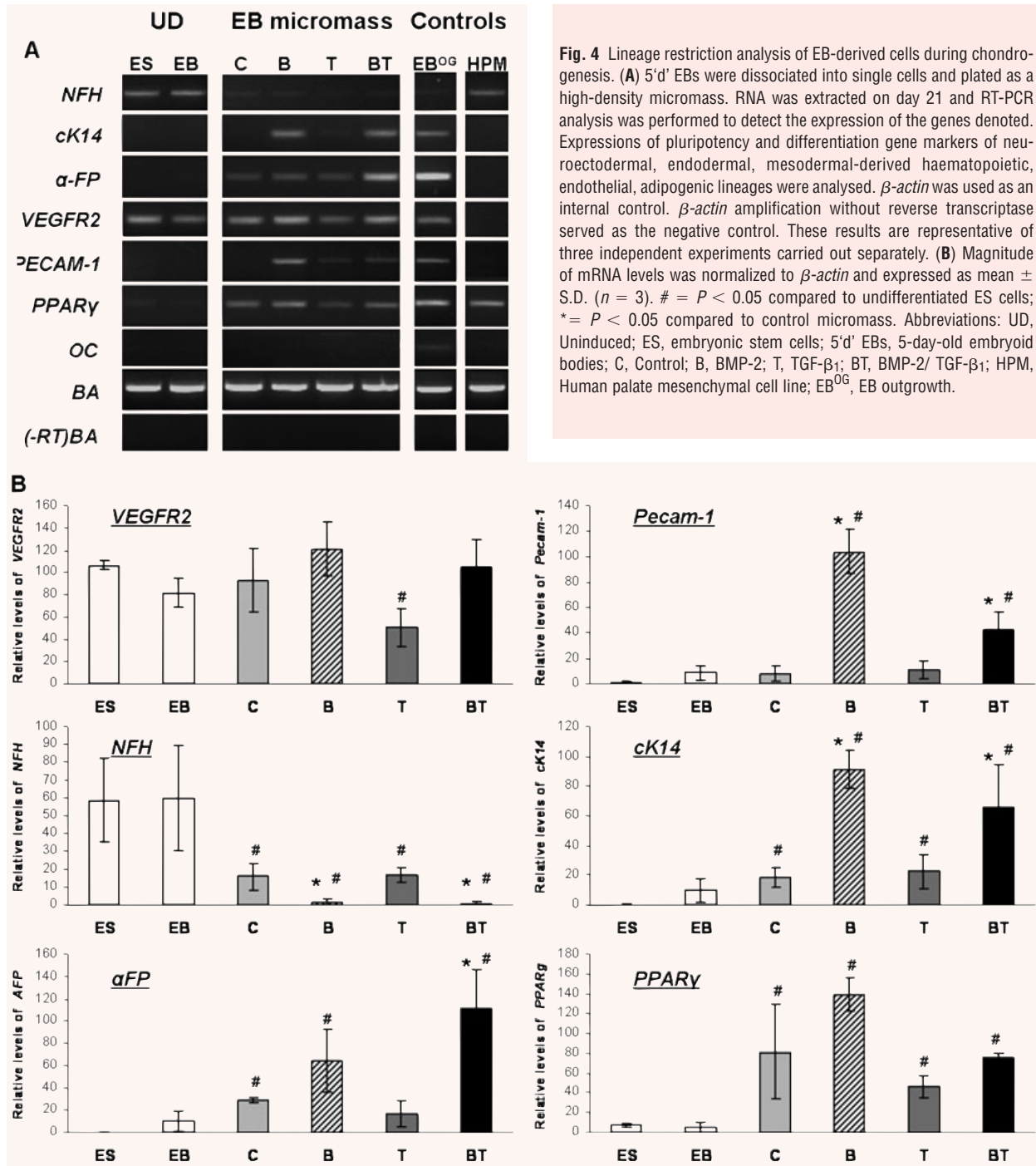
Fig. 3 Characterization of TGF- β_1 -induced chondrogenic differentiation. **(A)** Temporal expression pattern of chondrogenic genes *Col 1*, *Col 2* and *Sox9* in TGF- β_1 -induced chondrogenic differentiation of EB-derived cells. Results showed the mean magnitudes of mRNA levels normalized to *GAPDH*, and expressed relative to the expression levels of undifferentiated hESCs. Values are means \pm S.D. from three independent experiments with statistical significance at $*P < 0.05$, compared to the control. **(B)** Chondrogenic cells derived from micromass cultures of EB-derived cells at day 21 of TGF- β_1 -induced chondrogenic differentiation were double-stained with anti-collagen II (green) and anti-collagen I (red) specific antibodies and nuclear-counterstained with DAPI (blue) (a). Higher magnification images for phase-contrast (b), collagen I (red) (c), collagen II (green) (d), nuclear staining with DAPI (blue) (e) and merged images of collagen II and DAPI (f) and collagen I/II and DAPI (g). Single collagen II positive cells are highly enriched in the presence of TGF- β_1 (arrow) Scale bar = 200 μ m.

inhibition in development to other lineages, as compared to the EB outgrowth system in control chondrogenic condition. Under the micromass system, differentiation to the neuronal lineage (indicated by *NFH*) was greatly impeded. Differentiation to epidermal lineage, marked by the expression of *ck14*, was only observed in BMP-2 supplemented conditions. In the presence of TGF- β_1 alone, there was limited induction of extra-embryonic endoderm lineage as indicated by α -*FP*, and suppression or limited induction of haematopoietic/endothelial lineages as indicated by *VEGFR-2* and *PECAM-1*. Adipogenesis (indicated by *PPAR- γ*) was up-regulated in the presence of BMP-2 but was inhibited by TGF- β_1 in the micromass system. Osteogenesis (indicated by *OC*) was limited, with only low level detected in the EB outgrowth in chondrogenic condition without growth factor supplementation.

Generating expandable chondrogenic cells from micromass cultures

As chondrogenic cells were organized not only in nodules but also in filamentous collagen network in the peripheral outgrowth, we

attempted to enrich these chondrogenic cells by collagenase treatment and passing the cells through a 40- μ m mesh. Single cell suspensions derived from collagenase digestion were plated onto UC, GL or COL2-coated plates to test whether different substrates would influence the differentiation characteristics and marker gene expression of the isolated chondrogenic cells. Percentage of adhesion on UC, GL and COL2 surfaces were 35%, 65% and 51%, respectively (Fig. 5A). There was rapid de-differentiation of the chondrogenic cells upon monolayer attachment and expansion, yielding a homogenous population of collagen I positive cells regardless of the extracellular matrix (ECM) substrate that was used for expansion (Fig. 5B and C). These chondrogenic cells maintained high proliferative ability and chondrogenic differentiation potential when cultured in the presence of TFP growth factor supplementation. Proliferation was assessed by initial plating of the cells at 2×10^4 cells/cm² and monitoring the cell number at confluence every 72 hrs. When these chondrogenic cells were cultured in TFP-supplemented medium, a homogenous and expandable culture of spindle-shaped, fibroblast-like cells was generated within 2 weeks (Fig. 6A). Chondrogenic cells initially displayed a slow proliferation rate, but proliferation increased significantly



after passage 1. Chondrogenic cells were continuously expanded in the presence of TFP growth factor combination for 27 days (a total of 8 passages), with the total PDL being 13.47 (10^4 -fold increase in total cell number; doubling time, $t_d = 38.5$ hrs; Fig. 6B). Chondrogenic cells cultured in TFP-supplemented medium were

able to retain a higher *Sox9* expression with a significant 1.5-fold difference compared to the control (Fig. 6C). Pellet cultures of expanded chondrogenic cells demonstrated significant deposition of s-GAG only when differentiated in the presence of TGF- β 1 (Fig. 6D). In the presence of TGF- β 1, chondrogenic cells

expanded in TFP growth factor combination were able to generate cartilaginous tissues intensely and homogeneously stained for s-GAG by day 14 of differentiation. In contrast, chondrogenic cells expanded in control (DMEM containing 10% FBS) medium formed pellets faintly stained for s-GAG at the periphery of the pellets. s-GAG deposition observed from the histological staining was further confirmed using quantitative analysis. Chondrogenic cells expanded in control and TFP growth factor combination produced pellets with comparable, low amounts of s-GAG when differentiated in the absence of TGF- β_1 . The s-GAG/DNA content was significantly higher when pellets were cultured in presence of TGF- β_1 , and were the highest if pellets were based on TFP-expanded chondrogenic cells. Similar level of s-GAG production was maintained to 28 days of differentiation in pellet cultures (data not shown). For this reason, TFP-expanded chondrogenic cells or TC1 cells were more closely investigated. TC1 demonstrated the loss of pluripotency with no detectable alkaline phosphatase activity and the loss in expression of pluripotency-associated genes including *Oct4* and *Rex1* [34] (Fig. 7A). In addition, lineage restriction analysis by means of RT-PCR and specific immunostaining further confirmed that these cells are lineage-restricted and depleted of other cell types, particularly the undifferentiated and the endodermal cells (Fig. 7B). These cells are also highly proliferative with approximately 60% of cells being positive for Ki67. Upon differentiation in pellet culture in the presence of TGF- β_1 , these chondrogenic cells formed highly homogenous cartilaginous tissue with morphological similarities to neocartilage, such as round cells embedded in lacunae (Fig. 7B). Lineage restriction analysis by means of RT-PCR confirmed that the TC1-derived cartilaginous tissue was depleted of cells of ectodermal and endodermal lineages (data not shown). Absence of endodermal cells was further confirmed by AFP immunostaining (Fig. 7B). To further demonstrate the lineage restriction of TC1, osteogenic and adipogenic differentiations were performed with the established methods for primary adult MSCs [22]. In contrast to multipotent MSCs that have the capacity to differentiate along chondrogenic, osteogenic and adipogenic lineage pathways, TC1 is unipotential, being restricted to differentiation along the chondrogenic lineage pathway and is not responsive to osteogenic and adipogenic differentiation conditions (Fig. 7C). In addition, TC1 displays a somatic cell cycle kinetics (Fig. 7D) and a normal karyotype (Fig. 7E). As expected from the normal cell cycle profile and the loss of pluripotency, intramuscular injection of TC1 cells in SCID mice, contrary to undifferentiated hESCs which developed palpable teratomas after 6 weeks, did not produce teratomas even after more than 9 weeks after injection (Fig. 7F).

Extracellular matrix modulation of chondrogenic capacity of chondrogenic cells

TGF- β_1 demonstrated the most significant impact on chondrogenic differentiation of expanded chondrogenic cells. For this reason, prolonged pellet culture of chondrogenic cells was car-

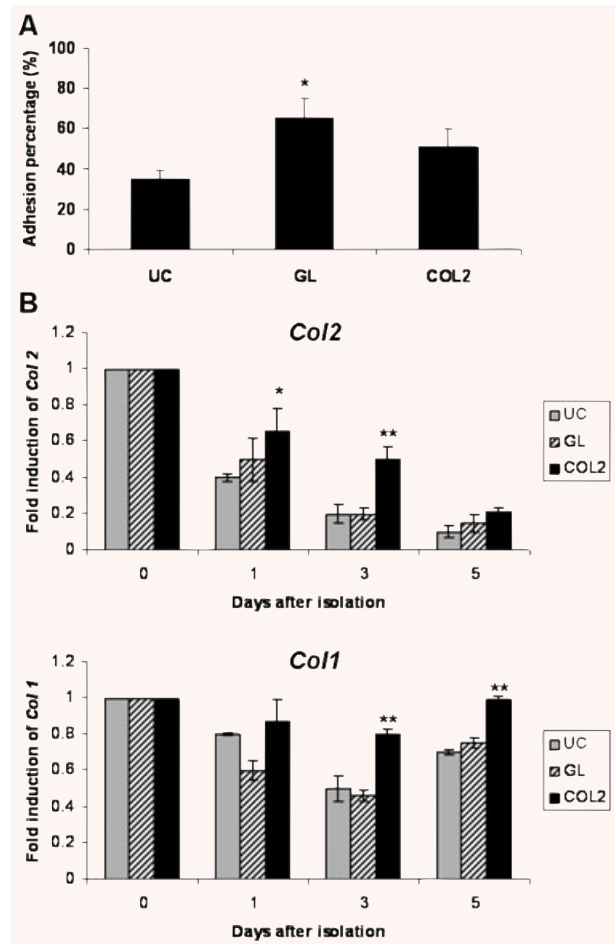


Fig. 5 Isolation and monolayer plating of chondrogenic cells. (A) Cell seeding efficacy on different surfaces. UC: uncoated; GL: gelatin-coated; COL2: type II collagen-coated surfaces. (B) Rapid de-differentiation of isolated chondrogenic cells occurred after collagenase treatment and monolayer plating, as observed from the gene expression profile of chondrogenic cells during expansion. Data reported were averaged from duplicates of two independent experiments, and expressed as mean \pm S.D. with statistical significance at * $P < 0.05$, compared to the uncoated control. (C) Immunostaining of collagen I and collagen II further confirmed the real-time PCR analysis. Isolated chondrogenic cells were double-stained with anti-collagen I (red) and anti-collagen II (green; arrow) specific antibodies and nuclear-counterstained with DAPI (blue). Eventual de-differentiation of the chondrogenic cells occurred regardless of the ECM substratum used for attachment and expansion. Scale bar = 200 μ m.

ried out in the presence of TGF- β_1 to assess possible hypertrophy maturation of these cells. After 4 weeks of pellet culture in chondrogenic medium in the presence of TGF- β_1 , TC1 cultured on COL2 substratum was able to generate cartilaginous tissues which more intensely and homogeneously stained for s-GAG and collagens. The staining intensity for s-GAG, collagen I and

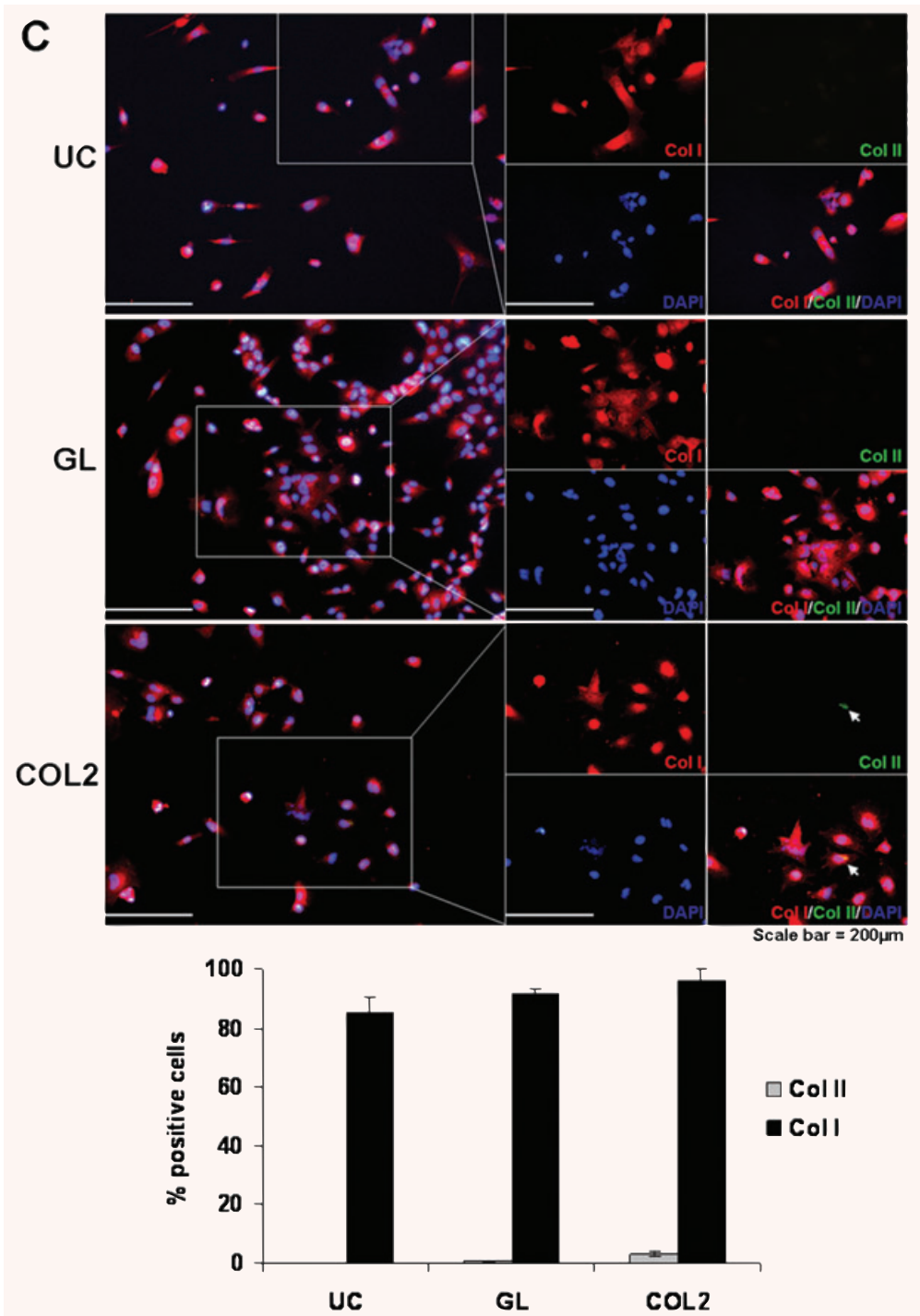


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collagen II appeared to be most intense in pellets generated by TC1 cultured on COL2 substratum (Fig. 7G). In contrast, TC1 cultured on UC and GL plates generated pellets that displayed heterogeneous or weak deposition of s-GAG and collagens, respectively. There was absence of collagen X in all pellets

generated by TC1 cells. s-GAG and collagen deposition observed from the histological staining were further confirmed using quantitative analyses. COL2-expanded TC1 cells produced pellets with the highest levels of s-GAG and total collagen ($*P < 0.05$).

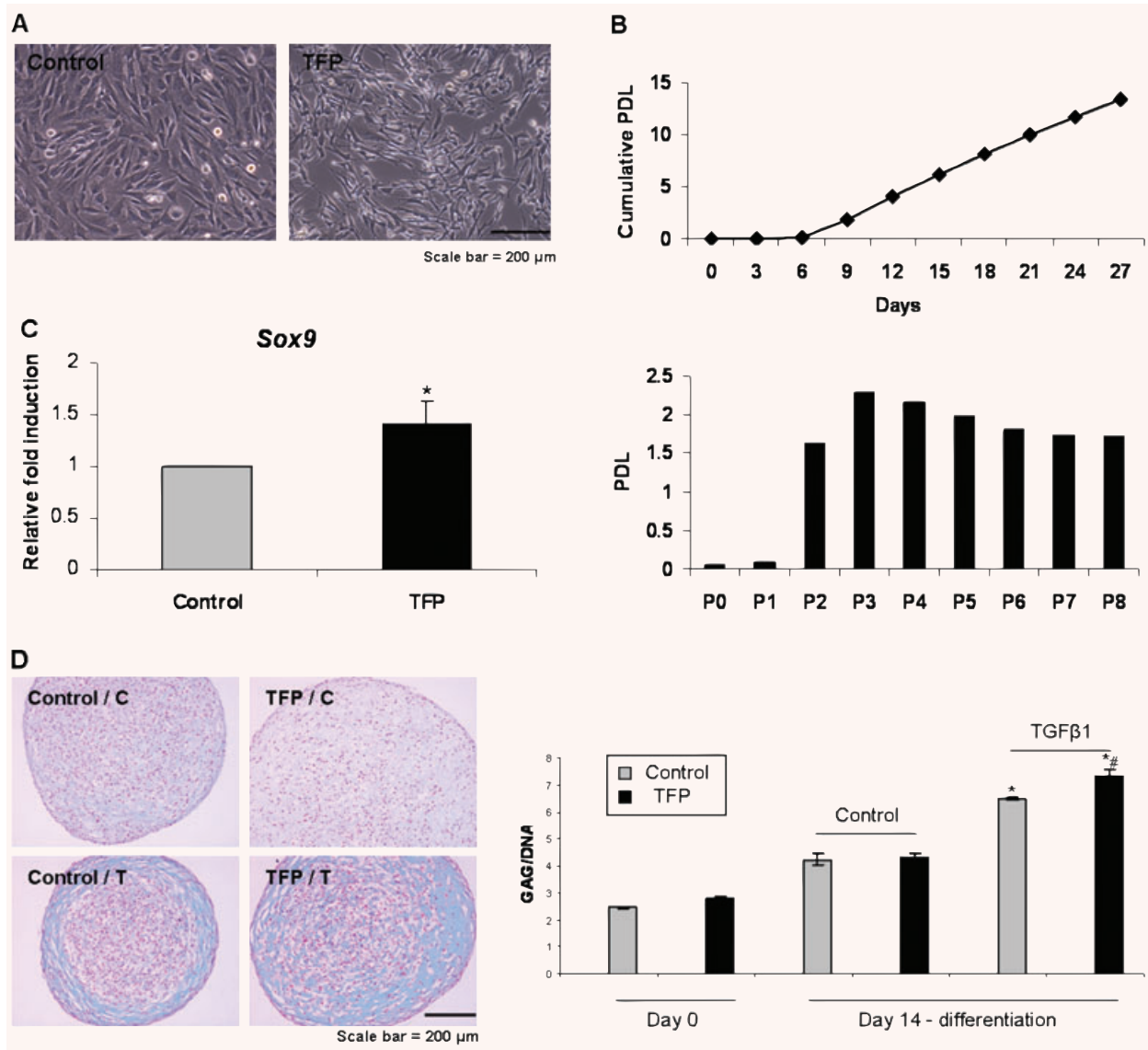


Fig. 6 Chondrogenic differentiation capability of expandable chondrogenic cells. (A) Cellular morphology and (B) growth characteristics of chondrogenic cells cultured in presence of TFP growth factor supplementation. (C) Chondrogenic cells showed enhanced *Sox9* expression when cultured in the presence of TFP growth factor supplementation. (D) Chondrogenic cells were expanded in DMEM/10%FBS (control) and TFP medium for 5 passages before induced to differentiate in pellet cultures for 14 days under control and TGF- β_1 supplemented chondrogenic conditions and assessed by histological and biochemical analysis. * = $P < 0.05$ versus control differentiated pellets; # = $P < 0.05$ versus control expanded, TGF- β_1 differentiated pellets.

In vivo cartilage regeneration

Cartilage tissue formation and repair of critical size defects by TC1 was evaluated in the knee joint of rats. Osteochondral defects (≈ 2 mm) were created on the patellar groove of the distal femur of Sprague-Dawley rats. Cells (5×10^5) were pellet-cultured in presence of TGF- β_1 for 14 days, and transplanted into the critical size defect site. The rats were killed at 6 weeks

after transplantation and the grafted sites were observed by histology (Fig. 8). The repaired tissue in the TC1-treated group demonstrated moderate surface regularity and displayed a greater area of cartilaginous tissue stained positive for s-GAG and collagen II, although staining was faint in some areas. In contrast, the empty defect control demonstrated poor healing with subchondral bone exposed to the articular cavity and little overlying cartilage layer was seen.

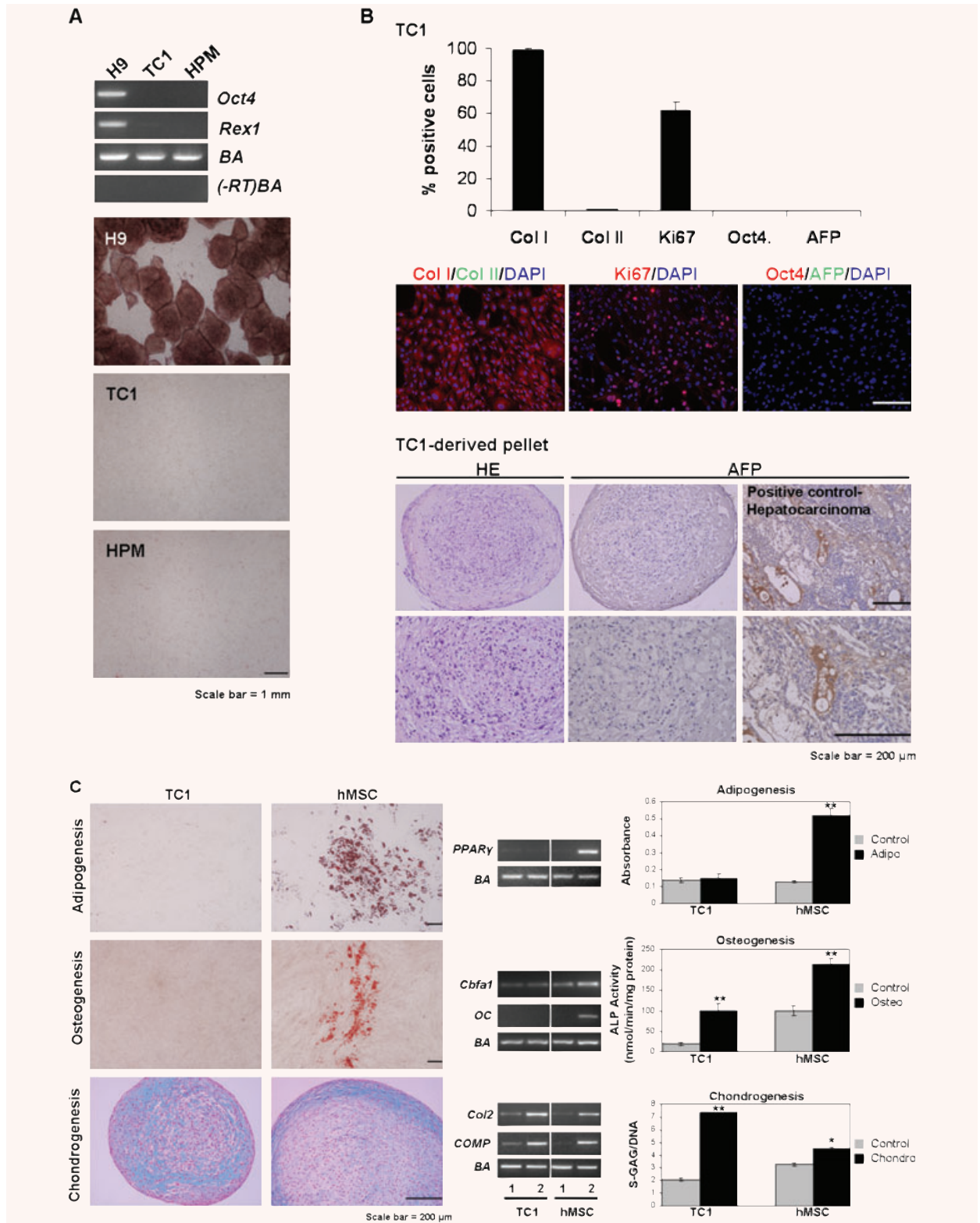


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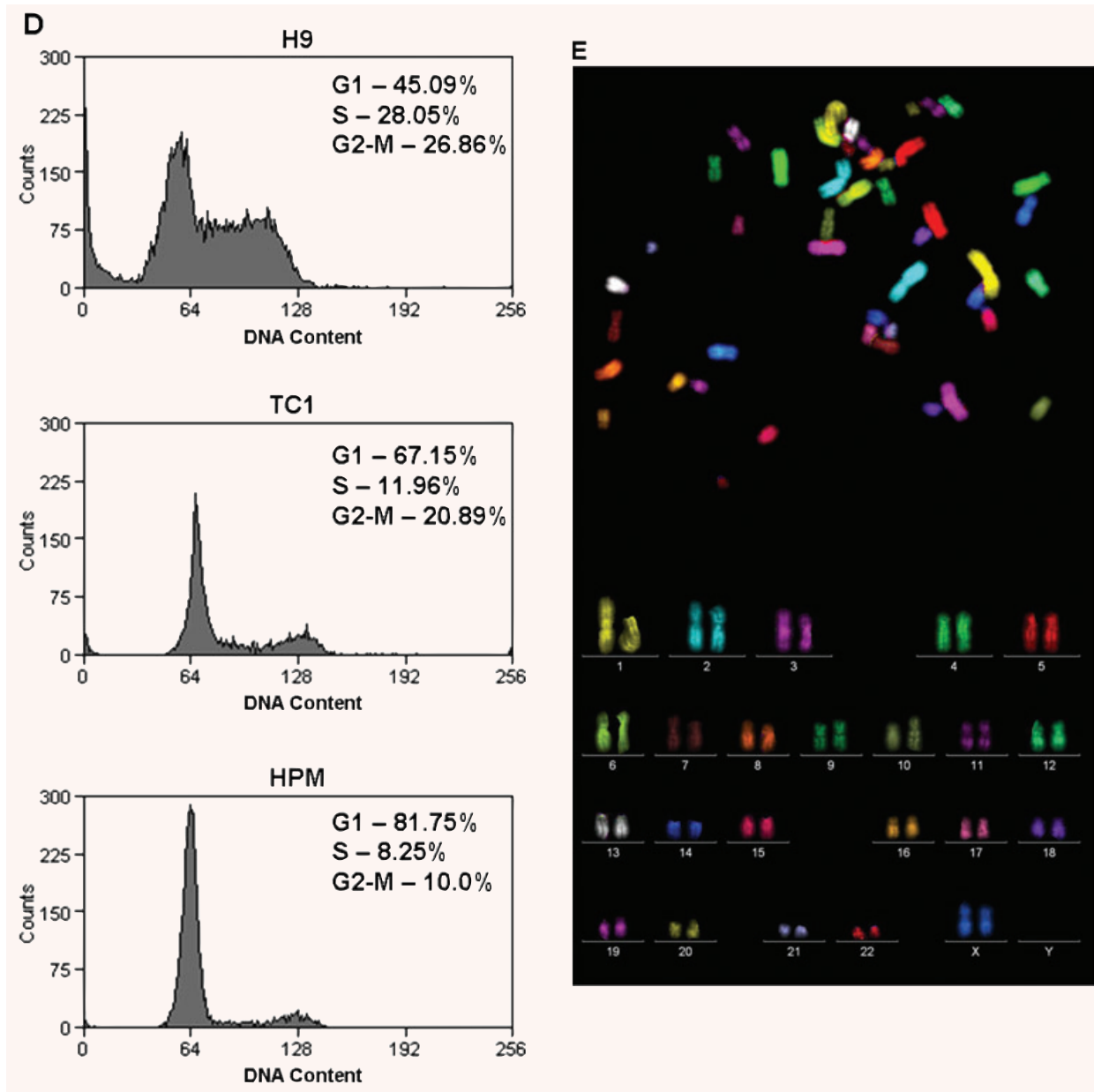


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Discussion

Directed chondrogenic differentiation of EB-derived cells in a high-density micromass system has been established in previous study [16]. In the first part of this study, we sought to understand the involvement of different growth factors (BMP-2 and TGF- β_1) in directing chondrogenic commitment of EB-derived cells. Next, we attempted to enrich these chondrogenic cells for further expansion and characterization of their chondrogenic differentiation capacity to form cartilaginous tissue.

Gene expression of cartilage-related matrix collagens and proteoglycans such as *LP*, *Col 9A1*, *COMP*, *Col 1* and *Col 2A/B* and cartilage-related receptors such as *CD44s* and *PDGFR α* were profiled for the micromass cultures of EB-derived cells undergoing chondrogenic differentiation for a period of 21 days. However, we observed that some of these markers such as *Col 9A1* and *PDGFR α* were also expressed in undifferentiated hESCs and 5'd' EBs. On the other hand, *Col 2* and *COMP* seem to be the most cartilage-specific with expression only in the differentiating EB-derived cells. Based on our findings, it is likely that BMP-2 and

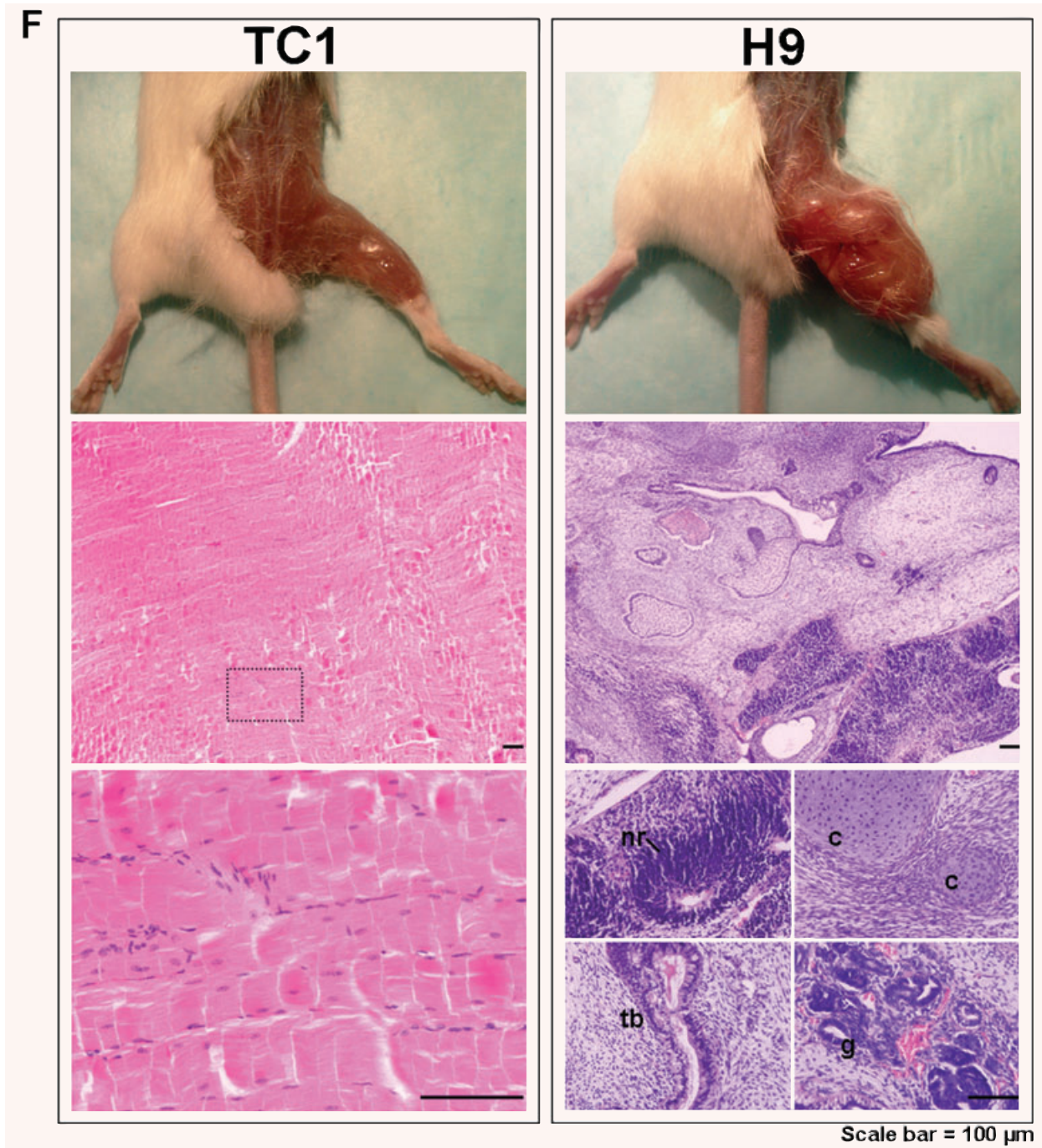


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TGF- β_1 modulate the expression of various protein components of cartilage differently. Although BMP-2 or TGF- β_1 alone was able to enhance expression of *COMP*, the combination of both factors was able to induce highest expression of *COMP* by day 21. This does not correlate to the *Col 2* expression whereby highest

expression was only observed in the presence of TGF- β_1 alone. This could be due to spatiotemporal differences in growth factor requirements and it is possible that sequential addition of growth factors may be necessary so as to cater to the differential requirement of each growth factor at different stages of chondrogenic

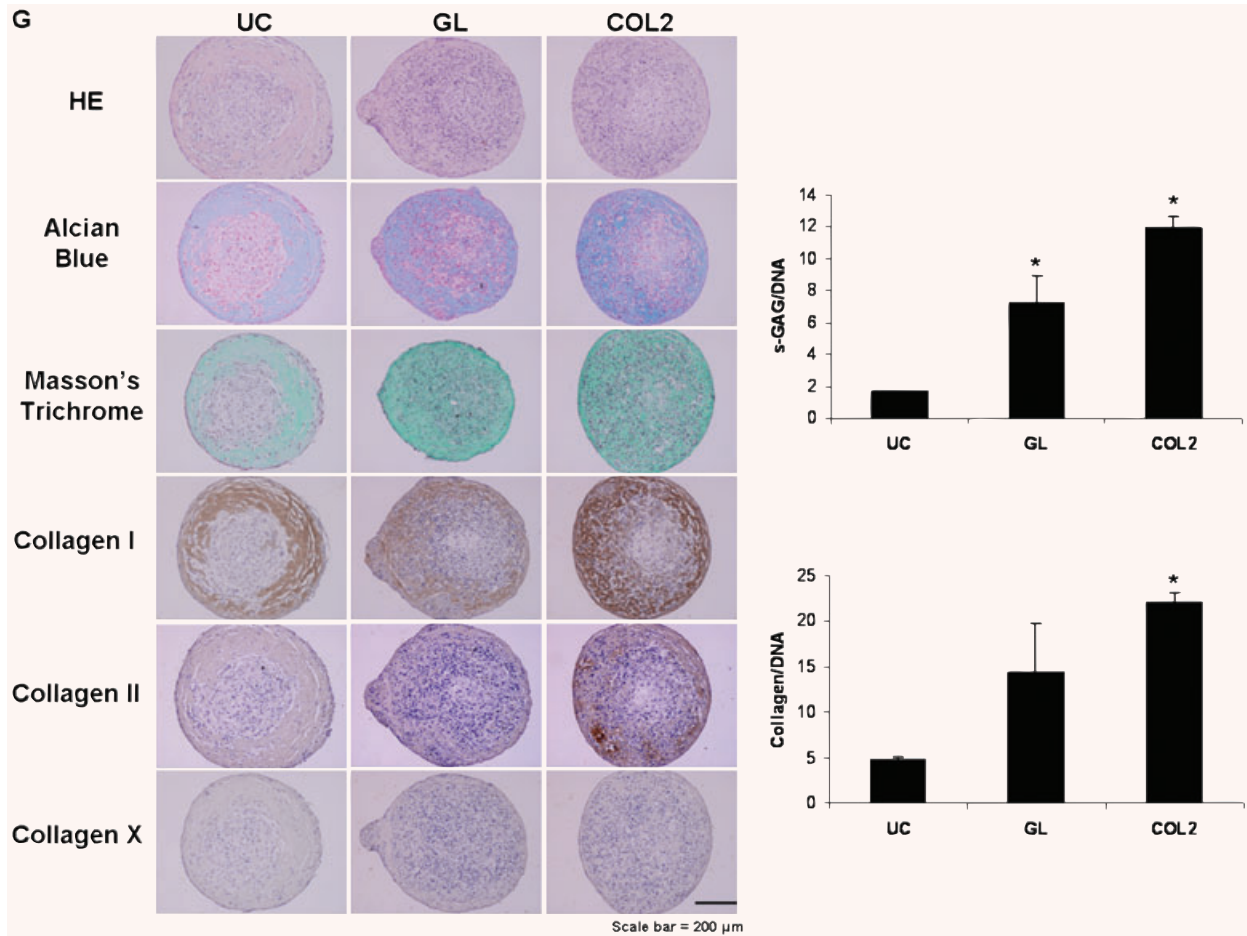


Fig. 7 Characterization of TC1. **(A)** Alkaline phosphatase activity and RT-PCR analysis of pluripotency-associated genes in H9, TC1 and HPM. **(B)** Morphological and lineage analysis of TC1 and differentiated cartilaginous pellet. **(C)** Comparative multilineage differentiation analysis of TC1 and hMSCs. Unlike hMSCs, TC1 cells did not differentiate to osteogenic and adipogenic lineages under inducing conditions, but only differentiate to the chondrogenic lineage. The mRNA expression of genes specific for adipogenesis (*PPAR γ*), osteogenesis (*Cbfa1* and *OC*), and chondrogenesis (*Col2a1* and *COMP*) are determined by RT-PCR. Lane 1 shows undifferentiated cells; lane 2 shows induced cells. **(D)** Comparative cell cycle analysis of undifferentiated H9, TC1 and HPM cells. **(E)** Chromosomal analysis of TC1 at passage 7. A total of 50 metaphase spreads were analysed and showed a normal karyotype of 46, XX for TC1. Metaphase spread (top panel) and karyotype (bottom panel) as observed under M-FISH. **(F)** Teratoma formation. Tumour observation and histological analysis at week 9 after injection with either TC1 (left) or parental undifferentiated H9 cells (right). Teratomas in SCID mice injected with H9 hESCs showed neural rosettes (nr), cartilage (c), tubular (tb) and glandular (g) structures. There is no observable teratoma formation in SCID mice injected with TC1. The boxed area in the top panel is shown at 200 \times magnification in the bottom panel. **(G)** ECM modulation of TC1. TC1 was expanded on UC, GL and COL2-coated surfaces for up to five passages before induce to differentiate in pellet cultures in the presence of TGF- β ₁ for 28 days. At the end of differentiation, the pellets were harvested for histological examination and biochemical analysis of s-GAG and total collagen. COL2-expanded TC1 formed cartilaginous tissue with significant higher levels of s-GAG and total collagen deposition, notably collagen I and II as observed histologically. Measurements reported were averaged from duplicates of two independent experiments, and data were analysed by one-way ANOVA and expressed as mean \pm S.D. and statistical significance at * $P < 0.05$, compared to the uncoated control.

development [35–38]. Whether addition of growth factors at specific time-points or stages of chondrogenesis would enhance differentiation in the micromass system is still currently under investigation.

Co-treatment with TGF- β ₁ and BMP-2 induced the formation of clusters of cells that are highly enriched in collagen I, and low in collagen II. This is consistent with the gene expression data with

highest level of *Col 1* relative to low level of *Col 2*, characteristic of chondrocytes of a fibrocartilage phenotype. This finding is somewhat similar to the observation by Koay *et al.* [19] whereby sequential induction by TGF- β ₃ followed by BMP-2 induced fibrocartilage-like formation of EB-derived cells. This is unlikely due to bone formation because we did not observe any gene expression

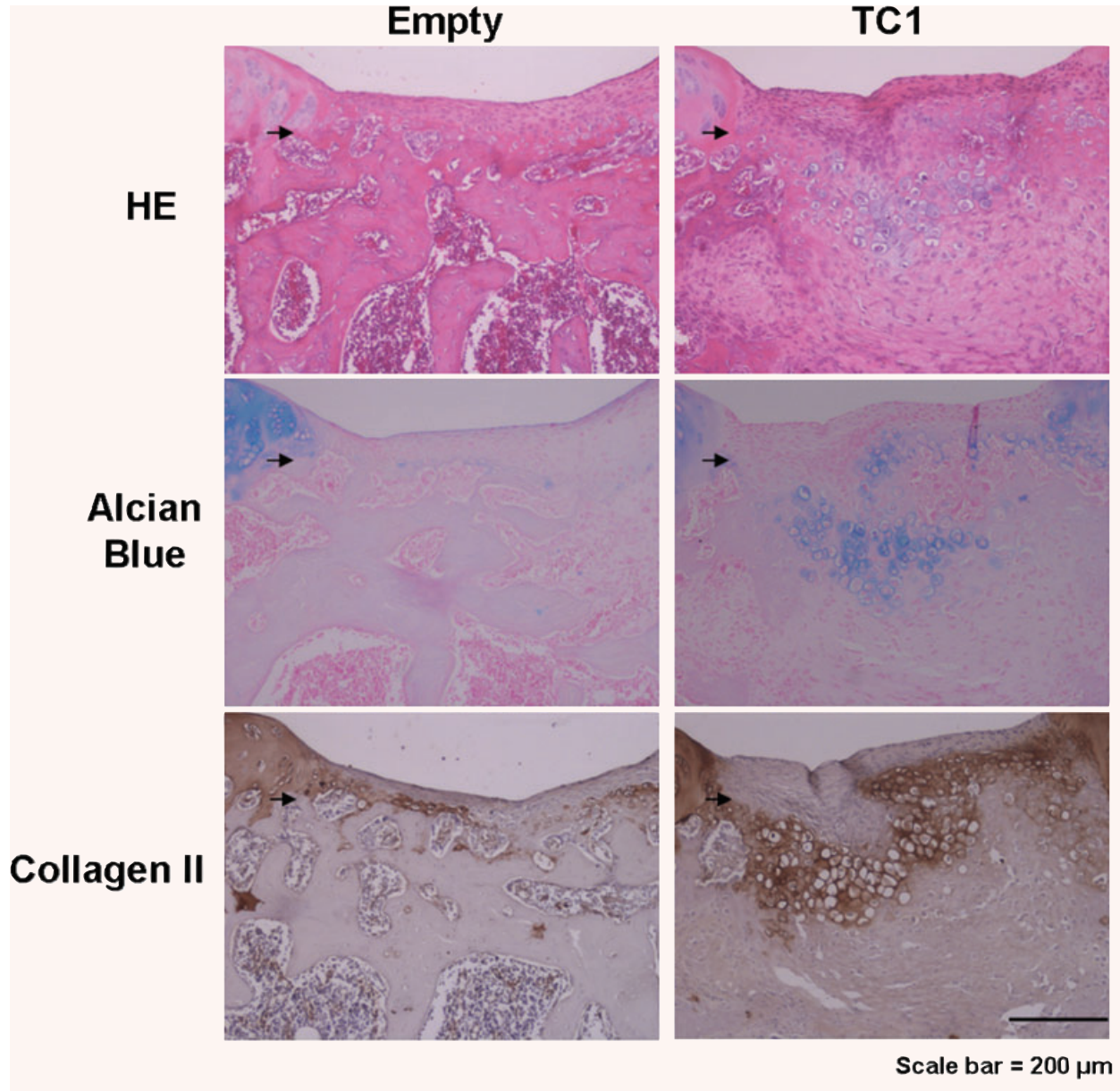


Fig. 8 *In vivo* cartilage regeneration with TC1 in rat osteochondral defect model. Alcian blue and collagen II positive cartilaginous tissue was observed in osteochondral defects repaired with TC1 pellets 6 weeks after transplantation. The empty defect control with no implant demonstrated poor healing with subchondral bone exposed to the articular cavity and little overlying cartilage layer was seen. Positions of defects created are indicated by arrows.

of the bone marker, *osteocalcin*, in our cultures. However, under this growth factor combination, there is still presence of differentiation to the ectodermal and endodermal lineages.

The gene expression profile of the chondrogenic differentiation induced under different growth factor treatments was further substantiated by determination of the s-GAG synthesis. It was observed that TGF- β_1 was able to induce the highest level of s-GAG/DNA ratio among the different growth factor treatments by

day 21 of differentiation, with a 2.5-fold up-regulation as compared to the control. Using *Col 2* as the cartilage-specific marker, time-course analysis using real-time PCR analysis of *Col 2* expression was performed. It was observed that TGF- β_1 was able to enhance chondrogenic differentiation of the EB-derived cells in the micromass system, with much intensified and prolonged expression of *Col 2* up to day 21. Furthermore, TGF- β_1 induced low levels of *Col 1* and yielded an overall highest differentiation

index denoted by *Col 2/Col 1* ratio. Consistently, we observed highest number of collagen II positive chondrogenic cells forming dense filamentous matrix network in the presence of TGF- β_1 .

Taken together, these results are consistent with findings reported for chondrogenic induction of human embryonic germ cells [39, 40] and murine embryonic stem cells [9], of which TGF- β_1 alone was sufficient to promote chondrogenesis of EB-derived cells in our adherent micromass system. In addition, the effect of TGF- β_1 on chondrogenesis may be correlated with enhanced chondrogenic commitment and the loss of propensity to ectodermal, haematopoietic and adipogenic lineages. In line with our previous report [16], the defaulted differentiation by EBs towards the extraembryonic endoderm differentiation, marked by the presence of cystic structures and expression of *AFP*, was moderately inhibited under high-density culture conditions and further reduced in the presence of TGF- β_1 . In addition, there was a substantial inhibition in differentiation to other mesodermal-derived lineages including haematopoietic, osteogenic and adipogenic lineages, suggesting indeed that the mesodermal progenitors derived in the presence of TGF- β_1 are likely to be chondroprogenitors committed to the chondrogenic lineage differentiation.

Next, we attempted to enrich these chondrogenic cells by means of collagenase treatment and selective plating on different ECM substratum. Isolated chondrogenic cells derived from the high-density cultures behave like primary chondrocytes in which they rapidly de-differentiate upon monolayer expansion [41]. This occurred regardless of the ECM substratum used for attachment. Similarly, these chondrogenic cells also tend to de-differentiate to a fibrochondrogenic lineage upon extensive monolayer expansion. When these chondrogenic cells were expanded in TFP-supplemented medium, they retain higher chondrogenic potential indicated by a higher *Sox9* expression, and form homogenous cartilaginous tissue rich in s-GAG by day 14 of culture. The TFP growth factor combination has been used to propagate human primary chondrocytes in monolayer while maintaining their differentiation potential [42]. This hESC-derived chondrogenic cell population (which we will refer to as TC1) is lineage-restricted with loss of pluripotency, highly expandable, and displays a normal karyotype and a somatic cell cycle kinetics [43]. In addition, the risk of teratoma formation is minimal as lineage-restricted TC1 did not induce tumour formation in *in vivo* SCID mouse model.

Despite the inability of ECM substratum to retain chondrocyte phenotype, the differentiation capacity of TC1 is largely modulated by the ECM during expansion. When expanded on COL2 substratum, TC1 formed a more homogenous cartilaginous tissue with increased collagen II deposition. There was also an overall enhancement in s-GAG and collagen I deposition. Interestingly, COL2-expanded chondrogenic cells cultured in DMEM/10% FBS in the absence of TFP growth factor supplementation did not display increase in s-GAG and collagen deposition, suggesting a synergistic requirement of both growth factors and ECM that modulate the phenotype of the cartilaginous tissue formed (data not shown). The mechanisms by which growth factors and ECM modulate the post-expansion differentiation capability of chondrogenic cells are not well understood. It is likely that provision of a carti-

lage-related ECM, type II collagen, in the initial plating and later in expansion helps in continuous selection of ECM-responsive subpopulations of cells with enhanced pre-existing commitment towards the chondrogenic lineage [44–45]. Indeed, chondrocytes were found to respond to ECM components, including collagen I [46], collagen II [44] and hyaluronic acid [47], resulting in the regulation of various chondrocyte behaviours and processes such as cell shape changes, chondrocytic phenotype maintenance, chondrogenic differentiation and re-differentiation. Most remarkably, no evidence of hypertrophy maturation was observed in TC1-derived pellets even after 4 weeks of pellet culture, in contrast to observations with *in vitro* differentiated MSC-derived pellets that displayed signs of hypertrophy, as early as 2 weeks onwards [48]. This reflects a fundamental difference in cell origin, and TC1 derived from hESCs may resemble more closely to chondrocytes in that they are not prone to hypertrophic differentiation during expansion [49] or in the presence of TGF- β_1 as an inhibitor of hypertrophy of chondrocytes [50].

TC1 may serve as a potential alternative cell source for cartilage repair where the current cell-based therapies involving MSCs and chondrocytes (autologous chondrocyte implantation) may have issues of limited availability of donor tissue and limited proliferative capacity that decreases with age. Indeed, preliminary studies utilizing TC1 in cartilage repair demonstrated reparative ability in cartilage regeneration with repaired cartilaginous tissue positive for s-GAG and collagen II. Long-term studies are currently ongoing and will be necessary to fully evaluate the cartilage repair and integration ability, as well as the cartilage phenotype stability of TC1, in comparison with MSCs and chondrocytes.

In summary, using high-density micromass cultures of EBs in the presence of specific growth factors, we have developed a method to induce chondrogenic commitment of the hESCs with generation of putative chondrogenic cells. We have also established a method to isolate and enrich the chondrogenic cells for expansion while still retaining the chondrogenic differentiation potential for re-differentiation to form cartilage-like tissue both in the pellet system *in vitro* and in the cartilage defect *in vivo*. This protocol enables the derivation of homogenous and potentially clinically compliant chondrogenic cells from hESCs in a defined manner that circumvents the need for transfection of any genetic material [15] and co-culture with chondrocytes [17, 18]. It also efficiently compensates for the lack of known surface markers specific for chondrogenic lineage. Although many questions remain to be answered regarding the biochemical, metabolic and biomechanical characteristics of the chondrogenic cells and the types of cartilage tissue formed, this defined protocol offers a simple and robust design that is amenable to further manipulations and modifications.

In conclusion, this work marks an important first step towards generating expandable chondrogenic cells from hESCs for cartilage tissue engineering and may also allow us to produce sufficient numbers of chondrogenic cells to develop a potential platform for drug testing [51], gene therapy [52], and cell-based therapy [53] for fibrocartilage repair in knee meniscus and temporomandibular joint disc as well as hyaline cartilage repair in articular cartilage.

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