Macroscopic cartilage formation with embryonic stem-cell-derived mesodermal progenitor cells

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Accepted 7 February 2003
Journal of Cell Science 116, 2015-2028 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00417

Summary

The totipotent embryonic stem cell generates various mesodermal cells when stimulated with BMP4. Among the resulting cells, those expressing flk-1 and/or PDGFRα displayed chondrogenic activity in the presence of TGFβ3 and expressed cartilage-specific genes in 7 to 16 day pellet cultures. Depositions of cartilage matrix and type II collagen were detected by day 14. TGFβ-stimulated chondrogenesis was synergistically enhanced by PDGF-BB, resulting in a larger cartilage particle filled with a cartilaginous area containing type II collagen, with a surface cell layer expressing type I collagen. In contrast, noggin inhibited both the TGFβ- and TGFβ+PDGF-stimulated cartilage formation, suggesting that a BMP-dependent pathway is involved. In fact, replacement of TGFβ3 with BMP4 on days 10 to 12 markedly elevated the cartilage matrix deposition during the following 7 to 8 days. Moreover, culture with TGFβ3 and PDGF-BB, followed by the incubation with BMP4 alone, resulted in a cartilage particle lacking type I collagen in the matrix and the surface layer, which suggests hyaline cartilage formation. Furthermore, such hyaline cartilage particles were mineralized. These studies indicate that the PDGFRα$^+$ and/or flk-1$^+$ cells derived from embryonic stem cells possess the full developmental potential toward chondrocytes, in common with embryonic mesenchymal cells.

Key words: Embryonic stem cells, Cartilage, BMP, TGFβ, PDGF

Introduction

Chondrogenesis is initiated during embryogenesis by the commitment of two types of mesoderm, paraxial mesoderm and lateral plate mesoderm, to differentiate into the sclerotome of the somite and limb mesenchyme, respectively. The former leads to the axial skeleton and the latter to the appendicular skeleton. A number of gene products playing important roles in skeletogenesis have been identified and characterized, and chondrogenesis culture methods using embryonic mesenchymes have also been established (Cancedda et al., 1995). However, the specific cellular events occurring during the transformation from a totipotent epiblast to a chondrocyte remain largely undefined. Taking advantage of the capacity of totipotent embryonic stem (ES) cells to differentiate in vitro into cell types derived from all three germ layers, we have begun to address the cellular events involved by selectively differentiating ES cells into mesodermal lineages and by identifying, isolating and characterizing various progenitor cells for their differentiation potential.

Upon differentiation of ES cells in a conventional serum-containing medium, some cells starts to express the vascular endothelial growth factor (VEGF) receptor-2 (VEGFR-2 or flk-1) protein, whose function is essential for embryonic hematopoiesis and vasculogenesis in mice (Shalaby et al., 1997; Shalaby et al., 1995), and some begin to express the platelet-derived growth factor receptor α (PDGFRα) protein (Kabrun et al., 1997; Nishikawa et al., 1998a). The ES-cell-derived flk-1$^+$ cells contain the hemangioblast, the common progenitor cell for both the hematopoietic and endothelial cell lineages (Choi et al., 1998; Faloon et al., 2000; Nishikawa et al., 1998a; Ogawa et al., 1999). Embryo-derived cells with similar cell-surface characteristics also show hemogenic and angiogenic activities (Nishikawa et al., 1998b; Ogawa et al., 1999). Consistently, both the extraembryonic mesoderm, the origin of the primitive endothelial cells and hematopoietic cells, and the intraembryonic mesoderm, which includes the presumptive endocardium and the proximal lateral mesoderm, express flk-1 (Dumont et al., 1995; Kataoka et al., 1997; Takakura et al., 1997; Yamaguchi et al., 1993). Therefore, flk-1$^+$ cells are considered to represent the hemoangiogenic lateral plate mesoderm, that is, the splanchnopleuric mesoderm. Another part of the lateral plate mesoderm, the somatopleuric mesoderm, participates in the formation of limb bone. However, the potential of flk-1$^+$ cells to generate cartilage and bone has not been reported yet.

During early mouse embryogenesis, the *PDGFRα* gene is expressed in most of mesodermal cells (Orr-Urtreger et al., 1992; Schatteman et al., 1992), and PDGFRα signaling is essential for normal development of many mesodermal tissues, including the axial skeleton (Soriano, 1997). Interestingly, protein expression appeared to be limited to the paraxial mesoderm, and later to the somite (Takakura et al., 1997). The strong expression of the *PDGFRα* gene in the somitic block is later restricted in the dermatome and sclerotome, but not in the myotome. *PDGFRα* is also expressed in the lateral mesoderm, the proximal limb mesenchyme, and the perichondrium. Thus, the ES-cell-derived PDGFRα$^+$ cells may form the paraxial mesoderm, somite cells or the chondrogenic mesenchymal cells, including the

sclerotome. However, the chondrogenic potential of the ES-cell-derived PDGFRα$^+$ cells has not yet been demonstrated.

Chondrogenesis from undifferentiated mesenchymal cells can be divided into several stages (Cancedda et al., 1995; Hall and Miyake, 2000). The first stage is precartilage condensation, in which the mesenchymal cells are closely packed and start to differentiate into chondroblasts and chondrocytes. Chondroblasts/chondrocytes proliferate and secrete increasing amounts of cartilage matrix macromolecules, until each single cell is completely surrounded by a matrix. Cells at the periphery of condensation are relatively undifferentiated and later form a sheath of spindle-shaped cells, which becomes the perichondrium, around the cartilage rudiment. Each stage requires a different and/or an overlapping set of extracellular factors. For example, transforming growth factor (TGF)β is a potent factor for driving the differentiation of mesenchymal cells toward chondrocytes in vitro (Kulyk et al., 1989). It seems to be necessary for the initial mesenchymal condensation stage (Leonard et al., 1991). In addition, TGFβ also plays an inhibitory role in chondrocyte maturation (Ballock et al., 1993; Kato et al., 1988; Serra et al., 1997; Yang et al., 2001), for which the importance of the perichondrium has been demonstrated (Alvarez et al., 2001). On the other hand, bone morphogenetic protein (BMP) function is also implicated in several steps in cartilage formation. Like TGFβ, BMP has early positive roles, that is, proliferation, maintenance and maturation of chondrocytes, and a negative role at a later stage, that is, delay of terminal hypertrophic differentiation in the growth plate (Capdevila and Johnson, 1998; Duprez et al., 1996; Enomoto-Iwamoto et al., 1998; Minina et al., 2001; Pathi et al., 1999; Pizette and Niswander, 2000; Zou et al., 1997). Different BMPs are detected around the mature chondrocytes in the mid to deep zone of the articular cartilage and the hypertrophic/calcifying zone of the growth plate (Anderson et al., 2000).

BMP4, required for mesoderm formation in mice (Winnier et al., 1995) and for hematopoietic cell genesis during the early embryogenesis of vertebrates, is a strong hematopoietic-cell-inducer for ES cells in a serum-free environment (Johansson and Wiles, 1995; Nakayama et al., 2000). We have previously demonstrated that treatments with BMP4 followed by VEGF, a flk-1 ligand, synergistically stimulate the generation of lymphohematopoietic progenitor cells from ES cells in a serum-free medium, which supports the idea that BMP4-induced mesoderm produces hematopoietic cell progenitors in a flk-1-dependent manner like it does in vivo (Nakayama et al., 2000). Here, we have demonstrated that two types of mesodermal cells, flk-1$^+$ cells and PDGFRα$^+$ cells, are induced by BMP4 in serum-free medium, from which macroscopic cartilage particles are generated in vitro. Hematopoietic progenitor cells were generated predominantly from the flk-1$^+$PDGFRα$^-$ cells. However, under the optimal conditions established with TGFβ3, BMP4 and PDGF-BB, the flk-1$^-$PDGFRα$^+$ cells, the flk-1$^+$PDGFRα$^+$ cells and the flk-1$^+$PDGFRα$^-$ cells produced hyaline cartilage particles, which were further mineralized. Therefore, the ES-cell-derived mesodermal cells seem to possess the full developmental potential to form mature cartilage.

Materials and Methods

Cells, factors and antibodies

The E14 ES cells and the OP9 stromal cell line were obtained and cultured as described previously, except that ES cells were cultured under 7.5% CO_2 (Nakayama et al., 1998). Fluorescein isothiocyanate (FITC)- or R-phycoerythrin (PE)-conjugated monoclonal antibodies and their isotype controls for the fluorescence-activated cell sorting (FACS), fetal calf serum (FCS), media, buffers, media supplements, cytokines and all of the tissue culture flasks and plates were obtained as described previously (Nakayama et al., 1998; Nakayama et al., 2000). Added to this study were human insulin-like growth factor-1 (IGF1), human PDGF-AA, human PDGF-BB, mouse thrombopoietin/megakaryocyte growth and differentiation factor (TPO) and human TGFβ3, purchased from R&D Systems (Minneapolis, MN), the rat monoclonal antibodies for mouse flk-1 (clone Ava12) conjugated with PE (Flk-1-PE), purchased from Pharmingen (San Diego, CA), and the monoclonal antibody for mouse PDGFRα (clone APA5), kindly provided by S. Nishikawa (Kyoto, Japan) and conjugated with biotin at Amgen (PDGFRα-bio). The PDGFRα$^+$ cells were detected with allophycocyanin-conjugated streptavidin (SA-APC), purchased from Pharmingen. For the collagen immunostaining analysis, the anti-type II collagen (anti-COL2) monoclonal antibodies (clones 2B1.5 and 6B3) were obtained from NeoMarker, the anti-type X collagen (anti-COL10) monoclonal antibody (clone X53) was from Quartett GmbH, the control mouse IgG was from Pharmingen, the rabbit anti-mouse type I collagen (anti-COL1) polyclonal antibody (AB765P) was from Chemicon (Temecula, CA) and the control rabbit IgG was from Sigma. Mouse noggin protein fused with the human IgG_1-Fc portion (noggin-Fc) was purchased from R&D, and human IgG_1-Fc fragment (IgG-Fc) was from Jackson ImmunoResearch Lab (West Grove, PA).

Induction of differentiation in a serum-free medium by the embryoid body formation method

Differentiation was performed as described previously (Nakayama et al., 2000). Briefly, E14 ES cells were cultured on a fibronectin-coated 60 mm plate (Becton Dickinson) for 2 days in the KnockOut-SR (Gibco)-based serum-free medium containing 10 ng/ml human leukemia inhibitory factor. The cells were then differentiated at 4500 cells/ml in the serum-free medium containing 0.9% methylcellulose (Stem Cell Technology) in the presence of 1.9 ng/ml BMP4 under 5% CO_2, 5% O_2. On days 3.6 and 4.6, embryoid bodies (EBs) were collected, combined and treated with 0.5 mM EDTA, and single cell suspensions were obtained by passing the EBs through a 22-gauge needle. The cells were then stained with 2 μg/ml PDGFRα-bio and 4 μg/ml Flk-1-PE or with 2 μg/ml SA-APC. The stained samples were analyzed and sorted using a Vantage SE cell sorter (Becton Dickinson).

Serum-free erythro-myeloid colony-forming cell analysis

Each sorted EB cell fraction was mixed with a serum-free colony-forming cell (CFC) medium, containing 1% methylcellulose and supplemented with a mixture of hematopoietic cytokines, and was distributed into two to four 35 mm bacterial-grade dishes at 2.5×10^4 cells/plate. The serum-free CFC medium was as described previously (Heyworth and Spooncer, 1993), except that linoleic acid and soybean lipids were replaced with 2% chemically defined lipids (Gibco). The cytokines included were the same as described before for the FACS-purified EB cells, except that 10 ng/ml TPO was added (Nakayama et al., 2000). On days 10 to 12, burst-forming-unit erythrocyte, colony-forming unit (CFU) macrophage/monocyte, CFU neutrophil, CFU mast cell and CFU mix were counted.

Co-culture method for developing erythro-myeloid CFCs, lymphokine-activated killer cells and pre-B cells from sorted EB cells

The OP9 cell co-culture method for generating the erythro-myeloid

CFC activity and the pre-B and/or lymphokine-activated killer (LAK) lymphocytes was as described previously (Nakayama et al., 1998). For the former, sorted EB cells were plated at 5×10^4 cells/well onto a confluent layer of OP9 cells within a six-well plate and were cultured for 3 days without interleukin (IL)-2 and IL-7. Both non-adherent cells and loosely attached cells were then mechanically harvested and subjected to CFC analysis. Lymphoid potential was detected with the same culture with IL-2 and IL-7, except that the sorted cells were plated at 1×10^4 cells/well. On days 10 to 12, both non-adherent cells and loosely attached cells were mechanically harvested, followed by FACS phenotyping (FACScan, Becton Dickinson) using the Sca-1-FITC and B220-PE antibodies to confirm the generation of $B220^+$ lymphocytes: pre-B cells ($Sca\text{-}1^-$) and LAK cells ($Sca\text{-}1^+$), as described previously (Nakayama et al., 1998).

Serum-free micromass cultures for chondrogenesis

The FACS-purified EB cells were re-suspended at 2×10^7 cells/ml in a serum-free chondrogenesis medium. The medium was a modification of that reported by Mackay et al. (Mackay et al., 1998), in that Dulbecco's modified Eagle's medium (DMEM) was replaced with DMEM (high glucose): Ham's F12=1:1 (Gibco), 0.3% glucose (Sigma), 50 μM monothioglycerol (MTG, Sigma) and 50 ng/ml IGF1 were added. Then, 7.5 μl (1.5×10^5 cells)/well of the cell suspensions were spotted onto a fibronectin-coated 24-well plate (Becton Dickinson), incubated for 1 hour, and cultured in 1 ml/well of the same medium under 5% CO_2, 5% O_2 for 7 to 8 days. The culture was washed twice with PBS and the total protein was extracted with LDS gel-loading buffer (Invitrogen) including the protease-inhibitor mix (Boehringer Mannheim) for western blot analysis. For the COL2 analysis, 1 μg/ml 6B3 was used. To detect the sulfated glycosaminoglycans, the culture was fixed with 10% formalin, stained with 1% Alcian blue at pH 1 (American Master Tech Scientific) and destained with acetic acid, according to Tallquist et al. (Tallquist et al., 2000).

The pellet culture was performed as described previously (Mackay et al., 1998) except that the serum-free chondrogenesis medium included 50 μM MTG. The $3\text{-}4\times10^5$ FACS-purified EB cells were first re-suspended in 0.5 ml of the chondrogenesis medium supplemented with or without 10 ng/ml TGFβ3 and/or other factors indicated. Cells were then centrifuged and cultured as a pellet. On days 14 to 20, each cartilage particle was fixed with 10% buffered zinc formalin (Anatech) or Gendre's fluid (Bedossa et al., 1987) for less than 24 hours at room temperature, paraffin-embedded, sectioned through the center part of each particle and stained with 0.1% Toluidine blue (Sigma) (Sheehan and Hrapchak, 1987). Two additional sections were also made through different parts of each particle, to confirm reproducibility. Some sections were immunostained with 4 μg/ml 2B1.5 for COL2 or with 2 μg/ml AB765P for COL1, according to the manufacturer's recommendation, and were counterstained with Gill 2 Hematoxylin (Shandon) (Sheehan and Hrapchak, 1987).

Mineralization was induced in the modified hypertrophic differentiation medium described by Mackay et al. (Mackay et al., 1998). We added 50 μM MTG and replaced 50 ng/ml thyroxine with 10 nM T3 (Sigma), as described previously (Alini et al., 1996). After 5 days of culture, the particle was fixed with 10% buffered zinc formalin, paraffin-embedded, sectioned, stained with von Kossa and counterstained with Nuclear FastRed (Sheehan and Hrapchak, 1987). Some sections were immunostained with 1:20 diluted X53 for COL10, according to the manufacturer's recommendations.

Cartilage-specific gene expression by the reverse transcriptase-polymerase chain reaction method

Two to five aggregates were harvested at each designated time point and were disrupted immediately in the guanidine isothiocyanate solution provided with the RNeasy Kit (Qiagen). Total RNAs were purified using the protocol recommended by the manufacturer, including the DNase I treatment step. Essentially the same materials and protocols were used for reverse transcription (RT) and nested polymerase chain reaction (PCR) as previously described (Nakayama et al., 1998). Modifications were (1) approximately 0.1 μg cDNA per reaction was used, (2) the annealing temperature was set at 68°C and (3) the cycle number was 21 with an outside primer set, followed by another 21 cycles with the corresponding inside primer set. The primers for cartilage–specific genes, including parathyroid hormone-related protein (PTHrP), aggrecan, cartilage oligomeric matrix protein (COMP), COL2, COL10 and Chordin-like 1 (CHL1), are shown in Table 1.

Results

BMP4 induced $flk\text{-}1^+$ and $PDGFR\alpha^+$ progeny from ES cells in a serum-free medium

First, using the EB formation method in a serum-free medium, we determined whether BMP4 would allow ES cells to generate the $flk\text{-}1^+$ cells and the $PDGFR\alpha^+$ cells, as demonstrated using the two-dimensional differentiation method in a serum-containing medium (Nishikawa et al., 1998a). As shown in Fig. 1A, 1.9 ng/ml of BMP4 induced $flk\text{-}1^+$ and $PDGFR\alpha^+$ cells from ES cells by day 4.6 of differentiation.

After FACS isolation, single positive cells ($flk\text{-}1^-PDGFR\alpha^+$ and $flk\text{-}1^+PDGFR\alpha^-$), double positive (DP) cells ($flk\text{-}1^+PDGFR\alpha^+$) and double negative (DN) cells ($flk\text{-}1^-PDGFR\alpha^-$) were individually examined for their erythro-myeloid CFC activities and lymphoid potentials. Prior to the OP9 culture, CFCs

Table 1. Oligonucleotide primers for nested RT-PCR analysis

Gene		Sequence	Product (bp)
Outside primers			
PTHrP	sense	5′-CGGTTTTCGCTCTTCTTTCAG-3′	798
	antisense	5′-GTTTCCTGGGGAGACAGTTTGAT-3′	
Aggrecan	sense	5′-CTTGGTGGCGCTGTAACAAAC-3′	1047
	antisense	5′-TTTCTTCTGCCCGAGGGTTCTA-3′	
COMP	sense	5′-ACAGATGCTGCGAGAACTTCA-3′	1683
	antisense	5′-TCATCAGTGGCGGTGTTTACAT-3′	
COL2	sense	5′-AGCAGGGCGAGAGGGGACT-3′	1224
	antisense	5′-GGGAGGACGGTTGGGTATCATC-3′	
COL10	sense	5′-GGCGGAAAGGGGAAACAGGATA-3′	1310
	antisense	5′-CATCGTAGGCGTGCCGTTCTTAT-3′	
CHL1	sense	5′-CATATTGCGTGTTTCAAGACAAGA-3′	817
	antisense	5′-GATTGGGGCAGTGGATTTTC-3′	
β-actin	sense	5′-GTGACGAGGCCCAGAGCAAGAG-3′	541
	antisense	5′-GAGGAAGAGGATGCGGCAGTG-3′	
Inside primers			
PTHrP	sense	5′-GCTGCGGAGGCTGGTTCA-3′	373
	antisense	5′-CTTCTTCCCGGGTGTCTTGAGTG-3′	
Aggrecan	sense	5′-CCGGAAGTGAGTGGAGAATCTA-3′	403
	antisense	5′-CAGGAAAGTGGCGGTAACAGT-3′	
COMP	sense	5′-GTTGCGACACGAGGTCAAGGAG-3′	410
	antisense	5′-GCTGCCCGGTCTCACACTCATT-3′	
COL2	sense	5′-TGCCAAGACCTGAAACTCTGC-3′	425
	antisense	5′-TCGGCCCTCATCTCTACATCAT-3′	
COL10	sense	5′-ACCCAGGGGCTCCAGGAATAG-3′	791
	antisense	5′-TACCCCGTGGTTAGCACTGACAA-3′	
CHL1	sense	5′-TGCGAATACAATGGAACCACTTA-3′	506
	antisense	5′-ACAATGCCAAATGCTCGTAGAT-3′	
β-actin	sense	5′-ACTGGGACGACATGGAGAAG-3′	336
	antisense	5′-TGAGGTAGTCCGTCAGGTCC-3′	

were hardly detected in any of these cell fractions. However, the CFC activities were strongly induced from the flk-1$^+$PDGFRα$^-$ cells and, to a lesser degree, from the DP cells in 3 days on OP9 (Fig. 1B). In contrast, the flk-1$^-$PDGFRα$^+$ and DN cell fractions were virtually devoid of such a CFC-generating activity. Similarly, the flk-1$^+$PDGFRα$^-$ cells and, to a lesser degree, the DP cells, eventually generated B220$^+$ lymphocytes during the 10 to 12 day culture on OP9 in the presence of IL-2 and IL-7 (Fig. 1C). The flk-1$^-$PDGFRα$^+$ and DN cell fractions never generated B220$^+$ pre-B or LAK cells. Endothelium-like cell clusters were

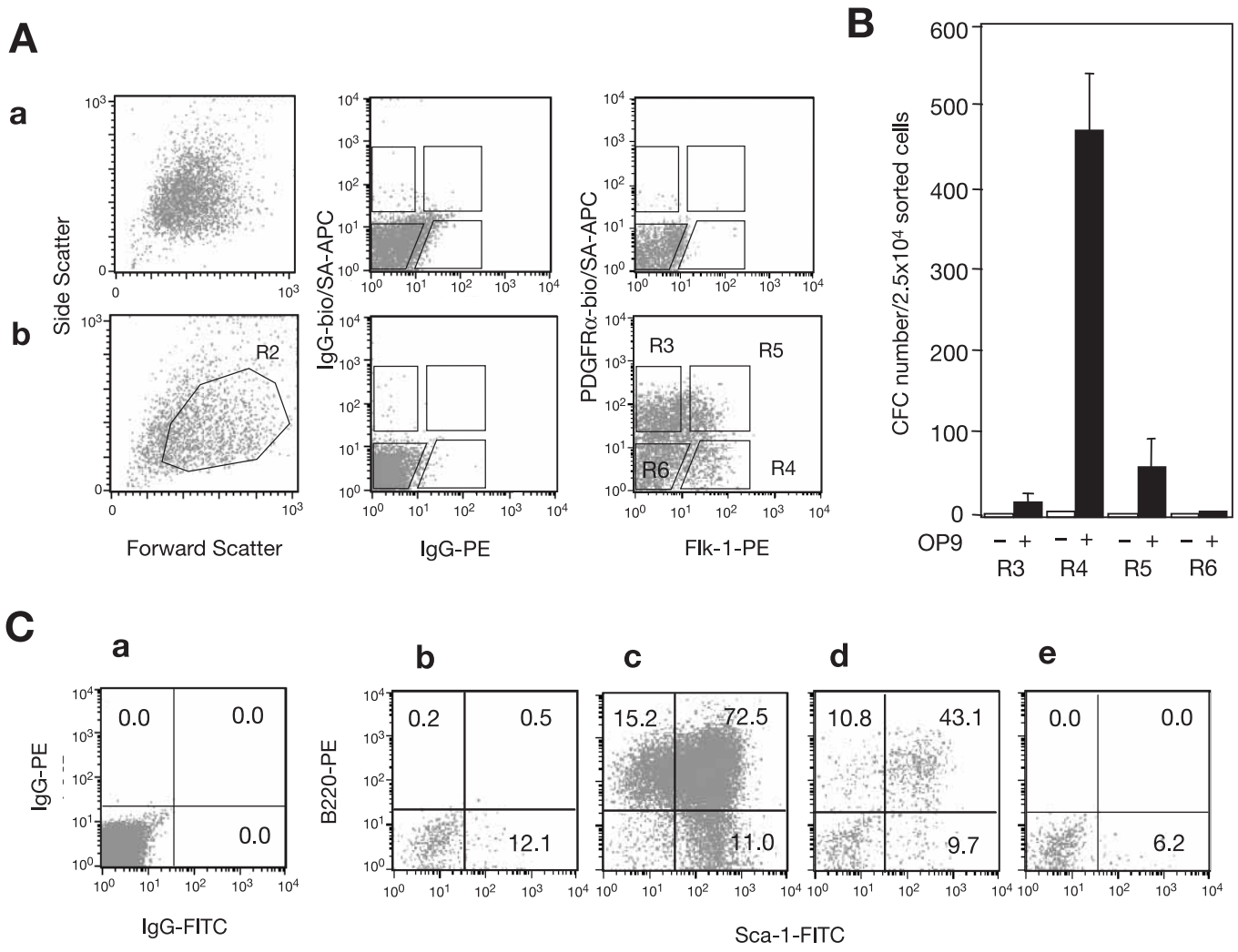

Fig. 1. Characterization of the flk-1$^+$ and PDGFRα$^+$ EB cells generated with BMP4 in the serum-free medium. (A) Generation of the flk-1$^+$ and PDGFRα$^+$ EB cells. The undifferentiated E14 ES cells (a) and the cells from EBs cultured for 4.6 days in the BMP4-containing serum-free medium (b) were stained with Flk-1-PE and PDGFRα-bio. The latter group was further stained with SA-APC. Single positive cells, such as flk-1$^-$PDGFRα$^+$ cells (R3) and flk-1$^+$PDGFRα$^-$ cells (R4), as well as DP cells (R5) and DN cells (R6), were then analyzed (a) and sorted (b) with Vantage SE. Viable cell populations were pre-enriched by propidium iodide exclusion (R1) as described previously (Nakayama et al., 2000). For sorting, stained EB cells were pre-gated with R2 to further reduce dead cell contamination (b, left). R6 was determined by the corresponding isotype control staining (a, middle; b, middle). Populations of R3 to R5, indicated as % total EB cells, are: (a) R3: 0.7, R4: 0.0, R5: 0.3 (right) and as for the isotype control, R3: 0.7, R4: 1.3, R5: 0.5 (middle); (b) R3: 26.4, R4: 7.7, R5: 9.2 (right) and as for the isotype control, R3: 0.5, R4: 0.0, R5: 0.2 (middle). These results are representative of 17 independent experiments. (B) Erythro-myeloid CFCs in the flk-1$^+$ and PDGFRα$^+$ EB cell fractions, with or without culturing on the OP9 cells. Individual FACS-purified cell fractions were either directly plated in the serum-free methylcellulose medium (–) or cultured for 3 days on OP9 (+). The latter cells were then plated into the same medium for hematopoietic colony formation. Total CFC numbers were averaged over four to six independent experiments and are shown with the corresponding s.d. (vertical line). R3: flk-1$^-$PDGFRα$^+$, R4: flk-1$^+$PDGFRα$^-$, R5: DP, R6: DN. (C) Lymphoid potentials of the flk-1$^+$ and PDGFRα$^+$ EB cells. Individual FACS-purified cell fractions were cultured for 12 days on OP9 in the presence of IL-2 and IL-7. The entire culture was then harvested, stained with B220-PE and Sca-1-FITC and analyzed by FACScan. Populations of B220$^+$Sca-1$^-$, B220$^-$Sca-1$^+$, and B220$^+$Sca-1$^+$ cells are indicated as % of total cells. The scatter patterns, the gate settings and the isotype control staining patterns (IgG-FITC/IgG-PE) were essentially the same as described previously (Nakayama et al., 1998). Note that the OP9 cells were B220$^-$Sca-1$^-$. Cellularity, reflected by the density of the plot, was highest in (a) and (c), owing to the massive accumulation of lymphocytic cells. In contrast, small numbers of lymphocytic cell foci were observed in d, and no such cells were detected in b and e. These results are representative of six independent experiments. (a), flk-1$^+$PDGFRα$^-$ (isotype control staining for c); (b), flk-1$^-$PDGFRα$^+$; (c), flk-1$^+$PDGFRα$^-$; (d), DP; (e), DN.

Table 2. Frequency of cartilage-like particle formation with sorted EB cells under different culture conditions

Factor* added	flk-1$^+$PDGFRα$^-$	flk-1$^+$PDGFRα$^+$ (DP)	flk-1$^-$PDGFRα$^+$	flk-1$^-$PDGFRα$^-$ (DN)
No factor	0/2	0/6	7/10 (0)[†]	ND[‡]
TGFβ3	26/30 (0)	11/17 (0)	44/46 (0)	2/2 (0)
BMP4	ND	ND	0/3	ND
PDGF	0/1[§]	0/2	3/3 (0)	ND
TGFβ3+BMP4	3/3 (3)	1/1 (1)[§]	5/5 (5)	ND
TGFβ3+PDGF	4/4 (3)	5/5 (1)	6/6 (5)	ND
TGFβ3+noggin	0/1[§]	ND	0/4	ND
TGFβ3+PDGF+noggin	0/1[§]	0/1[§]	0/2	ND

ES-cell-derived mesoderm cells cultured for 14 to 20 days were stained with Toluidine blue. The resulting particles containing cartilage nodules displaying weak to strong metachromatic staining were scored and are indicated per total number of particles.

*TGFβ3 10 ng/ml, BMP4 50 ng/ml, PDGF (i.e. PDGF-BB) 50 ng/ml, and noggin (i.e. noggin-Fc) 1 μg/ml.

[†]Parentheses indicate the number of 'full' cartilage particles, as shown in Figs 5, 6B, 7, 8A.

[‡]Not determined.

[§]Single experiment.

also observed during the culture of flk-1$^+$PDGFRα$^-$ cells and DP cells on OP9, whereas flk-1$^-$PDGFRα$^+$ cells never generated such cells (data not shown).

These results were consistent with the previous observations that the FACS-purified flk-1$^+$ cells have CFC-generating (hemogenic) activity and angiogenic activity. Thus, the mesodermal progenitor cells expressing flk-1 and/or PDGFRα generated with BMP4 in the serum-free medium are likely to be equivalent to those made in the serum-containing medium described before.

In vitro cartilage formation with the EB cells in the presence of TGFβ

Both the paraxial mesoderm and the lateral plate mesoderm form cartilage in vivo. Therefore, we addressed whether the ES-cell-derived mesodermal cells were also capable of forming cartilage. The sorted EB cell fractions were subjected to the serum-free pellet culture with 10 ng/ml TGFβ3, as described for mesenchymal stem cells (Mackay et al., 1998). 24 hours later, the cells pelleted in a tube at the beginning of the culture were detached from the bottom to form a round cell aggregate. By day 14, areas that stained light- to dark-blue with Alcian blue or light- to dark-red/purple with Toluidine blue (metachromatic staining) became noticeable within the cell particle, which is indicative of cartilage-like extracellular matrix accumulation.

At frequencies of 96%, 87% and 65% (Table 2), the flk-1$^-$PDGFRα$^+$ cells, flk-1$^+$PDGFRα$^-$ cells and DP cells, respectively, formed a particle containing one to three cartilage nodules that consisted of well separated, round cells and large intercellular spaces that stained metachromatically with Toluidine blue (Fig. 2, Fig. 3A). The other areas contained loosely attached, spindle-shaped cells and showed weak to no metachromatic staining with Toluidine blue. For further confirmation, the contiguous sections were stained with an anti-COL1 antibody as well as an anti-COL2 antibody (Fig. 3A). Most of the areas that stained weakly with Toluidine blue

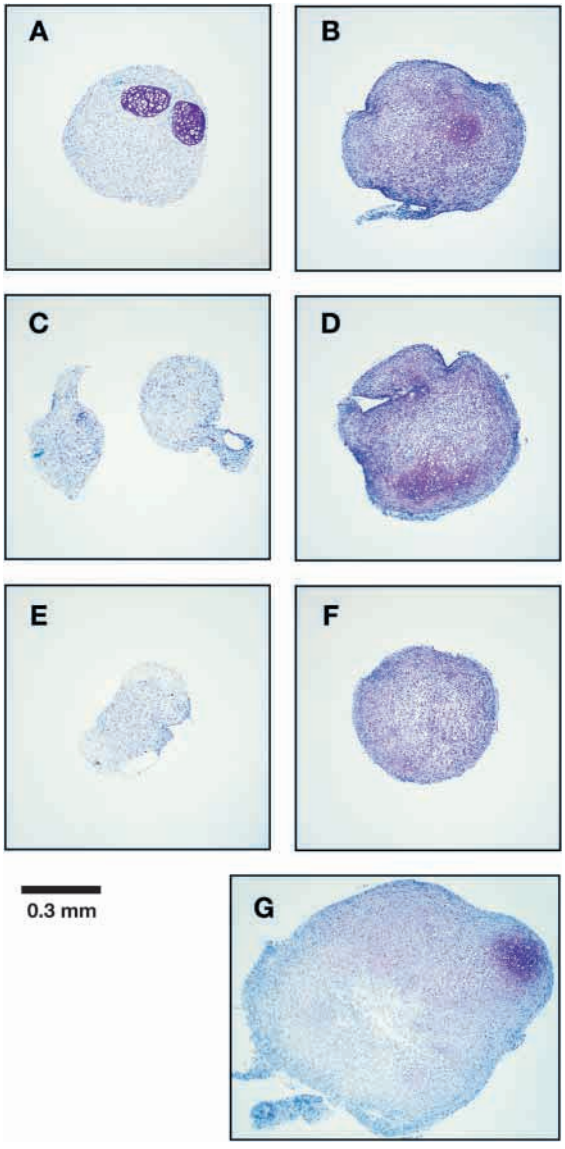

Fig. 2. Requirement of TGFβ for the in vitro chondrogenesis of the ES-cell-derived mesodermal cells. The sorted flk-1$^-$PDGFRα$^+$ cells (A,B), flk-1$^+$PDGFRα$^-$ cells (C,D), DP cells (E,F), and DN cells (G) were subjected to the pellet culture with (B,D,F,G) or without (A,C,E) TGFβ3. Particles formed in 15 days of culture were formalin-fixed, paraffin-embedded, sectioned and stained with Toluidine blue. These results are representative of three independent experiments.

expressed both COL1 and COL2, whereas some cartilage nodules that strongly stained with Toluidine blue consisted predominantly of COL2 (Fig. 3A), suggesting that the particles were heterogeneous, in that they consisted of hyaline cartilage nodules, fibro cartilage areas and non-cartilaginous areas. On the other hand, the DN cells formed a large particle, consisting mostly of a non-cartilaginous area that was not metachromatically stained (Fig. 2G). The DN cell population was expected to be heterogeneous, because APA5 is known to recognize part of the PDGFRα mRNA-expressing mesoderm (Takakura et al., 1997).

In conclusion, the pellet culture in the presence of TGFβ3 allowed fractions of ES-cell-derived mesodermal cells, such as flk-1$^-$PDGFRα$^+$ cells, flk-1$^+$PDGFRα$^-$ cells and DP cells, to form a cartilage-nodule-containing particles.

Cartilage-specific gene expression during the pellet culture of the ES-cell-derived mesodermal cells

For further validation, we investigated whether the in-vitro-formed cartilage expressed cartilage-specific genes by nested RT-PCR (Fig. 3B). Prior to the induction of chondrogenesis (day 0), transcripts for aggrecan COMP, COL10 and PTHrP were hardly detected in the flk-1$^-$PDGFRα$^+$ and flk-1$^+$PDGFRα$^-$ cell fractions, whereas the COL2 transcript was readily detectable. Upon differentiation, all of them were upregulated and became detectable by days 7 to 16. Interestingly, the CHL1 transcript, which is preferentially expressed in condensing mesenchymes as well as mature chondrocytes of the developing skeleton (Nakayama et al., 2001), and the PTHrP transcript were induced earlier (from day 3). Consistent with the previous histological observations, these results supported the idea that the ES-cell-derived mesodermal cells were capable of generating cartilage-containing particles in vitro.

TGFβ-dependent and TGFβ-independent chondrogenic activities

Without TGFβ3, the efficiency of the initial aggregate formation was significantly reduced (data not shown), and the size of the particles formed became smaller (Fig. 2A,C,E). Furthermore, in the case of flk-1$^+$PDGFRα$^-$ cells and DP cells, no area was stained metachromatically with Toluidine blue within the particles (Fig. 2C,E). In contrast, in the case of the flk-1$^-$PDGFRα$^+$ cells, seven out of 10 small particles formed contained one to two small cartilaginous nodules that stained strongly with Toluidine blue (Fig. 2A and Table 2).

To quantify the TGFβ-independent cartilage-forming activity, three chondrogenic EB cells, flk-1$^-$PDGFRα$^+$ cells, flk-1$^+$PDGFRα$^-$ cells and DP cells, were individually subjected to a serum-free micromass culture. In the presence

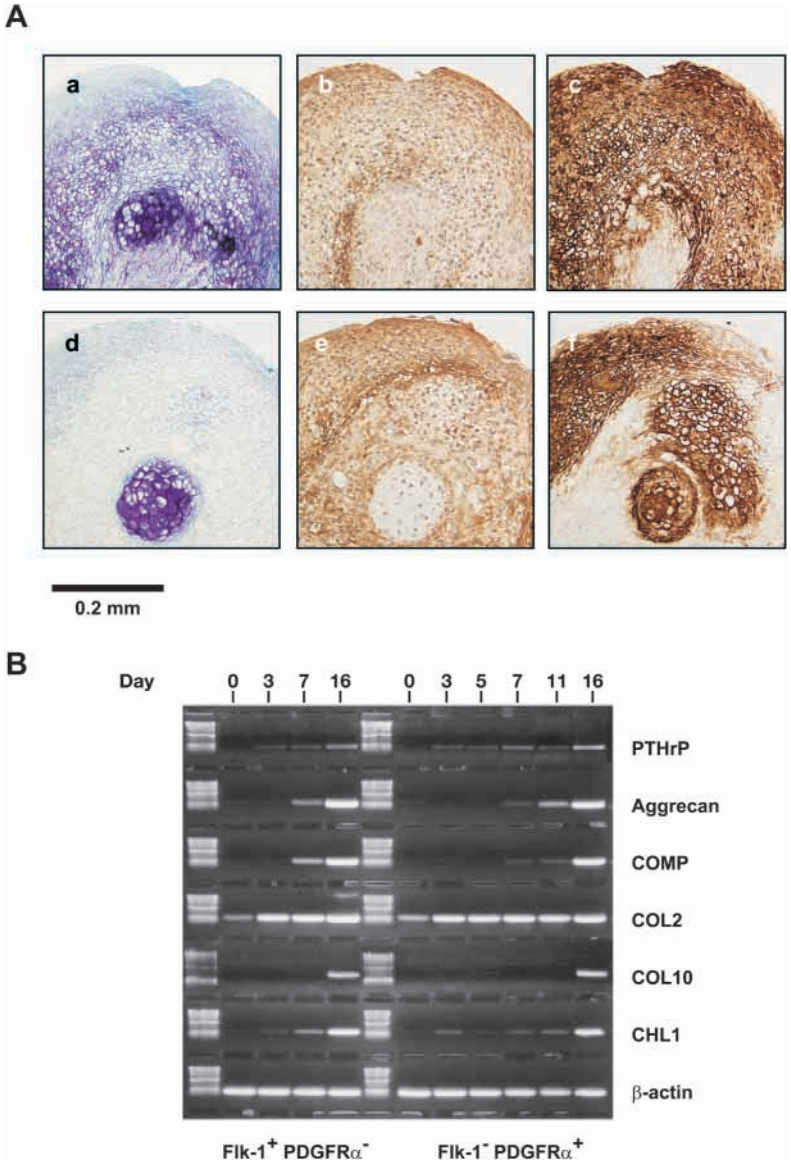

Fig. 3. TGFβ3-dependent chondrogenesis causes COL2-containing cartilage matrix accumulation and induces cartilage-specific gene expression. (A) Cartilage-like particle formation with single positive EB cells in vitro. The sorted flk-1$^-$ PDGFRα$^+$ cells (a-c) and flk-1$^+$PDGFRα$^-$ cells (d-f) were individually subjected to the pellet culture in the presence of TGFβ3. Particles formed in 17 days of culture were fixed with Gendre's fluid, paraffin-embedded, sectioned and stained with Toluidine blue (a,d). Two contiguous sections were also immunostained with AB765P (b,e) and 2B1.5 (c,f). Brown areas indicate the accumulation of COL1 (b,e) and COL2 (c,f). Immunostaining with rabbit IgG and mouse IgG did not yield any signals (data not shown). These results are representative of seven independent experiments. (B) Expression of cartilage-specific mRNAs during the TGFβ-dependent generation of cartilage-like particles from single positive EB cells. The sorted flk-1$^-$PDGFRα$^+$ cells and flk-1$^+$PDGFRα$^-$ cells were individually subjected to the TGFβ3-containing pellet culture. On the day indicated, three aggregates were collected and RNAs were isolated for nested RT-PCR. No signals were observed without reverse transcription or when the first 21 cycles were done with a primer set for a different gene, indicating that the positive signals originated from the corresponding transcripts (data not shown). Essentially the same results were obtained from three independent experiments.

of 50 ng/ml PDGF-BB, the cells survived or slowly proliferated during the first 8 days, as observed with the somite cells (Tallquist et al., 2000). Consistent with the results shown in Fig. 2, the addition of TGFβ3 stimulated the accumulation of sulfated glycosaminoglycan in all three cultures (Fig. 4A). Furthermore, the addition of TGFβ3 induced COL2 accumulation during the culture of the flk-1$^+$PDGFRα$^-$ cells and the DP cells (Fig. 4Ca). The flk-1$^-$PDGFRα$^+$ cell culture that accumulated low levels of COL2 without TGFβ3 also displayed an approximately three-fold increase in the COL2 level with TGFβ3.

Without TGFβ, however, approximately 79 small cartilage nodules that stained positively with Alcian blue were detected per 2×10^5 flk-1$^-$PDGFRα$^+$ cells initially seeded (Fig. 4B). Smaller cartilage nodules were detected in the DP cell fraction at a lower frequency (approximately 14 per 2×10^5 cells). No cartilage nodule was found with the flk-1$^+$PDGFRα$^-$ cells (Fig. 4B). Therefore, in contrast to the lymphohematopoietic potential, the TGFβ-independent chondrogenic activity was highly enriched in the flk-1$^-$PDGFRα$^+$ EB cell fraction and was absent from the flk-1$^+$PDGFRα$^-$ cell fraction.

Thus, there seem to be two modes of chondrogenesis. In one type, exogenous TGFβ is absolutely essential (for flk-1$^+$PDGFRα$^-$ as well as DP mesodermal cells) and in the other, chondrogenesis occurs as small, distinct nodules without the exogenous TGFβ, and the addition of TGFβ expanded the chondrogenic area (for flk-1$^-$PDGFRα$^+$ cells).

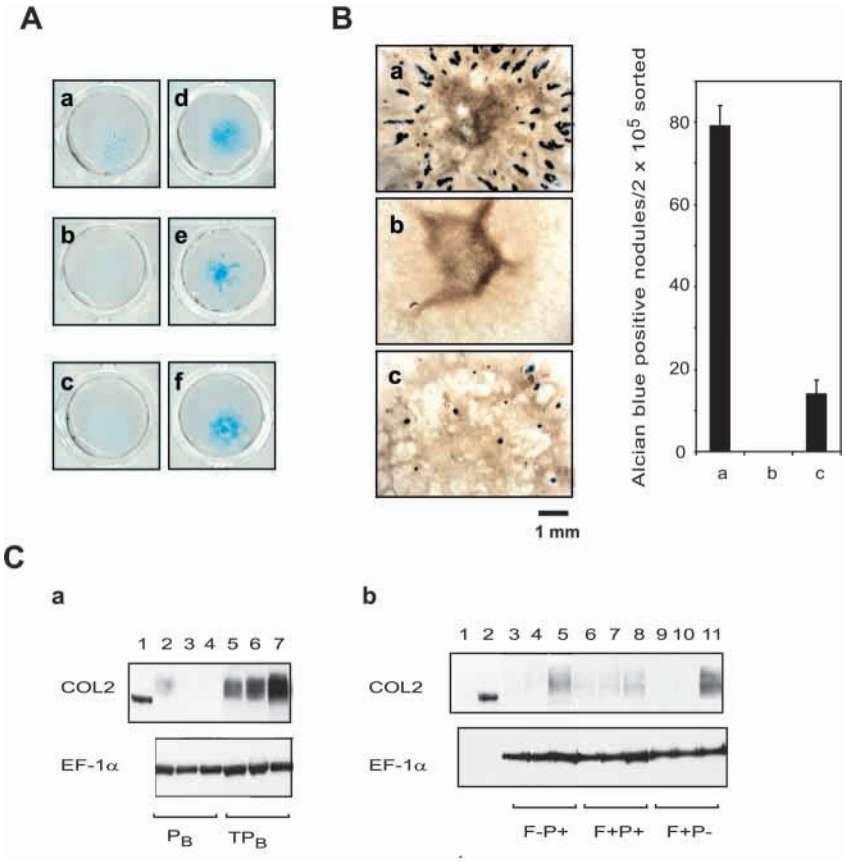

Fig. 4. TGFβ3 enhances the accumulation of sulfated glycosaminoglycans and COL2 during micromass culture. (A) Cartilage nodule formation in the absence (a-c) and presence (d-f) of TGFβ3. The sorted flk-1$^-$PDGFRα$^+$ cells (a,d), flk-1$^+$PDGFRα$^-$ cells (b,e) and DP cells (c,f) were individually spotted in two wells of a 24-well plate at 1.5×10^5 cells/7.5 μl/spot/well and were subjected to serum-free micromass culture. On day 8, cultures were formalin-fixed and stained with Alcian blue. (B) Higher power view of a-c. Cartilage nodules with a dark blue appearance were noted in the culture of flk-1$^-$PDGFRα$^+$ cells (a) and DP cells (c). The Alcian-blue-positive nodules were counted, and the numbers were normalized per 2×10^5 initial seeding cells and are displayed in the right graph. From three independent experiments, average numbers were calculated and are shown with the corresponding s.d. (vertical line). (C) COL2 accumulation during the micromass culture. (a) The sorted flk-1$^-$PDGFRα$^+$ cells (lanes 2,5), DP cells (lanes 3,6), and flk-1$^+$PDGFRα$^-$ cells (lanes 4,7) were individually spotted in 2 wells of a 24-well plate at 1.5×10^5 cells/7.5 μl/spot/well, and were subjected to the serum-free micromass culture with 50 ng/ml PDGF-BB (lanes 2-4, P_B) or TGFβ3 and 50 ng/ml PDGF-BB (lanes 5-7, TP_B). (b) The sorted flk-1$^-$PDGFRα$^+$ cells (lanes 3-5, F–P+), DP cells (lanes 6-8, F+P+), and flk-1$^+$PDGFRα$^-$ cells (lanes 9-11, F+P–**)** were also cultured in the same way with TGFβ3 (lanes 3,6,9), with TGFβ3 and 50 ng/ml PDGF-AA (lanes 4,7,10) or with TGFβ3 and 50 ng/ml PDGF-BB (lanes 5,8,11). On day 8, the cultures were harvested with 300 μl/well of loading buffer, and 40 μl aliquots were separated by SDS-PAGE, blotted and developed with 6B3. Soluble COL1 (pepsin-treated) was loaded in lane 1 (b), and soluble COL2 (pepsin-treated) was loaded in lane 1 (a) and lane 2 (b).

PDGF-BB augments TGFβ-induced chondrogenesis

TGFβ alone was not sufficient to form a particle filled with the cartilage matrix macromolecules (Figs 2, 3). Since PDGF-BB sustained the viability of the ES-cell-derived mesodermal cells during the serum-free micromass culture, we further investigated the effect of PDGF. The addition of 20 to 50 ng/ml PDGF-BB significantly elevated the COL2 accumulation during the micromass culture of all three cell types tested in the presence of TGFβ3 (Fig. 4Cb). Twenty to 100 ng/ml of PDGF-AA did not significantly elevate the COL2 level.

On the other hand, the addition of 50 ng/ml PDGF-BB to the pellet culture of flk-1$^-$PDGFRα$^+$ cells (Fig. 5A), flk-1$^+$PDGFRα$^-$ cells (Fig. 5B) or DP cells (data not shown) in the presence of TGFβ3 markedly enlarged the particle volume as well as the areas staining with the anti-COL2 antibody and metachromatically with Toluidine blue, so that, except for a thin surface cell layer, the cartilage matrix was distributed throughout the particle. We refer to such particles as ‘full’ cartilage in this report. The COL2$^+$ interior of the large ‘full’ cartilage particle formed in the presence of TGFβ3 and PDGF-BB (Fig. 5Ad-f, 5Bj-l) was weakly stained with the anti-COL1 antibody. However, a layer of cells on the surface of the particle showed relatively high levels of COL1, and COL2 was also detected at a level similar to that in the interior COL2$^+$ area. Thus, even in the presence of PDGF-BB, the ‘full’ cartilage was still fibro cartilage.

In contrast, PDGF-BB treatment alone resulted in small particles with either flk-1$^+$PDGFRα$^-$ cells (Fig. 5Bg-i) or DP cells (data not shown) that were devoid of cartilage matrix. The TGFβ-independent chondrogenesis of the flk-1$^-$PDGFRα$^+$ cells was still observed in the presence of PDGF-BB alone (Fig. 5Ba-c). However, a large 'full' cartilage particle was formed only with TGFβ3 (Table 2).

Thus, PDGF-BB seems to be a synergistic factor for the TGFβ-induced chondrogenesis of the ES-cell-derived mesodermal cells, leading to 'full' cartilage particle formation. Further analyses were performed, mostly with two interesting cell types that also efficiently formed 'full' cartilage (Table 2): the hemo-chondrogenic flk-1$^+$PDGFRα$^-$ cells (75%) and the chondrogenic flk-1$^-$PDGFRα$^+$ cells (83%).

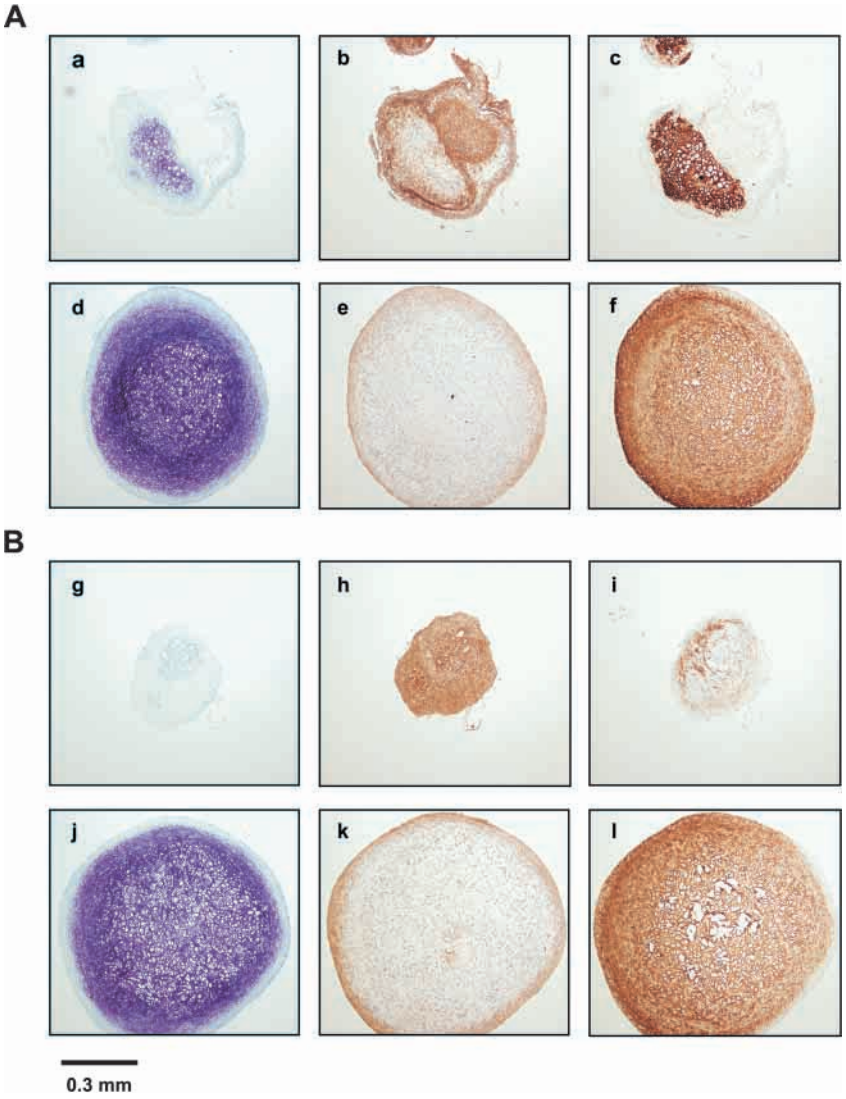

Fig. 5. Effects of PDGF-BB on cartilage particle development. The sorted flk-1$^-$PDGFR$^+$ cells (A) and flk-1$^+$PDGFR$^-$ (B) were subjected to the pellet culture in the presence of TGFβ3 (a-c,g-i), or TGFβ3 and 50 ng/ml PDGF-BB (d-f,j-l). Particles formed after 17 days of culture were fixed with Gendre's fluid, paraffin-embedded, sectioned and stained with Toluidine blue (a,d,g,j). A contiguous section of each particle was also immunostained with AB765P (b,e,h,k) or 2B1.5 (c,f,i,l). Brown areas indicate the accumulation of collagens. The immunostaining with control IgGs showed negative results (data not shown). Note that the addition of PDGF-BB resulted in a large aggregate filled with cartilage matrix and COL2 (d,f,j,l). These results are representative of four independent experiments.

Inhibitory effect of noggin

The BMP family is also implicated in chondrogenesis (Cancedda et al., 1995). To determine whether TGFβ-induced or TGFβ+PDGF-induced chondrogenesis involved BMP activity, we added a pan-BMP-binding inhibitor, noggin, to the pellet culture. Noggin interacts with BMP2, BMP4 and BMP7, but does not bind to Activin, TGFβ1 (Zimmerman et al., 1996) and TGFβ3 (data not shown).

When added at the beginning of the culture, noggin-Fc inhibited the TGFβ-induced cartilage formation in a dose-dependent manner (Fig. 6; Table 2). One μg/ml of noggin-Fc was sufficient for complete inhibition of the formation of areas stained metachromatically with Toluidine blue, and 0.1 μg/ml produced weaker inhibitory effects (Fig. 6A). A similar inhibitory effect on the TGFβ+PDGF-induced 'full' cartilage formation was observed (Fig. 6B), in that the area immunostained with the anti-COL2 antibody was also abolished. Moreover, the noggin-Fc at 1 μg/ml significantly retarded the growth of particles formed either with TGFβ3 alone or with TGFβ3 and PDGF-BB, resulting in a small particle. At 0.1 μg/ml, no effect on the particular volume was observed.

When TGFβ3 was removed on days 10 to 12, which otherwise would have resulted in a cartilage-nodule-containing particle, the addition of noggin-Fc at 1 μg/ml at this point prevented the formation of cartilaginous areas (0-20% of particles contained areas showing metachromatic staining) during the following 7 to 8 days (Fig. 7C; Table 3). An inhibitory effect on the particle growth was also noted.

Thus, the BMP family of proteins seems to be involved in the chondrogenesis of the ES-cell-derived mesodermal cells, probably downstream of TGFβ or TGFβ+PDGF.

Synergistic enhancement of the TGFβ-induced cartilage formation by BMP4

The roles of BMP were further investigated. First, 50 ng/ml BMP4, added in place of TGFβ3 at the beginning of the culture, resulted in a small particle without cartilage nodules, although it supported the initial aggregate formation. Rather, BMP4 prevented the TGFβ-independent formation of the small cartilage nodules in the flk-1$^-$PDGFRα$^+$-cell-derived particle (Fig. 7Ab; Table 2).

By contrast, the addition of 20 to 50 ng/ml BMP4 to the TGFβ-containing culture markedly enhanced the depositions of cartilage matrix (Fig. 7B) and COL2 (data not shown), leading to 'full' cartilage formation. The

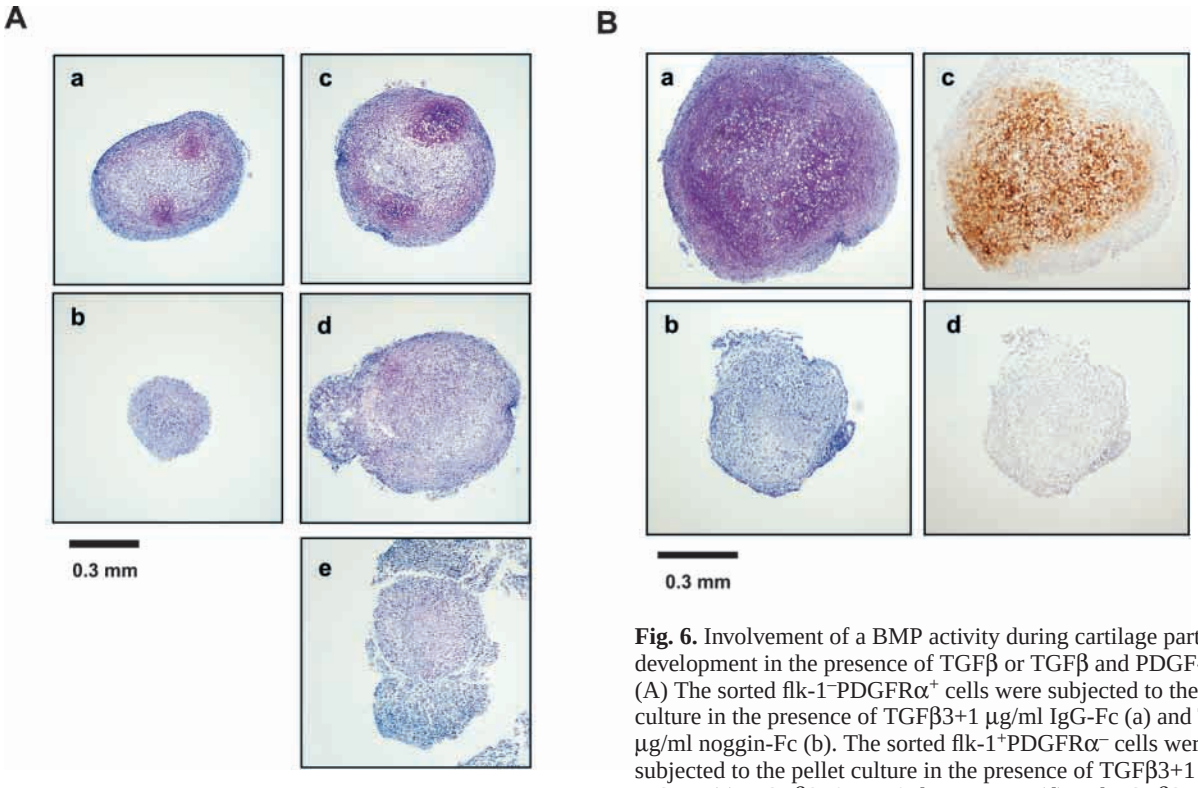

Fig. 6. Involvement of a BMP activity during cartilage particle development in the presence of TGFβ or TGFβ and PDGF-BB. (A) The sorted flk-1^{-}PDGFRα^{+} cells were subjected to the pellet culture in the presence of TGFβ3+1 μg/ml IgG-Fc (a) and TGFβ3+1 μg/ml noggin-Fc (b). The sorted flk-1^{+}PDGFRα^{-} cells were also subjected to the pellet culture in the presence of TGFβ3+1 μg/ml IgG-Fc (c), TGFβ3+0.1 μg/ml noggin-Fc (d) and TGFβ3+1 μg/ml noggin-Fc (e). Particles formed in 15 day (a,b) or 18 day (c-e) cultures were formalin-fixed, paraffin-embedded, sectioned and stained with Toluidine blue. These results are representative of three (c-e) to five (a,b) independent experiments. Note that the addition of 1 μg/ml IgG-Fc did not have any effect on the growth and maturation of the cartilage-containing particle (a,c). The addition of noggin-Fc resulted in a small aggregate and sometimes left part of the initial cell pellet unincorporated into the round cell aggregate, which occasionally attached to the particle throughout the culture (top and bottom cell debris in e). (B) The sorted flk-1^{-}PDGFRα^{+} cells were subjected to the pellet culture with TGFβ3 and 50 ng/ml PDGF-BB in the presence of 1 μg/ml IgG-Fc (a,c) and 1 μg/ml noggin-Fc (b,d). On day 18, particles were formalin-fixed, paraffin-embedded, sectioned and stained with Toluidine blue. A contiguous section of each particle was also immunostained with 2B1.5 (c,d). Brown areas indicate the accumulation of COL2. The immunostaining with mouse IgG showed negative results (data not shown). Note that the addition of 1 μg/ml noggin-Fc resulted in a small aggregate (b,d). These results are representative of two independent experiments.

enhancement was dose dependent, and 5 ng/ml of BMP4 were not effective (Fig. 7Bb). The frequency of 'full' cartilage formation reached 100% in the presence of TGFβ3 and 50 ng/ml BMP4 (Table 2). A similar synergistic effect was observed even when TGFβ3 was replaced with 50 ng/ml BMP4 after the cells were treated with TGFβ3 for 10 to 12 days (Fig. 7C). This resulted in a large 'full' cartilage particle filled with a cartilage matrix and COL2 at a frequency of 75-100% (Table 3) in the following 7 to 8 days of culture (Fig. 7B).

Thus, the exogenously added BMP4 synergistically enhanced the TGFβ-induced cartilage formation from the ES-cell-derived mesodermal cells. However, TGFβ treatment seemed to be pre-requisite for BMP4 to stimulate chondrogenesis.

Table 3. Frequency of cartilage-like particle formation with sorted EB cells in a switch culture

Factor* added	flk-1^{+}PDGFRα^{-}	flk-1^{+}PDGFRα^{+} (DP)	flk-1^{-}PDGFRα^{+}
TGFβ3†	26/30 (0)‡	11/17 (0)	44/46 (0)
TGFβ3, then none	12/17 (4)	5/6 (0)	19/19 (5)
TGFβ3, then BMP4	4/4 (3)	2/2 (2)	6/6 (6)
TGFβ3, then TGFβ3+BMP4	ND§	ND	3/3 (3)
TGFβ3, then noggin	0/3	0/2	1/5 (0)

Particles including areas morphologically resembling cartilage were scored, and are indicated per total number of particles examined, as in Table 2. This series of experiments involved a medium switch on days 10 to 12 of culture, in that the serum-free chondrogenesis medium with TGFβ3 was changed to medium alone, with BMP4, with TGFβ3+BMP4, or with noggin, and the culture was continued for another 7 to 8 days.

*TGFβ3 10 ng/ml, BMP4 50 ng/ml, and noggin (i.e. noggin-Fc) 1 μg/ml.

†This is a control for no medium change. The same data are shown in Table 2.

‡Definitions of numbers in parentheses are the same as those in Table 2.

§Not determined.

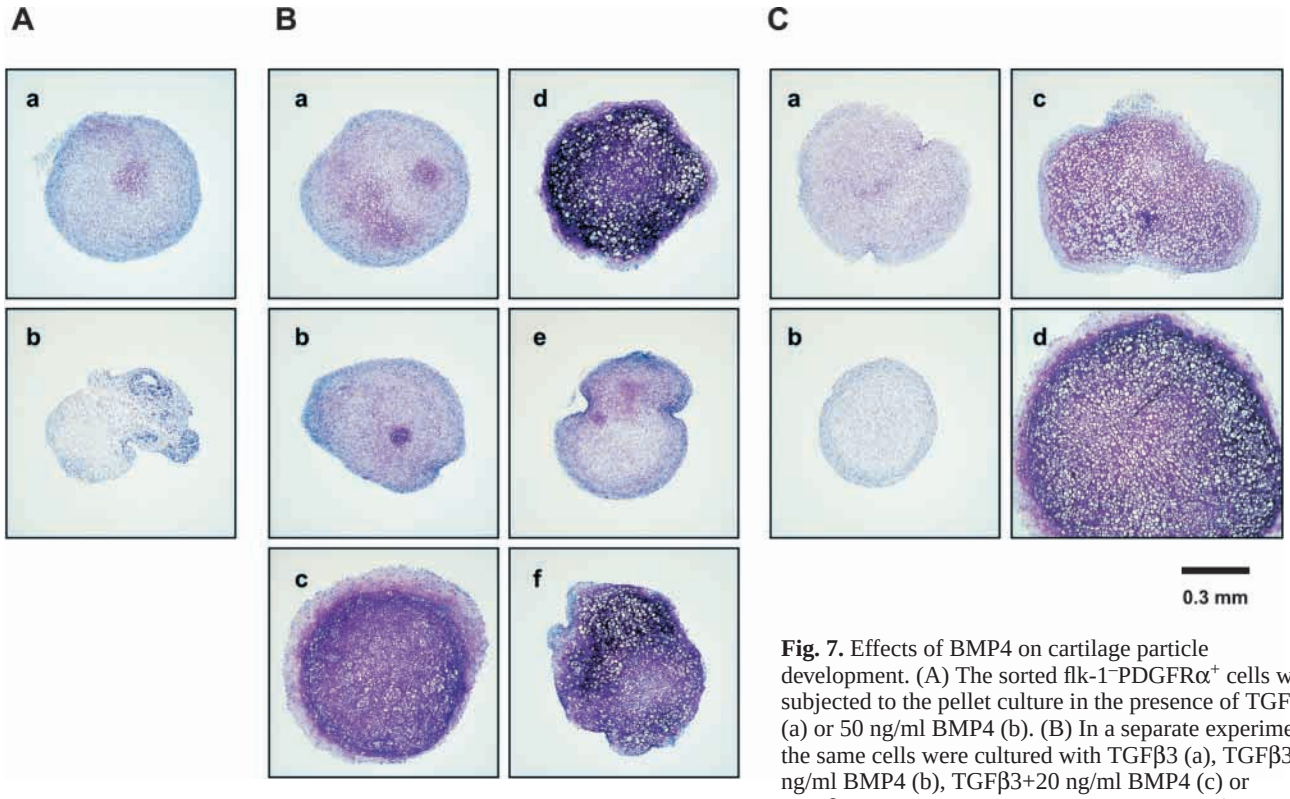

Fig. 7. Effects of BMP4 on cartilage particle development. (A) The sorted flk-1^{-}PDGFRα^{+} cells were subjected to the pellet culture in the presence of TGFβ3 (a) or 50 ng/ml BMP4 (b). (B) In a separate experiment, the same cells were cultured with TGFβ3 (a), TGFβ3+5 ng/ml BMP4 (b), TGFβ3+20 ng/ml BMP4 (c) or TGFβ3+50 ng/ml BMP4 (d). The sorted flk-1^{+}PDGFRα^{-} cells were also subjected to the pellet culture in the presence of TGFβ3 (e) or TGFβ3+50 ng/ml BMP4 (f). Particles formed during 16 days (Aa-b, Be-f) or 17 days (Ba-d) of culture were formalin-fixed, paraffin-embedded, sectioned and stained with Toluidine blue. These results are representative of three independent experiments. (C) Late removal of TGFβ3 and replacement with BMP4 during the in vitro development of cartilage particles. The sorted flk-1^{-}PDGFRα^{+} cells were individually subjected to the pellet culture in the presence of TGFβ3. On day 10, the TGFβ3 was removed (c), or was substituted with 1 μg/ml noggin-Fc (b) or 50 ng/ml BMP4 (d) in some cultures. On day 18, particles were formalin-fixed, paraffin-embedded, sectioned and stained with Toluidine blue. These results are representative of five independent experiments.

Potential inhibitory effects of TGFβ on chondrocyte maturation

The cartilage nodules formed in the presence of TGFβ3 stained fainter with Toluidine blue than those formed in the absence of it (Fig. 2A,B). This raised the possibility that TGFβ suppresses chondrocyte maturation at a later stage of cartilage formation, as is the case for the embryonic limb mesenchymes. Therefore, TGFβ3 was removed on days 10 to 12 of culture, and the resulting particles were examined on days 17 to 20 (Fig. 7C). Under these conditions, the flk-1^{-}PDGFRα^{+} cells gave rise to particles containing a larger cartilaginous area that consisted of larger chondrocytes and displayed stronger metachromatic staining with Toluidine blue. The ‘full’ cartilage was also obtained at a frequency of 26% (Table 3). The TGFβ+PDGF-induced chondrogenic culture gave rise to a ‘full’ cartilage particle in 17 to 20 days (Fig. 5). When both TGFβ3 and PDGF-BB were removed on days 10 to 12, no increase in either the COL2 level or the strength of Toluidine blue staining was noted on days 17 to 20. However, an increase in the size of the chondrocytes and a reduction in the COL1 level in the cartilaginous area were apparent (Fig. 8A).

These observations suggest that, in a later phase of the chondrogenic culture of the ES-cell-derived mesodermal cells, the continuous presence of TGFβ3 prevented or retarded the chondrocyte maturation and, even in the presence of PDGF-BB, maintained the COL1 expression within the cartilage matrix, thereby preventing the hyaline cartilage formation.

Hyaline cartilage formation and induction of mineralization

When TGFβ3 and PDGF-BB were removed, a distinct, thin layer of cells that strongly stained with the anti-COL1 antibody, but did not stain with either Toluidine blue or the anti-COL2 antibody, became apparent on the surface of a particle (Fig. 7Cc, Fig. 8Ad-f). It was composed of spindle-shape cells resembling undifferentiated mesenchymal cells. Upon the addition of 50 ng/ml BMP4, the thin cell layer became a thicker layer containing round chondrocytic cells and a matrix that was rich in COL2 and stained lightly with Toluidine blue (Fig. 7Cd, Fig. 8Ag,i). Moreover, BMP treatment resulted in a reduced level of COL1 in the surface cell layer as well as in the interior cartilaginous area (Fig. 8Ah), resulting in a hyaline cartilage particle. A similar thick layer that stained lightly with Toluidine blue was evident when the cells were cultured from the beginning with TGFβ3 and 20-50 ng/ml BMP4 (Fig. 7Bc,d,f).

The hyaline cartilage particles formed by culturing the flk-1^{-}PDGFRα^{+} cells or the flk-1^{+}PDGFRα^{-} cells with TGFβ3 and PDGF-BB for 10 days, and with BMP4 alone for the following

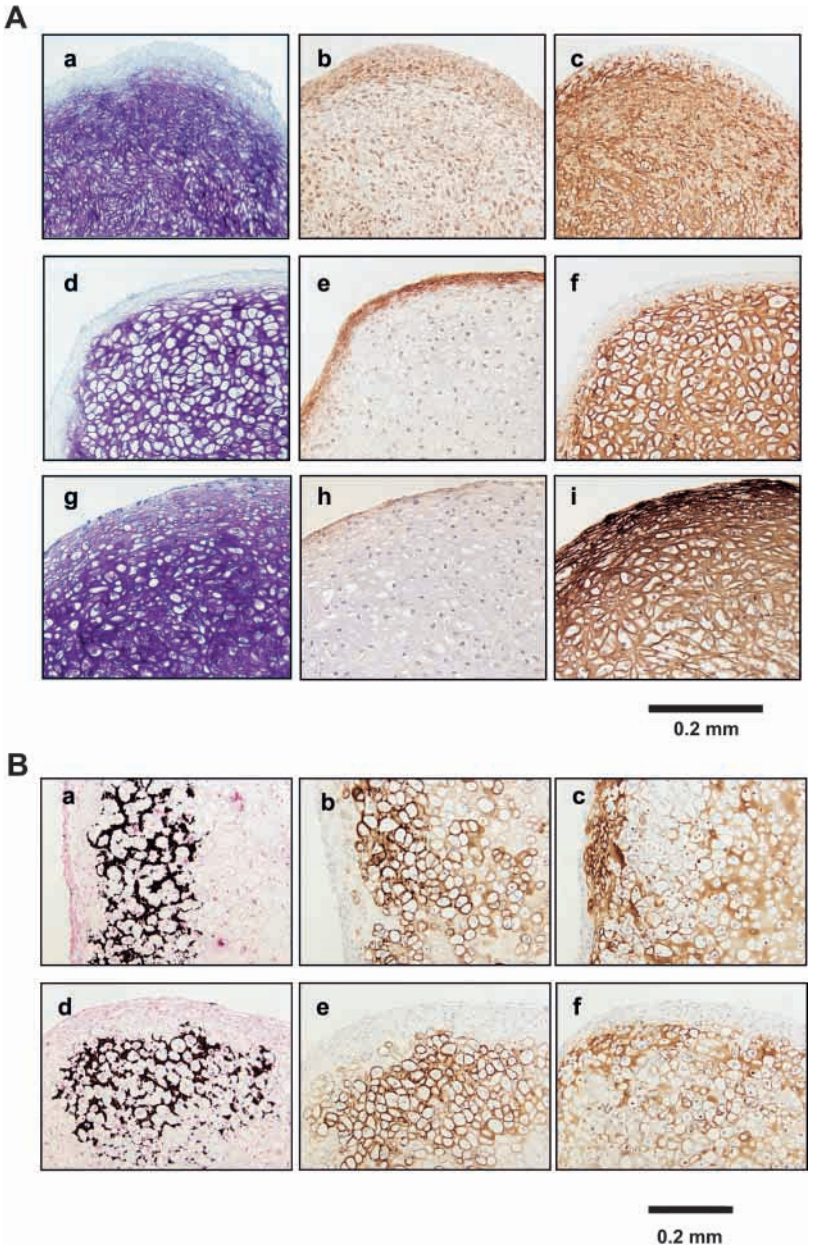

Fig. 8. Hyaline cartilage formation and mineralization. (A) Late removal of TGFβ3 and PDGF-BB, and replacement with BMP4 during the in vitro development of cartilage particles. The sorted flk-1$^-$PDGFRα$^+$ cells were subjected to the pellet culture in the presence of TGFβ3 and 50 ng/ml PDGF-BB. On day 10, the TGFβ3and PDGF-BB were removed (d-f) or were substituted with 50 ng/ml BMP4 (g-i) in some cultures. On day 18, particles were fixed with Gendre's fluid, paraffin-embedded, sectioned and stained with Toluidine blue (a,d,g) or immunostained with AB765P (b,e,h) or 2B1.5 (c,f,i). The results are representative of five independent experiments. (B) The sorted flk-1$^-$PDGFRα$^+$ cells (a-c) and flk-1$^+$PDGFRα$^-$ cells (d-f) were pellet-cultured with TGFβ3 and 50 ng/ml PDGF-BB for 10 days, with 50 ng/ml BMP4 for 6 days and then in the hypertrophic differentiation medium for 5 days. Particles were formalin-fixed, paraffin-embedded, sectioned and stained individually with von Kossa (a,d), with X53 (b,e) and with 2B1.5 (c,f). The results are representative of three independent experiments. Black staining indicates mineral deposition (Ba,d), and brown areas indicate the accumulation of collagens. Immunostaining with control IgGs showed negative results (data not shown).

6 days, were further tested for mineralization. In the presence of T3 and β-glycerophosphate an area in which the intercellular space was stained with von Kossa became apparent within the particle after 5 days of culture, indicating that mineral deposits in the cartilage matrix were induced (Fig. 8B). The anti-COL10 antibody detected COL10 deposition as a larger area, in which the mineralizing matrix was included and a lower level of COL2 was accumulated.

These observations indicate that the ES-cell-derived mesodermal cells are able to form a hyaline cartilage particle and that the hyaline cartilage generated with the ES-cell-derived mesodermal cells mature and mineralize the matrix. They also suggest that the COL1$^+$ mesenchymal cells in the surface layer could be BMP-sensitive chondroprogenitors that serve as the source of chondrocytes during the growth of the cartilage particle, which is equivalent to the perichondrium.

Discussion

We have demonstrated that the ES-cell-derived mesodermal cells are chondrogenic and that TGFβ and PDGF synergistically function in in vitro development of cartilage through BMP activity. As far as we know, this is the first report demonstrating the full chondrogenic activity of ES-cell-derived mesodermal cells in vitro.

In vitro chondrogenesis of the ES-cell-derived mesodermal cells as a faithful culture model for chondrogenesis

First, to address whether chondrogenesis was achieved through a developmental pathway similar to that in vivo, we examined the roles of three factors that are known to be involved in embryonic chondrogenesis: TGFβ, PDGF and BMP.

TGFβ and BMP are implicated in several steps during limb cartilage development, and their effects are both positive and negative, depending on the developmental stage. For example, TGFβ has an early positive function and a late negative function. Our pellet culture of either flk-1$^+$PDGFRα$^-$ or flk-1$^-$PDGFRα$^+$ EB cells produced similar TGFβ effects: (1) supporting aggregate growth and formation of cartilaginous areas within the particle generated (Figs 2, 5) and (2) inhibiting of the generation of hypertrophic chondrocytes and the formation of hyaline cartilage, both of which were induced by removing TGFβ later (Fig. 7C, Fig. 8A). However, the detection of the COL10 transcript by PCR suggested that some degree of hypertrophic differentiation might occur in the presence of TGFβ (Fig. 3). BMP is also involved in multiple steps of chondrogenesis. We provided evidence that (1) BMP

is involved in TGFβ-induced as well as TGFβ+PDGF-induced chondrogenesis (Fig. 6, Fig. 7C), (2) exogenous BMP enhanced the cartilage matrix deposition in the TGFβ-treated particles, leading to a dramatic increase in the volume of the cartilage particles (Fig. 7), and (3) BMP suppressed the COL1 level and elevated COL2 expression, resulting in a hyaline cartilage particle (Fig. 8A), thus supporting the positive roles of BMP. It is worth mentioning that no significant changes in mineral deposition within such hyaline cartilage particles were observed after 5 days of hypertrophic differentiation induced with or without 100 ng/ml BMP6, although COL10 accumulation was enhanced by BMP6 (data not shown).

Despite the positive effects of BMP4 at a late stage of chondrogenesis culture, BMP4 added alone from the beginning was rather detrimental (Fig. 7A), which implies that chondrogenesis of the ES-cell-derived mesodermal cells occurs through two steps requiring different proteins: TGFβ followed by BMP. This two-step model agrees with the previous notion that, during the micromass culture of chicken limb mesenchymal cells, a stimulatory effect of BMP4 is observed 24-48 hours after culture, whereas that of TGFβ3 was readily detectable from the beginning of the culture (Roark and Greer, 1994).

PDGF-BB also has striking synergy with TGFβ and forms a large 'full' cartilage particle (Fig. 5) through BMP action (Fig. 6B). Therefore, the role of PDGF may also be placed upstream of that of BMP. The direct effect of PDGF on chondrogenesis has been demonstrated with primary mouse somitic cells (Tallquist et al., 2000). PDGF-BB, and to a lesser degree PDGF-AA, increased the accumulation of Alcian-blue-positive cartilage matrix in the absence of TGFβ without stimulating cell proliferation. Enhancement of COL2 accumulation by PDGF-BB but not by PDGF-AA (Fig. 4C) is consistent with this observation, although our experiment was done in the presence of TGFβ3.

Thus, as judged by the effects of TGFβ, BMP and PDGF, the serum-free chondrogenesis culture using isolated ES-cell-derived mesodermal cells seems to reproduce some, if not all, aspects of the in vivo process.

The ES-cell-derived PDGFRα$^+$ mesodermal cells are chondrogenic but not hemogenic

The TGFβ-independent chondrogenic activity was highly enriched in the flk-1$^-$PDGFRα$^+$ EB cell fraction (Fig. 4), although no lymphohematopoietic cell potential was detected (Fig. 1), suggesting that the flk-1$^-$PDGFRα$^+$ cells are committed to non-hematopoietic cell lineages, including the chondrocyte lineage. This is consistent with the notion that the PDGFRα signaling is essential for normal axial skeletogenesis (Soriano, 1997) and that PDGFRα is highly expressed in the somites and later in the sclerotomal mesenchymes, the condensing limb mesenchymes and the perichondrium, which are considered to be non-hematopoietic, chondrogenic cells (Orr-Urtreger et al., 1992; Schatteman et al., 1992).

The biological significance of the two types of chondrogenic activities, the TGFβ-independent activity and the TGF-dependent activity, is not clear. The flk-1$^-$PDGFRα$^+$ EB cell fraction may contain TGFβ-producing cells or mature chondroprogenitors that no longer require TGFβ to form cartilage nodules. Candidates for the latter are the PDGFRα$^+$ sclerotomal mesenchymes and the limb mesenchymes. Nevertheless, we have concluded that the PDGFRα$^+$ EB cell fraction contains chondrogenic mesoderm.

The ES-cell-derived flk-1$^+$ mesodermal cells containing the hemangioblasts are also chondrogenic

The ES-cell-derived flk-1$^+$ hemangioblast is characterized by its ability to form a blast cell colony in the presence of VEGF and to generate hematopoietic CFCs (Faloon et al., 2000; Kabrun et al., 1997; Nishikawa et al., 1998a). In this respect, flk-1$^+$PDGFRα$^-$ is likely to be the hemangioblast fraction, because it was enriched for CFC-generating activity (Fig. 1B), whereas no spontaneous chondrogenic activity was detected in it (Figs 2, 4, 5). The TGFβ-dependent chondrogenic activity of the flk-1$^+$PDGFRα$^-$ cells thus implies that the hemangioblast may be multi-potential at this stage of development. The observations that the ES-cell-derived flk-1$^+$ cells produce vascular smooth muscle-like cells in the presence of PDGF-BB (Yamashita et al., 2000), and that a purer preparation of hemangioblasts (flk-1$^+$VE-cadherin$^+$PDGFRα$^-$ cells) was also chondrogenic in the presence of TGFβ3 and BMP4 (data not shown), supported this possibility.

Another possibility is that the flk-1$^+$PDGFRα$^-$ cell fraction is a heterogeneous mixture of cell types that are differentially committed to either the hematopoietic/endothelial cell lineages or the chondrogenic cell lineage. However, the result that flk-1$^-$PDGFRα$^+$ cells, DP cells and flk-1$^+$PDGFRα$^-$ cells accumulated similar levels of COL2 during 8 days of micromass culture in the presence of TGFβ3 and PDGF-BB (Fig. 4C) would argue against the possibility that cross contamination by neighboring cells, such as DP cells, accounts for the TGFβ-dependent cartilage forming activity of the flk-1$^+$PDGFRα$^-$ cells.

An appropriate expansion culture method may have to be established to answer these questions definitively and is currently under way.

In vitro chondrogenesis in the whole EB and with the ES-cell-derived mesodermal cells

Kramer et al. demonstrated that chondrogenesis can be performed directly within an EB (Kramer et al., 2000). The culture medium contained 15% FCS, unlike ours, and a high concentration of BMP (100 ng/ml) stimulated chondrogenesis when added within the first 5 days of culture. In contrast, TGFβ displayed either no effect or an inhibitory effect. These results are inconsistent with our observation that chondrogenesis with the FACS-purified mesodermal cells from day 3.6-4.6 EBs either required TGFβ or was augmented by TGFβ (Fig. 2) and that the effect of BMP4 (50 ng/ml) was observed even after 10 days of the pellet culture (Fig. 7C; Table 3). The discrepancy can be explained by the serum effect as well as by the difference in the cell populations used. For example, the requirement of TGFβ in our culture condition may be caused by the removal of TGFβ-producing cells and/or a TGFβ-inducing factor, FCS (Mummery and van den Eijnden-van Raaij, 1999). Nevertheless, as judged by the temporal order of the factor requirements, the serum-free chondrogenesis culture using isolated ES-cell-derived mesodermal cells seems to represent the in vivo process better. Furthermore, considering

the heterogeneity in the DN cell population (Fig. 2G), macroscopic cartilage containing a homogeneous population of chondrocytes would be formed easier with isolated mesodermal cells.

By taking advantage of the ease of obtaining large numbers of purified mesodermal cells, the ES cell differentiation culture described here would provide an important research platform for molecular and cellular dissections of the chondrogenesis pathway in the context of macroscopic cartilage. This method may lead to a better strategy for cartilage repair and cartilage engineering. Applications to the human ES cells may provide an important tissue source for cartilage replacement/repair therapy.

We would like to acknowledge D. Chang for the biotinylation of APA5. We would also like to give warm thanks to W. Boyle and S. Simonet for support.

References

Alini, M., Kofsky, Y., Wu, W., Pidoux, I. and Poole, A. R. (1996). In serum-free culture thyroid hormones can induce full expression of chondrocyte hypertrophy leading to matrix calcification. *J. Bone Miner. Res.* **11**, 105-113.

Alvarez, J., Horton, J., Sohn, P. and Serra, R. (2001). The perichondrium plays an important role in mediating the effects of TGF-beta 1 on endochondral bone formation. *Dev. Dyn.* **221**, 311-321.

Anderson, H. C., Hodges, P. T., Aguilera, X. M., Missana, L. and Moylan, P. E. (2000). Bone morphogenetic protein (BMP) localization in developing human and rat growth plate, metaphysis, epiphysis, and articular cartilage. *J. Histochem. Cytochem.* **48**, 1493-1502.

Ballock, R. T., Heydemann, A., Wakefield, L. M., Flanders, K. C., Roberts, A. B. and Sporn, M. B. (1993). TGF-beta 1 prevents hypertrophy of epiphyseal chondrocytes: regulation of gene expression for cartilage matrix proteins and metalloproteases. *Dev. Biol.* **158**, 414-429.

Bedossa, P., Bacci, J., Lemaigre, G. and Martin, E. (1987). Effects of fixation and processing on the immunohistochemical visualization of type-I, -III and -IV collagen in paraffin-embedded liver tissue. *Histochemistry* **88**, 85-89.

Cancedda, R., Descalzi-Cancedda, F. and Castagnola, P. (1995). Chondrocyte differentiation. *Int. Rev. Cytol.* **159**, 265-358.

Capdevila, J. and Johnson, R. L. (1998). Endogenous and ectopic expression of noggin suggests a conserved mechanism for regulation of BMP function during limb and somite patterning. *Dev. Biol.* **197**, 205-217.

Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J. C. and Keller, G. (1998). A common precursor for hematopoietic and endothelial cells. *Development* **125**, 725-732.

Dumont, D. J., Fong, G. H., Puri, M. C., Gradwohl, G., Alitalo, K. and Breitman, M. L. (1995). Vascularization of the mouse embryo: a study of flk-1, tek, tie, and vascular endothelial growth factor expression during development. *Dev. Dyn.* **203**, 80-92.

Duprez, D., Bell, E. J., Richardson, M. K., Archer, C. W., Wolpert, L., Brickell, P. M. and Francis-West, P. H. (1996). Overexpression of BMP-2 and BMP-4 alters the size and shape of developing skeletal elements in the chick limb. *Mech. Dev.* **57**, 145-157.

Enomoto-Iwamoto, M., Iwamoto, M., Mukudai, Y., Kawakami, Y., Nohno, T., Higuchi, Y., Takemoto, S., Ohuchi, H., Noji, S. and Kurisu, K. (1998). Bone morphogenetic protein signaling is required for maintenance of differentiated phenotype, control of proliferation, and hypertrophy in chondrocytes. *J. Cell Biol.* **140**, 409-418.

Faloon, P., Arentson, E., Kazarov, A., Deng, C. X., Porcher, C., Orkin, S. and Choi, K. (2000). Basic fibroblast growth factor positively regulates hematopoietic development. *Development* **127**, 1931-1941.

Hall, B. K. and Miyake, T. (2000). All for one and one for all: condensations and the initiation of skeletal development. *Bioessays* **22**, 138-147.

Heyworth, C. M. and Spooncer, E. (1993). In vitro clonal assays for murine multipotential and lineage restricted myeloid progenitor cells. In *Haemopoiesis, a practical approach* (eds N. G. Testa and G. Molineux), pp. 37-53. New York: Oxford University Press.

Johansson, B. M. and Wiles, M. V. (1995). Evidence for involvement of activin A and bone morphogenetic protein 4 in mammalian mesoderm and hematopoietic development. *Mol. Cell. Biol.* **15**, 141-151.

Kabrun, N., Buehring, H. J., Choi, K., Ullrich, A., Risau, W. and Keller, G. (1997). Flk-1 expression defines a population of early embryonic hematopoietic precursors. *Development* **124**, 2039-2048.

Kataoka, H., Takakura, N., Nishikawa, S., Tsuchida, K., Kodama, H., Kunisada, T., Risau, W., Kita, T. and Nishikawa, S. I. (1997). Expressions of PDGF receptor alpha, c-Kit and Flk1 genes clustering in mouse chromosome 5 define distinct subsets of nascent mesodermal cells. *Dev. Growth Differ.* **39**, 729-740.

Kato, Y., Iwamoto, M., Koike, T., Suzuki, F. and Takano, Y. (1988). Terminal differentiation and calcification in rabbit chondrocyte cultures grown in centrifuge tubes: regulation by transforming growth factor beta and serum factors. *Proc. Natl. Acad. Sci. USA* **85**, 9552-9556.

Kramer, J., Hegert, C., Guan, K., Wobus, A. M., Mueller, P. K. and Rohwedel, J. (2000). Embryonic stem cell-derived chondrogenic differentiation in vitro: activation by BMP-2 and BMP-4. *Mech. Dev.* **92**, 193-205.

Kulyk, W. M., Rodgers, B. J., Greer, K. and Kosher, R. A. (1989). Promotion of embryonic chick limb cartilage differentiation by transforming growth factor-beta. *Dev. Biol.* **135**, 424-430.

Leonard, C. M., Fuld, H. M., Frenz, D. A., Downie, S. A., Massague, J. and Newman, S. A. (1991). Role of transforming growth factor-beta in chondrogenic pattern formation in the embryonic limb: stimulation of mesenchymal condensation and fibronectin gene expression by exogenenous TGF-beta and evidence for endogenous TGF-beta-like activity. *Dev. Biol.* **145**, 99-109.

Mackay, A. M., Beck, S. C., Murphy, J. M., Barry, F. P., Chichester, C. O. and Pittenger, M. F. (1998). Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Eng.* **4**, 415-428.

Minina, E., Wenzel, H. M., Kreschel, C., Karp, S., Gaffield, W., McMahon, A. P. and Vortkamp, A. (2001). BMP and Ihh/PTHrP signaling interact to coordinate chondrocyte proliferation and differentiation. *Development* **128**, 4523-4534.

Mummery, C. L. and van den Eijnden-van Raaij, A. J. (1999). Developmental tumours, early differentiation and the transforming growth factor beta superfamily. *Int. J. Dev. Biol.* **43**, 693-709.

Nakayama, N., Fang, I. and Elliott, G. (1998). Natural killer and B-lymphoid potential in CD34+ cells derived from embryonic stem cells differentiated in the presence of vascular endothelial growth factor. *Blood* **91**, 2283-2295.

Nakayama, N., Lee, J. and Chiu, L. (2000). Vascular endothelial growth factor synergistically enhances bone morphogenetic protein-4-dependent lymphohematopoietic cell generation from embryonic stem cells in vitro. *Blood* **95**, 2275-2283.

Nakayama, N., Han, C. E., Scully, S., Nishinakamura, R., He, C., Zeni, L., Yamane, H., Chang, D., Yu, D., Yokota, T. et al. (2001). A novel chordin-like protein inhibitor for bone morphogenetic proteins expressed preferentially in mesenchymal cell lineages. *Dev. Biol.* **232**, 372-387.

Nishikawa, S. I., Nishikawa, S., Hirashima, M., Matsuyoshi, N. and Kodama, H. (1998a). Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. *Development* **125**, 1747-1757.

Nishikawa, S.-I., Nishikawa, S., Kawamoto, H., Yoshida, H., Kizumoto, M., Kataoka, H. and Katsura, Y. (1998b). In vitro generation of lymphohematopoietic cells from endothelial cells purified from murine embryos. *Immunity* **8**, 761-769.

Ogawa, M., Kizumoto, M., Nishikawa, S., Fujimoto, T., Kodama, H. and Nishikawa, S. I. (1999). Expression of alpha4-integrin defines the earliest precursor of hematopoietic cell lineage diverged from endothelial cells. *Blood* **93**, 1168-1177.

Orr-Urtreger, A., Bedford, M. T., Do, M. S., Eisenbach, L. and Lonai, P. (1992). Developmental expression of the alpha receptor for platelet-derived growth factor, which is deleted in the embryonic lethal Patch mutation. *Development* **115**, 289-303.

Pathi, S., Rutenberg, J. B., Johnson, R. L. and Vortkamp, A. (1999). Interaction of Ihh and BMP/Noggin signaling during cartilage differentiation. *Dev. Biol.* **209**, 239-253.

Pizette, S. and Niswander, L. (2000). BMPs are required at two steps of limb chondrogenesis: formation of prechondrogenic condensations and their differentiation into chondrocytes. *Dev. Biol.* **219**, 237-249.

Roark, E. F. and Greer, K. (1994). Transforming growth factor-beta and bone morphogenetic protein-2 act by distinct mechanisms to promote chick limb cartilage differentiation in vitro. *Dev. Dyn.* **200**, 103-116.

Schatteman, G. C., Morrison-Graham, K., van Koppen, A., Weston, J. A. and Bowen-Pope, D. F. (1992). Regulation and role of PDGF receptor alpha-subunit expression during embryogenesis. *Development* **115**, 123-131.

Serra, R., Johnson, M., Filvaroff, E. H., LaBorde, J., Sheehan, D. M., Derynck, R. and Moses, H. L. (1997). Expression of a truncated, kinase-defective TGF-beta type II receptor in mouse skeletal tissue promotes terminal chondrocyte differentiation and osteoarthritis. *J. Cell Biol.* **139**, 541-552.

Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X.-F., Breitman, M. L. and Schuh, A. C. (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* **376**, 62-66.

Shalaby, F., Ho, J., Stanford, W. L., Fischer, K. D., Schuh, A. C., Schwartz, L., Bernstein, A. and Rossant, J. (1997). A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. *Cell* **89**, 981-990.

Sheehan, D. C. and Hrapchak, B. B. (1987). *Theory and Practice of Histotechnology.* Columbus: Battelle Press.

Soriano, P. (1997). The PDGF alpha receptor is required for neural crest cell development and for normal patterning of the somites. *Development* **124**, 2691-2700.

Takakura, N., Yoshida, H., Ogura, Y., Kataoka, H., Nishikawa, S. and Nishikawa, S. (1997). PDGFR alpha expression during mouse embryogenesis: immunolocalization analyzed by whole-mount immunohistostaining using the monoclonal anti-mouse PDGFR alpha antibody APA5. *J. Histochem. Cytochem.* **45**, 883-893.

Tallquist, M. D., Weismann, K. E., Hellstroem, M. and Soriano, P. (2000). Early myotome specification regulates PDGFA expression and axial skeleton development. *Development* **127**, 5059-5070.

Winnier, G., Blessing, M., Labosky, P. A. and Hogan, B. L. (1995). Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes Dev.* **9**, 2105-2116.

Yamaguchi, T. P., Dumont, D. J., Conlon, R. A., Breitman, M. L. and Rossant, J. (1993). flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. *Development* **118**, 489-498.

Yamashita, J., Itoh, H., Hirashima, M., Ogawa, M., Nishikawa, S., Yurugi, T., Naito, M., Nakao, K. and Nishikawa, S. (2000). Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature* **408**, 92-96.

Yang, X., Chen, L., Xu, X., Li, C., Huang, C. and Deng, C. X. (2001). TGF-beta/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage. *J. Cell Biol.* **153**, 35-46.

Zimmerman, L. B., de Jesus-Escobar, J. M. and Harland, R. M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* **86**, 599-606.

Zou, H., Wieser, R., Massague, J. and Niswander, L. (1997). Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. *Genes Dev.* **11**, 2191-2203.