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Enhanced Cartilage Tissue Engineering by Sequential Exposure of Chondrocytes to FGF-2 During 2D Expansion and BMP-2 During 3D Cultivation

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Abstract Bovine calf articular chondrocytes, either primary or expanded in monolayers (2D) with or without 5 ng/ml fibroblast growth factor-2 (FGF-2), were cultured on three-dimensional (3D) biodegradable polyglycolic acid (PGA) scaffolds with or without 10 ng/ml bone morphogenetic protein-2 (BMP-2). Chondrocytes expanded without FGF-2 exhibited high intensity immunostaining for smooth muscle α-actin (SMA) and collagen type I and induced shrinkage of the PGA scaffold, thus resembling contractile fibroblasts. Chondrocytes expanded in the presence of FGF-2 and cultured 6 weeks on PGA scaffolds yielded engineered cartilage with 3.7-fold higher cell number, 4.2-fold higher wet weight, and 2.8-fold higher wet weight glycosaminoglycan (GAG) fraction than chondrocytes expanded without FGF-2. Chondrocytes expanded with FGF-2 and cultured on PGA scaffolds in the presence of BMP-2 for 6 weeks yielded engineered cartilage with similar cellularity and size, 1.5-fold higher wet weight GAG fraction, and more homogenous GAG distribution than the corresponding engineered cartilage cultured without BMP-2. The presence of BMP-2 during 3D culture had no apparent effect on primary chondrocytes or those expanded without FGF-2. In summary, the presence of FGF-2 during 2D expansion reduced chondrocyte expression of fibroblastic molecules and induced responsiveness to BMP-2 during 3D cultivation on PGA scaffolds. J. Cell. Biochem. 83: 121–128, 2001.

Key words: chondrocyte differentiation; growth factors; smooth muscle alpha actin; polymer scaffolds

Chondrocytes in articular cartilage synthesize collagen type II and large sulfated proteoglycans, whereas the same cells cultured in monolayers (2D) dedifferentiate into fibroblastic cells and express collagen type I and small proteoglycans [von der Mark et al., 1977]. Chondrocyte dedifferentiation can be prevented or reversed by culturing cells in a 3D environment supporting spherical cell morphology, such as agarose gels [Benya and Shaffer, 1982; Bonaventure et al., 1994] or biodegradable polyglycolic acid (PGA) scaffolds [Freed et al., 1994; Martin et al., 1999].

We recently showed that bovine calf articular chondrocytes expanded in 2D in the presence of FGF-2 fully redifferentiated and deposited collagen type II when cultured on 3D PGA scaffolds, whereas the same cells expanded without FGF-2 remained elongated, developed thick F-actin structures, and induced shrinkage of the same scaffolds [Martin et al., 1999]. Canine chondrocytes expanded in 2D without FGF-2 also induced shrinkage of sponges made of collagen and GAG [Nehrer et al., 1997; Lee et al., 2000]. Chondrocyte-mediated extracellular matrix contraction was recently proposed to be related to the expression of smooth muscle α-actin (SMA) [Lee et al., 2000], a contractile actin isoform typically expressed by specialized fibroblastic cells capable of applying tractional forces [Sappino et al., 1990].

Bone morphogenetic proteins (BMPs) specifically regulate the early commitment of mesenchymal cells toward the chondrogenic and osteogenic lineages [Celeste et al., 1990]. In

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particular, BMP-2 was demonstrated to be a key chondrogenic factor for a pluripotent mesenchymal cell line [Ahrens et al., 1993], mouse embryonic stem cells [Kramer et al., 2000], embryonic cells at a pre-chondrogenic stage [Quarto et al., 1997], and undifferentiated mesenchymal cells expressing SMA [Aikawa et al., 1996].

In the present study, we tested the hypothesis that the presence of FGF-2 during 2D chondrocyte expansion modulates (i) expression of molecules typically expressed by contractile fibroblastic cells (e.g., collagen type I and SMA) and (ii) cellular responsiveness to chondrogenic factors (e.g., BMP-2) during subsequent 3D cultivation.

MATERIALS AND METHODS

Chondrocyte Culture

Bovine articular chondrocytes were isolated from the femoropatellar grooves of 2 to 3-week-old calves by incubation in 0.15% collagenase type II (Worthington, Freehold, NJ) at 37°C with orbital mixing (40 rpm) for 14–16 h [Freed et al., 1993]. The cells were plated on Petri dishes at 4×10^3cells/cm^2 in Dulbecco's modified Eagle medium containing 10% fetal bovine serum (FBS), 0.1 mM nonessential amino acids, 0.4 mM proline, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.5 μg/ml fungizone (hereafter referred to as expansion medium), with or without 5 ng/ml FGF-2 (R&D Systems, Minneapolis, MN), and passaged twice, undergoing a total of 10–11 doublings and an expansion of 1000 to 2000-fold [Martin et al., 1999]. Second passage (P2) chondrocytes expanded with or without FGF-2 were trypsinized off the dishes and dynamically seeded [Vunjak-Novakovic et al., 1998] onto biodegradable 3D PGA scaffolds [Freed et al., 1994] at an initial density of 4×10^6 cells/5 mm diameter × 2 mm thick disc; control studies were done using primary chondrocytes. In brief, PGA discs were pre-wetted with medium containing 10% FBS and threaded onto needles embedded in the stopper of a spinner flask that was filled with 120 ml of medium. Cells were inoculated into the flask and incubated with stirring (60 rpm) for 3 days, then cell-polymer constructs were transferred to Petri dishes coated with a thin layer of 1% agarose (1 construct in 3 ml of medium/1.5 cm diameter well) and incubated with orbital mixing (60 rpm) in a 37°C/5% CO_2 incubator [Martin et al., 1999]. Constructs were cultured in expansion medium supplemented with 5 μg/ml insulin (Sigma, St Louis, MO) and 50 μg/ml ascorbic acid, with or without 10 ng/ml BMP-2 (kindly provided by Genetics Institute, Cambridge, MA). Medium was completely replaced three times/week for up to 6 weeks.

Analytical Methods

Histology and fluorescence microscopy. Cell-polymer constructs (n = 3/group) were bisected, extensively rinsed with phosphate buffered saline (PBS), fixed in 4% neutral buffered formalin for 24 h at 4°C, dehydrated, embedded in paraffin, and cross-sectioned (5 μm thick). For histological evaluation, sections were stained with safranin O for sulfated GAGs. Cross-sectional areas were quantified by computer-assisted image analysis of sections acquired by a 3 color charge-coupled camera (Sony) mounted on a Nikon Diaphot microscope. Using Image Pro software (Media Cybernetics, Silver Spring, MD), construct cross-sectional areas were measured after manual identification of the section profile. For immunohistochemical analyses, sections were incubated with 0.25% ammonia (NH_3) in 70% ethanol for 1 h during deparaffinization. Sections were sequentially treated with 10 mg/ml sodium borohydride ($NaBH_4$) for 40 min at 4°C, 1 mg/ml testicular hyaluronidase for 30 min at 37°C, and normal goat serum diluted 1:10 in PBS for 15 min at room temperature (RT, ~22°C). Sections were labeled by incubation with a monoclonal antibody against collagen type I (Biodesign International, Kennebunk, ME) or smooth muscle α-actin (kindly provided by G. Gabbiani, University of Geneva, Switzerland) for 1 h at RT, with Alexa 488 (Molecular Probes) diluted 1:800 in PBS for 1 h at RT, and with 0.1% Sudan Black in 70% ethanol for 30 min at RT. NH_3, $NaBH_4$, and Sudan Black were used to reduce background fluorescence (Baschong, unpublished data). Specimens were imaged using a confocal laser scanning microscope (TCS 4-D CLSM, Leica AG, Heidelberg) using Argon laser excitation and an Apochromat 25 × 0.75 NA oil immersion objective. Optical sections were acquired at 0.5 μm increments and rendered using Imaris software (Bitplane, Zürich, CH).

Biochemical analyses. Cell-polymer constructs (n = 3/group) were frozen, lyophilized, and papain-digested [Freed et al., 1993]. The number of cells per construct was assessed from the

DNA content using a spectrofluorometer and conversion factors of 7.7 pg DNA/chondrocyte [Kim et al., 1988] and 10^{-10} g dry weight/chondrocyte [Freed et al., 1994]. GAG contents were measured spectrophotometrically using dimethylmethylene blue [Farndale et al., 1986] and bovine chondroitin sulfate as a standard. Total collagen contents were determined spectrophotometrically from the hydroxyproline content after acid hydrolysis and reaction with p-dimethylaminobenzaldehyde and chloramine-T [Woessner, 1961], using a hydroxyproline to collagen ratio of 1:10 [Hollander et al., 1994].

Statistical analysis. All values are presented as mean±standard deviation. Statistics were assessed using a student's *t*-test, assuming double-sided independent variance and with $P < 0.05$ considered to be significant.

RESULTS

Constructs based on primary chondrocytes (PRI) and those expanded in the presence of FGF-2 (EXP+) immunostained less intensively for SMA and collagen type I than constructs based on chondrocytes expanded without FGF-2 (EXP−) (Fig. 1). After 1 week of culture, SMA and collagen type I were distributed throughout the cross-sectional areas of constructs from all experimental groups (Fig. 1A,C). By 6 weeks of culture, SMA and collagen type I staining decreased in intensity and became localized at the periphery of PRI and EXP+ constructs (Fig. 1B I&III, D I&III), whereas SMA staining remained intense peripherally and collagen type I staining remained intense throughout EXP− constructs (Fig. 1 BII, DII). BMP-2 did not appear to affect SMA or collagen type I staining intensity in any experimental group (data not shown).

PRI and EXP+ constructs maintained their initial dimensions during the first week of culture, and their cross-sectional areas almost doubled over 6 weeks (Fig. 2). In contrast, EXP− constructs contracted such that their cross-sectional areas were reduced by 50% within 1 week of culture and did not change further in size for the remainder of the study (Fig. 2). BMP-2 did not affect construct cross-sectional area in any experimental group (Fig. 2).

PRI constructs and EXP+ constructs cultured in the absence of BMP-2 were similar at 1 week, whereas after 6 weeks the PRI constructs had higher cell number (1.7-fold), higher wet weight (1.6-fold), and similar wet weight fractions of GAG and total collagen (Table IA). EXP+ constructs cultured without BMP-2 for 6 weeks were superior to the corresponding EXP− constructs with respect to cell number (3.7-fold), wet weight (4.2-fold), and wet weight GAG fraction (2.8-fold) (Table IA).

PRI constructs and EXP+ constructs cultured in the presence of BMP-2 were similar at 1 week, whereas after 6 weeks the PRI constructs had higher cell number (1.6-fold) and lower wet weight fractions of GAG (77% as high) (Table IB). EXP+ constructs cultured with BMP-2 for 6 weeks were superior to the corresponding EXP− constructs with respect to cell number (3.4-fold), wet weight (4.4-fold), and wet weight GAG fraction (3.1-fold) (Table IB). EXP+ constructs cultured with BMP-2 for 6 weeks had higher wet weight GAG fraction than the corresponding EXP+ constructs cultured without BMP-2 (1.5-fold), whereas PRI and EXP− constructs were not affected by BMP-2 in any of the assessed parameters (Table 1A vs. B).

PRI and EXP+ constructs cultured in the absence of BMP-2 contained significantly more GAG on a per cell basis than EXP− constructs (Fig. 3A,D). In response to BMP-2, the amount of GAG per cell was highest in EXP+ constructs, reaching levels that after 6 weeks were 1.7- and 3.8-fold higher than in PRI and EXP− constructs, respectively (Fig. 3B). EXP+ constructs cultured with BMP-2 contained ~0.75 ng GAG/cell (Fig. 3), as compared to ~1 ng GAG/cell previously measured for native bovine calf articular cartilage [Freed et al., 1998].

Sequential exposure of chondrocytes to FGF-2 during 2D expansion and BMP-2 during 3D culture improved the histological appearance of 6 week constructs, which showed a cartilaginous matrix continuously stained by safranin-O (Fig. 4F). In contrast, all other groups exhibited a 100–200 μm thick surface region containing elongated cells and very little GAG (Fig. 4A–E), and EXP− constructs displayed less intense and less homogeneous GAG stain (Fig. 4B,E).

DISCUSSION

The present study demonstrates that the presence of FGF-2 during monolayer expansion of chondrocytes (i) reduces the expression of molecules typically expressed by contractile

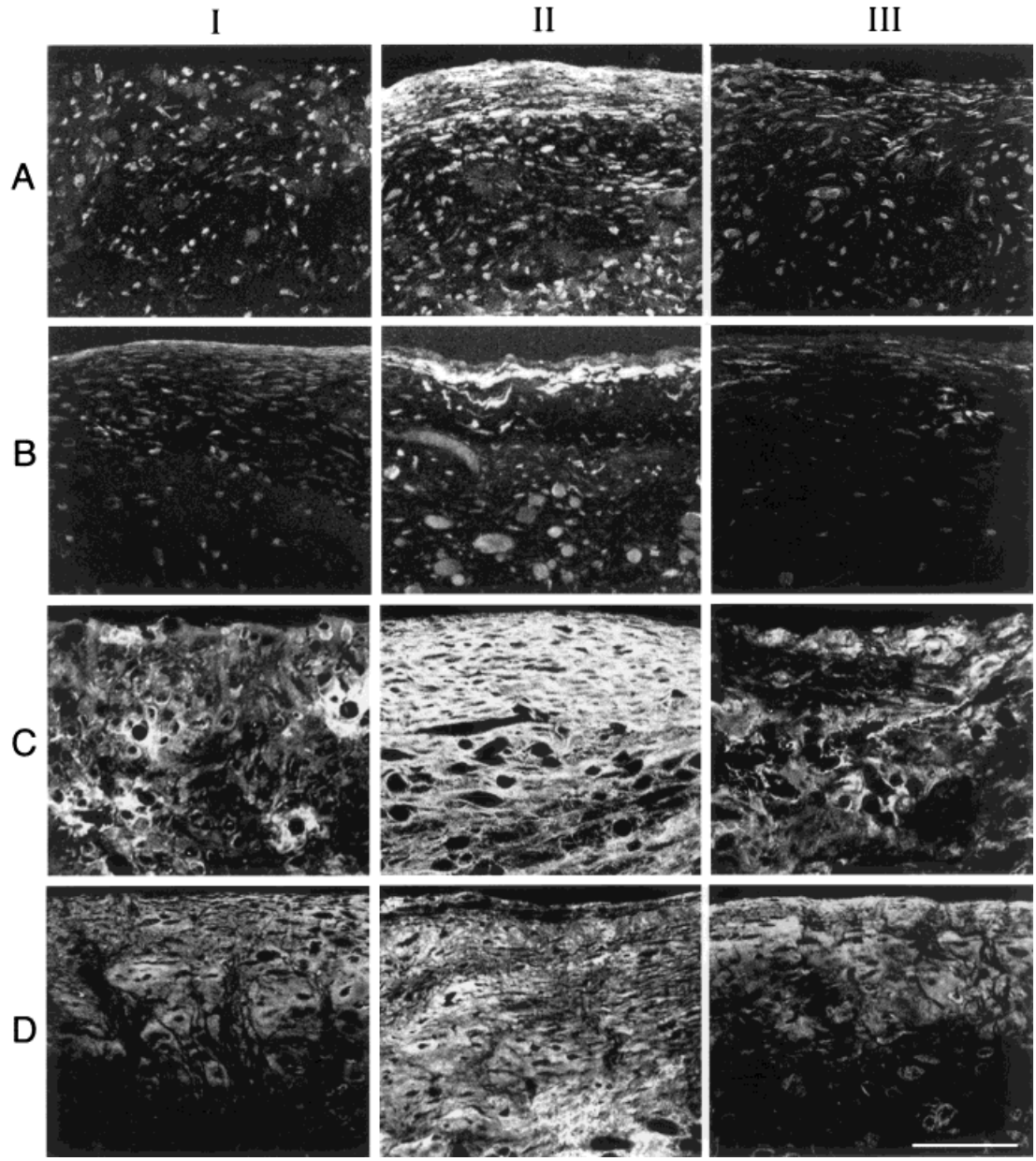

Fig. 1. Alpha smooth muscle actin (SMA) and collagen type I staining of chondrocyte polymer constructs. Constructs based on primary chondrocytes (I, PRI), chondrocytes expanded without FGF-2 (II, EXP−) and chondrocytes expanded with FGF-2 (III, EXP+), after 1 week (**A**,**C**) and 6 weeks (**B**,**D**) of cultivation. Histological sections were stained with monoclonal antibodies to SMA (A,B) or collagen type I (C,D). Scale bar = 100 μm.

fibroblastic cells (e.g., collagen type I and SMA) and (ii) enhances the responsiveness to chondrogenic factors (e.g., BMP-2), thus improving the size and composition of engineered cartilage generated by subsequent 3D cultivation on PGA scaffolds.

Microfilaments of SMA normally develop in fibroblastic cells during experimental wound healing [Darby et al., 1990]. These structures, which are considered instrumental in wound contraction [Gabbiani et al., 1971], are one of the most distinct features of a particular stage of fibroblast differentiation, generally referred to as the myofibroblast [Skalli et al., 1988]. The persistence of SMA-containing microfilaments in fibroblastic cells after the wound is closed is associated with various pathologies [Skalli et al., 1989] and clinical disorders including fibromatosis and fibrosis [Seemayer et al., 1981; Brenner et al., 2000]. In the present study, we

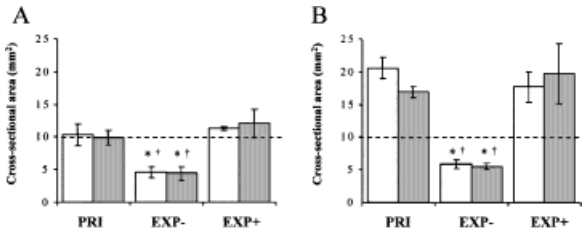

Fig. 2. Size of chondrocyte-polymer constructs. Cross-sectional areas (mm^2) are shown for constructs based on primary chondrocytes (PRI), chondrocytes expanded without FGF-2 (EXP−), and chondrocytes expanded with FGF-2 (EXP+), cultured for 1 week (**A**) and 6 weeks (**B**) without (unfilled bars) and with (filled bars) BMP-2. Dashed lines indicate initial construct area. *Statistically different from the corresponding PRI constructs. †Statistically different from the corresponding EXP+ constructs.

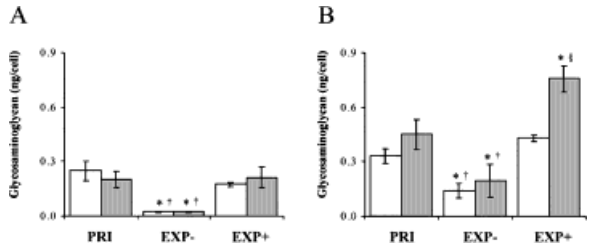

Fig. 3. Glycosaminoglycan (GAG) content of chondrocyte-polymer constructs. Amount of GAG (ng/cell) for constructs based on primary chondrocytes (PRI), chondrocytes expanded without FGF-2 (EXP−), and chondrocytes expanded with FGF-2 (EXP+), cultured for 1 week (**A**) and 6 weeks (**B**) without BMP-2 (unfilled bars) and with BMP-2 (filled bars). Native bovine calf cartilage contains ~1.0 ng of GAG/cell [Freed et al., 1998]. *Statistically different from the corresponding PRI constructs. †Statistically different from the corresponding EXP+ constructs. § Statistically different from constructs based on the same cells and cultured without BMP-2.

detected SMA in engineered cartilage based on primary or expanded articular chondrocytes and 3D scaffolds made of PGA, consistent with recent findings of SMA expression by articular chondrocytes in situ [Wang et al., 2000] and in vitro, on 3D scaffolds made of collagen and GAG [Lee et al., 2000]. We further showed that intense immunostain for SMA in EXP− constructs (Fig. 1 AII, BII) was associated with intense immunostain for collagen type I (Fig. 1 CII, DII), reduction of construct size (Fig. 2; Table I), and low GAG fraction (Table I), indicating a reduced cell ability to acquire a chondrocytic phenotype. In contrast, less intense stain for SMA in PRI and EXP+ constructs (Fig. 1A I&III, BI&III) corresponded to the formation of larger tissues (Fig. 2; Table I) that contained only peripherally localized collagen type I (Fig. 1D I&III), abundant GAG (Table I), and cartilaginous histomorphology

TABLE I. Size and Composition of Engineered Cartilage[a]

	Experimental group					
	A. −BMP-2			B. +BMP-2		
Time	PRI	EXP−	EXP+	PRI	EXP−	EXP+
1 week						
Cells (millions/construct)	10.5±1.9	6.6±1.3[b,c]	10.2±0.9	10.4±1.7	6.9±0.3	9.7±2.3
Wet weight (ww)	86±7	37±4[b,c]	86±6	79±14	35±3[b,c]	89±1
GAG (%ww)	1.69±0.45	0.40±0.04[b,c]	2.06±0.06	1.69±0.55	0.48±0.08[b,c]	2.23±0.05[d]
Collagen (total, %ww)	1.51±0.10	2.18±0.18[b,c]	0.84±0.09[b]	1.71±0.38	2.04±0.68	1.15±0.32
4 weeks						
Cells (millions/construct)	24.6±4.6	4.7±0.8[b,c]	14.6±3.9[b]	21.4±1.6	4.9±0.7[b,c]	16.1±1.9[b]
Wet weight (ww)	218±27	37±4[b,c]	139±14[b]	165±16	41±4[b,c]	159±10
GAG (%ww)	2.77±0.33	1.03±0.06[b,c]	2.76±0.16	2.39±0.48	1.18±0.11[b,c]	3.84±0.45[b,d]
Collagen (total, %ww)	1.59±0.14	2.76±0.19[b,c]	1.90±0.13	1.61±0.35	2.56±0.21[b]	2.09±0.04
6 weeks						
Cells (millions/construct)	22.8±4.4	3.6±0.9[b,c]	13.2±0.2[b]	19.5±2.8	3.6±0.5[b,c]	12.1±1.7[b]
Wet weight (ww)	268±25	40±2[b,c]	169±11[b]	222±14	41±1[b,c]	180±24
GAG (%ww)	2.92±0.48	1.20±0.01[b,c]	3.37±0.14	3.88±0.55	1.66±0.53[b,c]	5.09±0.52[b,d]
Collagen (total, %ww)	2.17±0.28	2.60±0.06	2.45±0.25	1.88±0.30	2.88±0.23[b]	2.46±0.13

[a]Engineered cartilage was based on primary chondrocytes (PRI), chondrocytes expanded without FGF-2 (EXP−), and chondrocytes expanded with FGF-2 (EXP+), cultured on PGA scaffolds without (A) or with (B) BMP-2.
[b]Statistically different from the corresponding constructs in the PRI group.
[c]Statistically different from the corresponding constructs in the EXP+ group.
[d]Statistically different from the constructs based on the same cells and cultured without BMP-2.

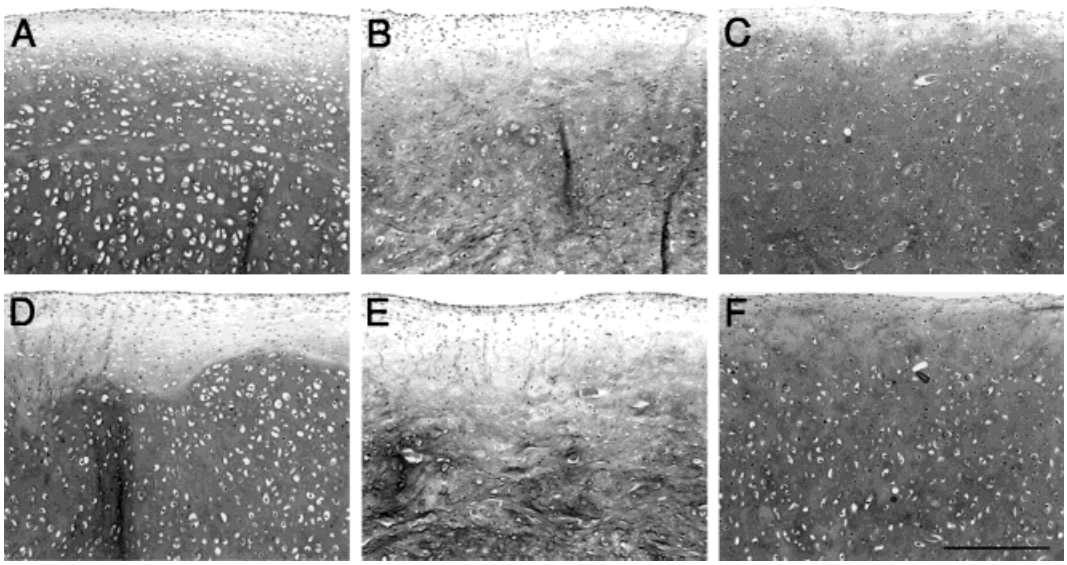

Fig. 4. Glycosaminoglycan (GAG) stain of chondrocyte-polymer constructs. Constructs based on primary chondrocytes (**A**,**D**) and chondrocytes expanded for two passages without (**B**,**E**) and with (**C**,**F**) FGF-2 cultured on PGA meshes for 6 weeks without (A–C) and with (D–F) BMP-2. Stain = safranin O. Scale bar = 200 μm.

(Fig. 4A,C). In our previous studies, collagen type II represented the predominant fraction ($>84\%$) of the total collagen, as assessed by inhibition ELISA, Western dot blot, and a SDS–PAGE method based on CNBr fragments [Freed et al., 1998; Riesle et al., 1998].

The main reported effects of FGF-2 on chondrocytes are (i) increased proliferation, and (ii) reduced differentiation [Wroblewski and Edwall-Arvidsson, 1995], including decreased expression of cartilage-specific genes [Jingushi et al., 1990; Shida et al., 1996]. In the present study, the culture medium was first supplemented with 5 ng/ml FGF-2 during 2D chondrocyte expansion, based on our previous report that these conditions yielded a population of cells with superior differentation ability [Martin et al., 1999], and then with 10 ng/ml BMP-2 during 3D culture on PGA scaffolds cells in an attempt to further promote differentiation. We found that the presence of FGF-2 during 2D chondrocyte expansion modulated the subsequent responsiveness of the cells to BMP-2. In particular, EXP+ constructs cultured with BMP-2 had higher wet weight fractions of GAG (Table I), higher amounts of GAG/cell (Fig. 3), and more homogeneously distributed GAG (Fig. 4) than the corresponding constructs cultured without BMP-2. In contrast, EXP– constructs did not appear to be responsive to BMP-2 with respect to any of the assessed parameters. Chondrocyte expansion without FGF-2 thus yielded a population of fibroblasts with a contractile phenotype and very limited chondrogenic potential, whereas expansion with FGF-2 generated a population of cells that appeared highly dedifferentiated in 2D culture [Martin et al., 1999], but maintained their chondrogenic ability and responded to BMP-2 during 3D culture on PGA scaffolds (Table I, Figs. 3 and 4F). We could thus speculate that the presence of FGF-2 during monolayer expansion prevented chondrocyte trans-differentiation into a terminal contractile fibroblast-like phenotype, recently referred to as a 'myochondrocyte' [Wang et al., 2000] or a 'myofibrochondrocyte' [Kambic et al., 2000] and instead supported chondrocyte de-differentiation into a committed chondroprogenitor cell. It would be interesting to investigate whether FGF-2 supported a selective expansion of specific sub-populations of primary chondrocytes, or whether it stimulated all primary cells to reach a similar developmental stage.

Primary chondrocytes cultured on PGA scaffolds were not responsive to BMP-2 with respect to any of the assessed parameters, and the resulting engineered cartilage had a lower GAG fraction than that based on FGF-2-expanded cells. Previously established relationships be-

tween the GAG fraction and compressive modulus in engineered cartilage [Vunjak-Novakovic et al., 1999] suggest that constructs based on FGF-2-expanded chondrocytes may also have improved mechanical properties as compared to constructs based on primary chondrocytes.

The present study thus demonstrates that engineered cartilage tissue can be improved by sequential exposure of bovine calf articular chondrocytes to FGF-2 during 2D expansion and to BMP-2 during 3D culture on PGA scaffolds. We recently demonstrated enhanced chondrogenesis of human adult articular chondrocytes expanded in the presence of a combination of factors including FGF-2 and differentiated with factors belonging to the same superfamily of BMPs [Jakob et al., 2001], implying that a similar approach can potentially improve the engineering of human cartilage grafts starting from a small biopsy specimen. Our findings also outline a general paradigm for tissue engineering, whereby mature cells are first dedifferentiated by specific regulatory factors (e.g., FGF-2) into committed progenitor cells, and then redifferentiated using other regulatory factors (e.g., BMP-2) that have higher efficacy on progenitor than mature cells [Kramer et al., 2000]. This paradigm, which was recently demonstrated for central nervous system cells [Kondo and Raff, 2000], could open new opportunities for tissue engineering by recapitulation of embryonic events.

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