Cells and Materials, Volume 8, 1998 (Pages 99-122) 1051-6794/98$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

MESENCHYMAL CELL-BASED REPAIR OF CONNECTIVE TISSUE DEFECTS: APPLICATION OF TRANSFORMING GROWTH FACTOR-ß SUPERFAMILY MEMBERS AND BIODEGRADABLE POLYMER SCAFFOLDS

S.B. Nicoll[1], A.E. Denker[2] and R.S. Tuan*

Department of Orthopaedic Surgery, Thomas Jefferson University, Philadelphia, PA
Current addresses: [1]Univ. California, Berkeley/San Francisco; and [2]Washington Univ. Sch. Med., St. Louis, MO

(Received for publication September 16, 1997 and in revised form December 10, 1997)

Abstract

Mesenchymal stem cells are characterized by their ability to differentiate into multiple differentiated cellular phenotypes, including connective tissue cells, such as osteoblasts and chondrocytes. The pluripotent nature of these progenitor cells is consistent with their involvement in developmental and biological repair processes, such as embryonic skeletal formation and fracture healing. Experimental analyses of mesenchymal stem cells have made use of both primary cells as well as clonal cell lines derived from bone marrow, muscle, and other tissue sources. The commitment and differentiation of mesenchymal cells is regulated *in vivo* and *in vitro* by complex effectors, most notably, members of the transforming growth factor-ß (TGF-ß) superfamily. Specifically, several members of the TGF-ß superfamily induce osteo- and chondro-differentiation of mesenchymal stem cells. This review summarizes recent studies which explore the application of mesenchymal cells as a source of bone and cartilage-forming cells, upon seeding within resorbable polymeric scaffolds in the presence of bioactive growth factors. Such cell-polymer-growth factor composites may be fabricated for use as templates for the engineering and repair of bone and cartilage tissues.

Key Words: Bone, cartilage, tissue engineering, transforming growth factor-ß, mesenchymal cells, poly(L-lactic acid), C3H10T1/2.

*Address for correspondence:
Rocky S. Tuan
Department of Orthopaedic Surgery
Thomas Jefferson University
501 Curtis Bldg., 1015 Walnut St.
Philadelphia, PA 19107

Telephone number: 215-955-5479
FAX number: 215-955-9159
E-mail: Rocky.S.Tuan@mail.tju.edu

Introduction

Mesenchymal stem cells are pluripotent progenitor cells which give rise to a variety of specialized connective tissue cell types including chondrocytes, osteoblasts, adipocytes and myocytes (Caplan, 1991). The functional role of mesenchymal stem cells in bone and cartilage formation is the primary focus of this review. The biology of mesenchymal cells within the context of embryonic skeletal development and fracture repair is presented, as well as a historical review of the various primary mesenchymal stem cell populations and multipotential clonal cell lines. The regulation of these cells by potent bioactive factors, specifically, members of the transforming growth factor (TGF-ß) gene superfamily, is also discussed. In the concluding section, the application of mesenchymal cells to connective tissue engineering is addressed. Specific topics include the use of biodegradable polymers as delivery vehicles for bioactive agents and as cellular substrates for the promotion of bone and cartilage tissue repair.

Mesenchymal Stem Cells in Embryonic Skeletal Development and Fracture Repair

Skeletal formation in the vertebrate embryo involves two distinct developmental processes, endochondral ossification and intramembranous bone formation. The endochondral pathway, which occurs primarily in the long bones, vertebral bodies, and pelvic bones, is characterized by bone formation via a cartilaginous intermediate or template (see Fig. 1; Reddi, 1981; Rosen and Thies, 1992; Erlebacher *et al.*, 1995; Gilbert, 1997). The cascade begins with the proliferation of undifferentiated mesenchymal cells which migrate to specific sites in the embryo. The cells condense into closely-packed aggregates and deposit copious amounts of cartilaginous matrix components, such as type II collagen and aggrecan. Layers of fibroblast-like cells then form a sheath around each of the cartilage nodules; this sheath, the perichondrium, separates the anlagen from the surrounding tissue. The chondrocytes in the center of the cartilage proliferate, mature, and eventually become

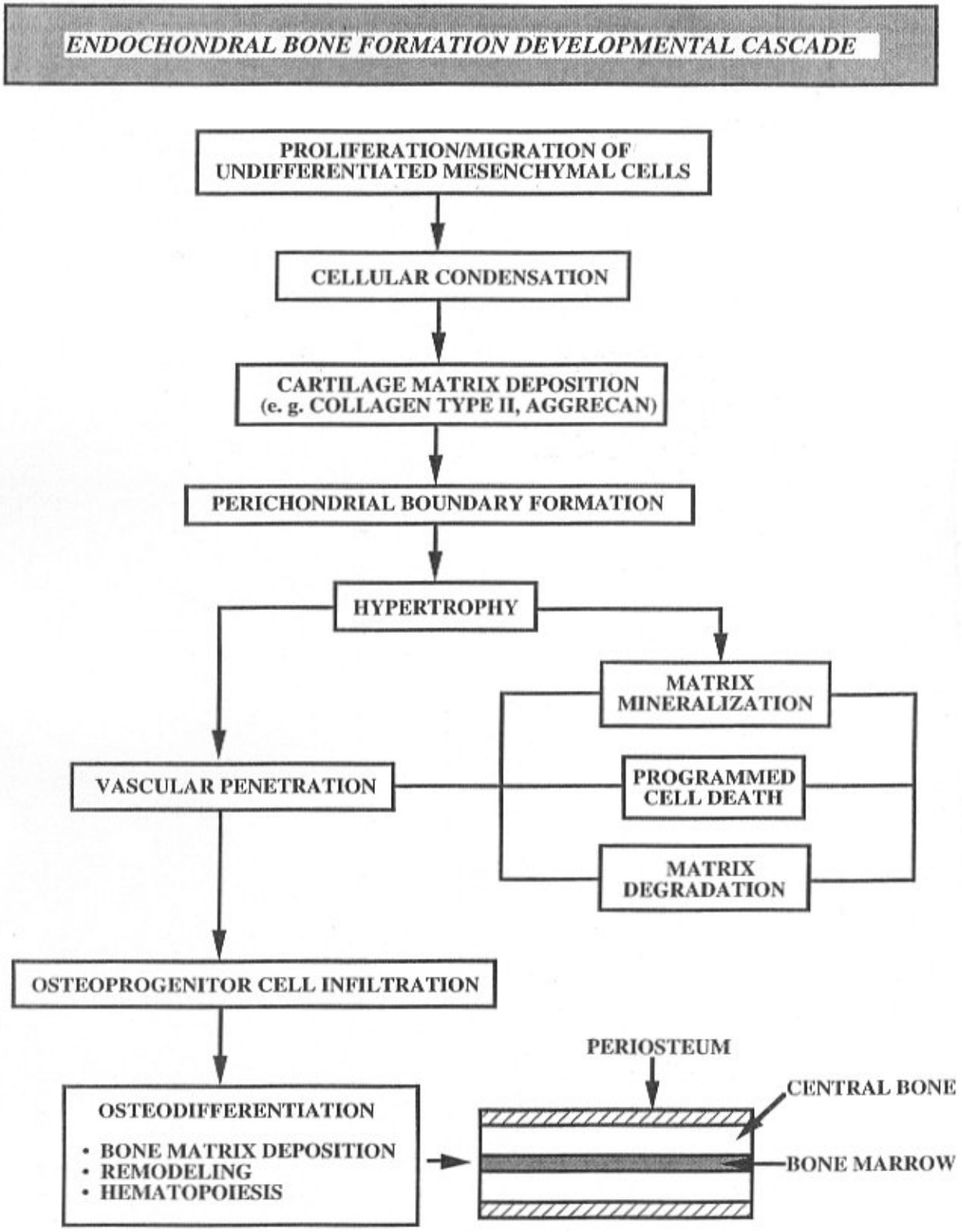


Figure 1. Diagrammatic representation of the endochondral ossification sequence. Endochondral bone formation in the vertebrate embryo occurs via a spatially and temporally regulated developmental program involving mesenchymal differentiation into a cartilage intermediate prior to the final bony element.

hypertrophic as vasculature begins to penetrate the region, and the matrix becomes mineralized. As the hypertrophic chondrocytes start to undergo programmed cell death, the cartilaginous matrix is degraded by invading cells. Osteoprogenitor cells, likely originating from either the perichondrium or the invading blood vessels, differentiate into osteoblasts and secrete new bone matrix. Eventually, bone tissue is formed complete with a marrow cavity, an inner core, and an outer periosteal layer.

Intramembranous bone formation, on the other hand, occurs predominantly in the craniofacial flat bones and involves the direct conversion of mesenchymal cells to osteoblasts (Reddi, 1981; Erlebacher *et al*., 1995; Gilbert, 1997). Progenitor cells migrate to designated sites in the embryo, condense, and differentiate into osteoblasts which deposit bone matrix.

The events observed in embryonic skeletal development are recapitulated in adult animals during the fracture repair processes. The repair of outer bone tissue occurs by a mechanism resembling intramembranous bone formation (Sevitt, 1981; Rosen and Thies, 1992). Osteoprogenitor cells in the periosteum proliferate, undergo osteodifferentiation, and secrete matrix materials that bridge the gap at the cortical surface of the bone. The central core of bone is repaired in a series of events analogous to endochondral ossification (Sevitt, 1981; Rosen and Thies, 1992). Mesenchymal cells migrate into a fracture site and undergo chondrogenic differentiation, soon followed by calcification of the cartilaginous matrix, osteodifferentiation, and bone and marrow formation. These mesenchymal cells thus possess stem cell characteristics, i.e., the ability to give rise to multiple mesenchymal cell lineages (see reviews by Bruder *et al*., 1994 and Prockop, 1997). The origin and distribution of these precursor cells is described in the next section.

Primary Mesenchymal Stem Cell Populations

In adult animals, mesenchymal stem cells have been shown to exist in a number of tissues, with bone marrow serving as perhaps the most abundant reservoir of these progenitor cells. A variety of transplantation and diffusion chamber experiments have established the existence of a self-renewing population of mesenchymal stem cells within bone marrow stroma. This was first demonstrated by Friedenstein and co-workers in experiments in which bone marrow was transplanted under the renal capsule in mice (Friedenstein *et al*., 1968). After a period of forty days, bone tissue containing marrow had formed, indicating the presence of osteogenic precursor cells in bone marrow stroma. Subsequently, he and others, most notably Owen and colleagues (Ashton *et al*., 1980, 1984; Bab *et al*., 1986; Owen, 1988), demonstrated *in vivo* that marrow explants implanted either in diffusion chambers or subcutaneously in rabbits gave rise to bone and cartilage, as well as adipose and fibrous tissue. Direct *in vitro* evidence of marrow-derived osteoprogenitor cells was also reported, as fibroblast-like cells isolated from marrow suspensions were shown to form bone nodules after two weeks in culture (Howlett *et al*., 1986; Maniatopoulos *et al*., 1988).

Similar experiments confirmed that mesenchymal stem cells reside in the periosteum. In a classic study by Dame Honor Fell, periosteal explants from the limb bones of embryonic fowl exhibited cartilage nodule and osteoid tissue formation when cultured *in vitro* (Fell, 1932), proving that mesenchymal stem cells exist in the periosteum. Subsequent *in vivo* studies in which periosteal grafts were implanted in a variety of anatomical sites in rodents including the anterior eye chamber (Urist and Mclean, 1952) and under the renal capsule (Cohen and Lacroix, 1955) corroborated Fell's earlier findings. An extension of this earlier work using periosteal grafts explanted into diffusion chambers and inserted subcutaneously into rodents also resulted in bone and cartilage formation (Rosin *et al*., 1963). More recent and convincing *in vitro* evidence by Tenenbaum and associates showed that periosteal tissue cultured in the presence of ß-glycerophosphate formed mineralized osteoid tissue which appeared virtually identical to bone formed *in vivo* (Tenenbaum and Heersche, 1982, 1986). Caplan and co-workers further demonstrated the pluripotent nature of periosteum-derived cells using subcultured cell populations. When seeded into diffusion chambers and implanted intraperitoneally in mice, these cells gave rise to cartilage after just four days, which was eventually replaced by bone from between 2 to 4 weeks (Nakahara *et al*., 1990a,b).

In addition to bone marrow and periosteum, another important source of mesenchymal stem cells is muscle tissue. The pioneering work of Marshall Urist (Urist, 1965) showed that intramuscular implantation of demineralized bone matrix (DBM) in rodents induced ectopic bone formation. This indicated not only that mesenchymal cells exist within muscle, but that bioactive factors capable of affecting osteo-chondrogenic differentiation are present in bone matrix. These findings were confirmed in later studies by Urist and colleagues (Nogami and Urist, 1970, 1974; Terashima and Urist, 1977) and by Reddi and co-workers (Sampath *et al*., 1984), wherein minced muscle tissue grown on a substratum consisting of cross-hatched hemicylinders of HCl-demineralized diaphyseal bone produced cartilage tissue after 7 days. Recent work in our laboratory, which supports the previous findings, has shown the formation of ectopic cartilage in poly-L-lysine injected muscle grafts (Tuan *et al*., 1991) and in micromass cultures derived from mixed bone- or muscle-derived cells and limb bud mesenchymal cells (Stringa and Tuan, 1996; Tuan *et al*.,

1996). Interestingly, subcutaneous implantation of DBM into soft connective tissue has also been reported to stimulate ectopic cartilage and bone formation in a manner similar to that observed in muscle tissue (Reddi and Huggins, 1972; Urist *et al*., 1983), suggesting that mesenchymal stem cells in several connective tissues may be converted into specialized cell types by exogenous inducing agents.

Mesodermal cells of the embryonic limb bud have also been shown to possess multiple differentiation potentials. This is to be expected since limb bud mesenchyme are destined to form cartilage tissue which serves as a template for long bone formation in the developing embryo. Embryonic chick limb bud cells are the most extensively studied of these mesodermal cells, and have been shown to possess myogenic as well as osteogenic and chondrogenic potential. The classic work by Fell first demonstrated that embryonic chick limbs give rise to bone and cartilage tissue in organ culture (Fell, 1925). Later studies by Searls and co-workers showed that limb bud mesenchymal cells are also capable of differentiating into muscle, and further demonstrated that the multipotential behavior of limb bud mesenchymal cells depends upon the developmental stage of the embryo, with mesenchyme from embryos after stage 24 no longer capable of differentiating into both muscle and cartilage tissue (Searls, 1965; Searls and Janners, 1969). Expanding on this earlier work, Caplan and colleagues illustrated that several factors, in addition to the maturation stage of the embryo, influence the differentiated phenotype of chick limb bud cells (Caplan, 1970, 1977; Caplan and Koutroupas, 1973; Osdoby and Caplan, 1979). These include (1) vascularization, (2) oxygen tension, and (3) initial cell plating density. In particular, it was established that high cell density and low oxygen tension promote the cartilage phenotype. Related studies by the late Michael Solursh and associates using both chick and mouse limb bud mesenchyme introduced a novel high density cell culturing technique referred to as micromass culture, which confirmed the role of high cell density in cartilage induction (Ahrens *et al*., 1977; Owens and Solursh, 1981). More recent work performed in our laboratory demonstrated the requirement of high cell density (San Antonio and Tuan, 1986), calcium-mediated cell-cell interactions (Oberlender and Tuan, 1994), and specific cell-matrix interactions (Gehris *et al*., 1996, 1997) for optimal chondrogenesis in embryonic chick limb bud mesenchymal cells.

Multipotential Clonal Cell Lines

Along with the various primary mesenchymal stem cell populations, a number of stable, multipotential clonal cell lines have been established from connective tissues, providing further *in vitro* evidence for the existence of pluripotent mesenchymal cells. Of these, the fibroblast-like C3H10T1/2 Clone 8 cell line is the most extensively studied. Originally established from early mouse embryos, C3H10T1/2 cells were first identified based on their sensitivity to density-dependent growth inhibition (Reznikoff *et al*., 1973). Subsequent studies have shown that 10T1/2 cells express multiple phenotypes when treated with 5-azacytidine, a nucleoside analog of cytidine (Constantinides *et al*., 1977; Taylor and Jones, 1979; Konieczny and Emerson, 1984). Specifically, the formation of striated muscle cells, adipocytes, and chondrocytes has been reported, although the chondrocyte phenotype was infrequently expressed (Taylor and Jones, 1979; Konieczny and Emerson, 1984).

Another multipotential clonal cell population, RCJ 3.1, was isolated from 21-day fetal rat calvaria by limiting dilution cloning (Grigoriadis *et al*., 1988, 1990; Aubin *et al*., 1993). When cultured under conditions that favor bone formation, i.e., in the presence of ascorbic acid and ß-glycerophosphate, and with the synthetic glucocorticoid dexamethasone, these cells differentiate in a time-dependent manner into multinucleated muscle cells, adipocytes, chondrocytes and osteoblasts. Interestingly, RCJ 3.1 cells do not respond to 5-azacytidine treatment in the same manner as C3H1OT1/2 cells, indicative of the complex, heterogeneous nature of multipotential mesenchymal cells. Neonatal rat calvarium is also the source of ROB-C26 cells, a clonal osteoblastic cell line which exhibits a markedly different capacity to differentiate along the various specialized connective tissue pathways (Yamaguchi and Kahn, 1991; Yamaguchi, 1995). For example, monolayer cultures express an osteoblastic phenotype, whereas myotube formation is observed in confluent cultures and adipocytes are formed with dexamethasone treatment.

Recently, two less characterized clonal cell lines have been described with pluripotent activity. W-20-17 is a bone marrow stromal cell line isolated from a mouse strain that expresses the osteoblastic phenotype when exposed to bone growth factors (see Table 1; Thies *et al*., 1992). RMD-1, a cell line derived from the skeletal muscle of a 20-day fetal rat, also undergoes chondrogenic differentiation when cultured with similar growth factors in agarose gels, but when left untreated, displays an undifferentiated mesenchymal cell-type morphology (Aikawa *et al*., 1996).

Transforming Growth Factor-ß Gene Superfamily: Effectors of Mesenchymal Cell Commitment and Differentiation

A variety of factors have been shown to modulate the commitment and differentiation of mesenchymal cells, including epithelial-mesenchymal interactions (Kosher and Church, 1975; Lash and Vasan, 1978; for review, see Hay,

Table 1. Regulation of cell differentiation in primary and clonal lines of mesenchymal stem cells by TGF-ß superfamily members.

Mesenchymal Cell Type	TGF-β Family Member	Induced Phenotype - Biological Marker(s)	Reference
Bone Marrow Stromal	BMP-2	Osteoblast - ALP, OP, OC	Rickard *et al*., 1994
	BMP-3	Osteoblast - ALP	Vukicevic *et al*., 1989
Periosteum-Derived	BMP-3	Osteoblast - ALP, PTH, Type I Col	Vukicevic *et al*., 1989
	TGF-β1	Chondrocyte - Type II Col	Izumi *et al*., 1992
	TGF-β1	Chondrocyte - PG, Type II Col	Iwasaki *et al*., 1993
Chick Limb Bud	BMP-2	Chondrocyte - Alcian	Duprez *et al*., 1996
	BMP-2	Chondrocyte - $^{35}SO_4$, Alcian, N-cad	Tyndall and Tuan, 1994, 1996
	BMP-3	Chondrocyte - $^{35}SO_4$, Alcian, Type II Col	Carrington *et al*., 1991
	BMP-4	Chondrocyte - $^{35}SO_4$, Alcian, Type II Col	Chen *et al*., 1991
	TGF-β1	Chondrocyte - Alcian	Leonard *et al*., 1991
	TGF-β1	Chondrocyte - PG, $^{35}SO_4$, Alcian, Type II Col	Kulyk *et al*., 1989
	TGF-β1	Chondrocyte - $^{35}SO_4$, Alcian, N-cad	Tyndall and Tuan, 1994, 1996
	TGF-β2	Chondrocyte - PG, $^{35}SO_4$, Alcian, Type II Col	Kulyk *et al*., 1989
	TGF-β3	Chondrocyte - $^{35}SO_4$, Alcian	Roark and Greer, 1994
Mouse Limb Bud	BMP-2	Chondrocyte - PG, $^{35}SO_4$, Alcian, Type II Col Osteoblast - ALP, PTH, BGP	Rosen *et al*., 1994
	TGF-β1	Chondrocyte - PG, LP, Type II Col	Chimal-Monroy and
	TGF-β2	Chondrocyte - PG, LP, Type II Col	De Leon, 1997
	TGF-β3	Chondrocyte - PG, LP, Type II Col	
ROB-C26	BMP-2	Osteoblast - ALP, PTH, OC	Yamaguchi *et al*., 1991b
	BMP-6	Osteoblast - ALP	Gitelman *et al*., 1995
W - 20 -17	BMP-2	Osteoblast - ALP, PTH, OC	Thies *et al*., 1992
RMD - 1	BMP-2	Chondrocyte - PG, $^{35}SO_4$, Toluidine, Types II & IX Col	Aikawa *et al*., 1996
C3H10T1/2	BMP-2	Osteoblast - ALP, PTH	Katagiri *et al*., 1990
Clone 8	BMP-2	Osteoblast - ALP, BGP Chondrocyte - Alcian, Type II Col Adipocyte - Oil Red O, ICN	Wang *et al*., 1993
	BMP-2	Chondrocyte - $^{35}SO_4$, Alcian, Type II Col	Denker *et al*., 1994a,b, 1995b
	BMP-7	Osteoblast - ALP Chondrocyte - Alcian Adipocyte - Oil Red O, GPDH	Asahina *et al*., 1996
	TGF-β1	Chondrocyte - $^{35}SO_4$, Alcian, Type II Col	Denker *et al*., 1995a

ALP: alkaline phosphatase activity; PTH: cAMP response to parathyroid hormone; OC: osteocalcin expression; OP: osteopontin expression; Col: collagen; BGP: bone Gla protein expression; PG: cartilage proteoglycan expression; LP: link protein expression; N-cad: N-cadherin expression; Alcian: Alcian blue staining foci/nodules; Toluidine: toluidine blue staining foci/nodules; $^{35}SO_4$: sulfate incorporation; Oil Red O: Oil Red O staining deposits; ICN: insulin-regulatable glucose transporter; GPDH: glycerophosphate dehydrogenase activity.

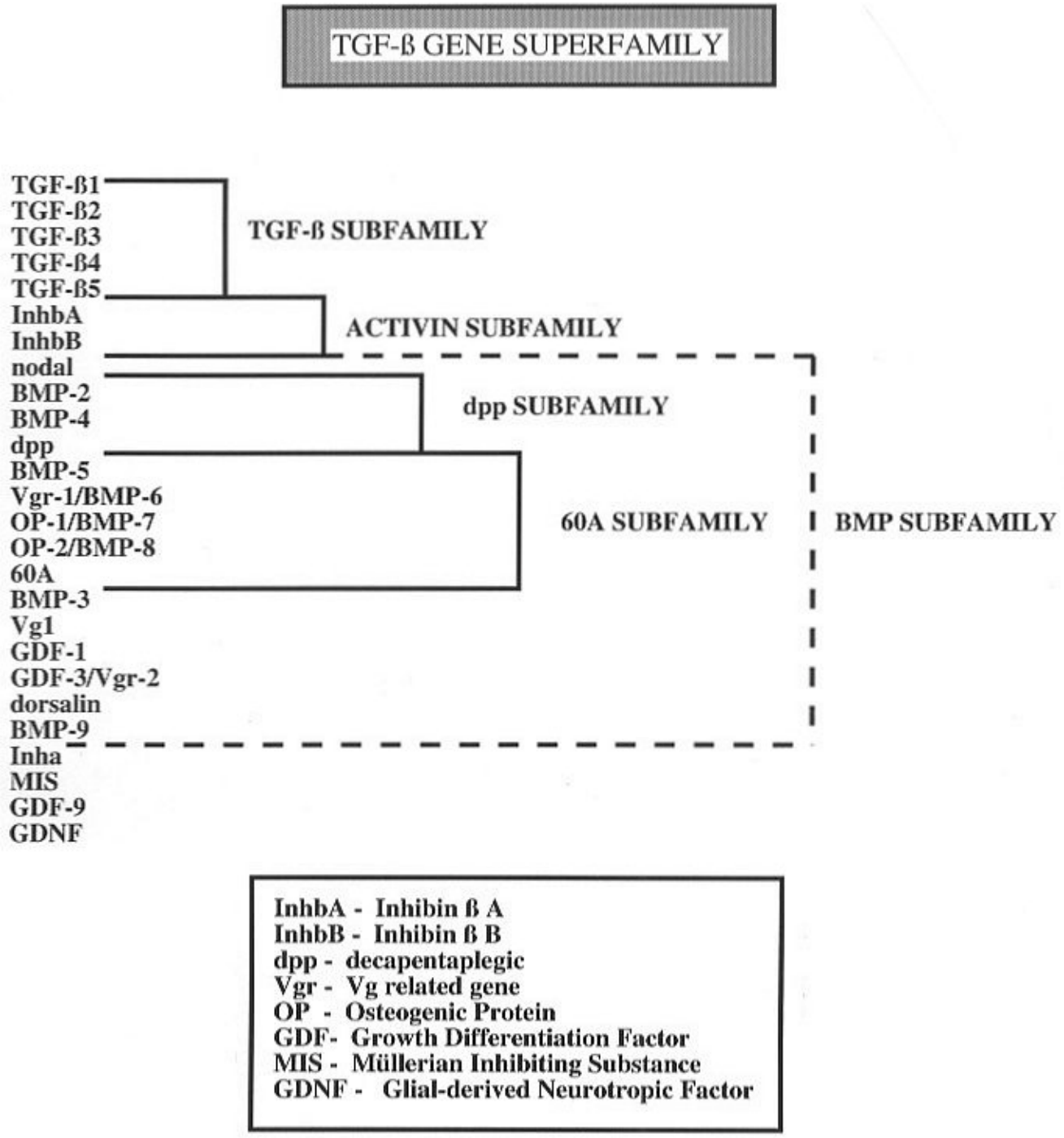


Figure 2. Structural relationship among members of the TGF-ß gene superfamily and subfamilies.

1981), oncogene expression (e.g., Ras) (Lu *et al.*, 1992) and growth factors. Members of the TGF-ß family of growth factors have been identified as key regulators of mesenchymal cell maturation. The TGF-ß family is composed of several closely related proteins termed TGF-ß1 through TGF-ß5 in addition to several homologous growth regulators (Massague, 1990; Jakowlew, 1993; Kim and Ballock, 1993; Kingsley, 1994). Family members exist as active hetero- and homodimers which share 30-40% sequence homology including conservation of seven of the nine cysteine residues in TGF-ß. They are ubiquitous in nature, having been identified in a multitude of tissues (i.e.,

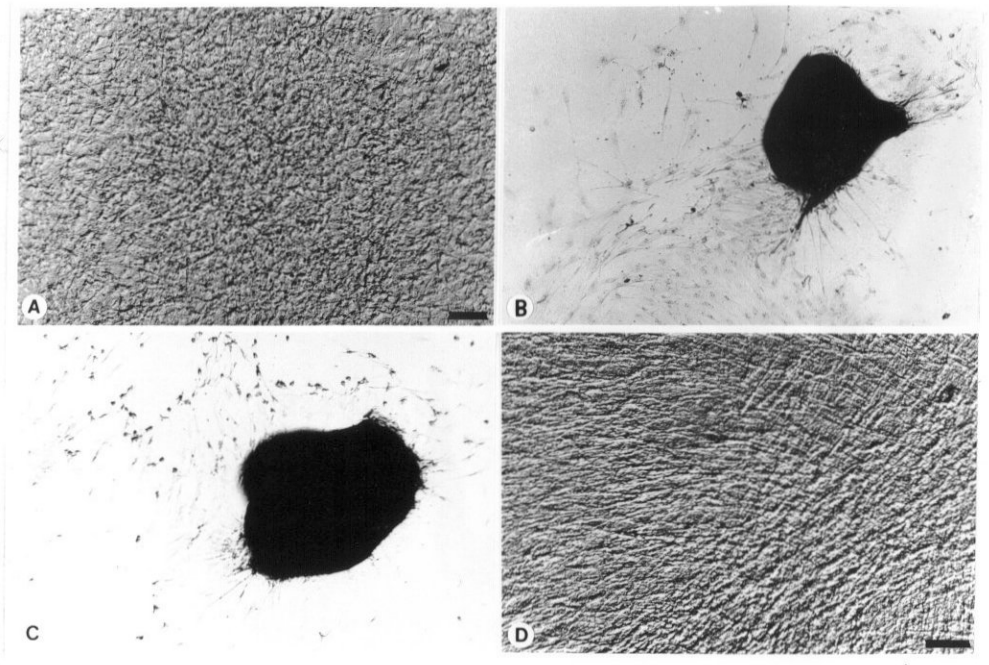


Figure 3. Spheroid formation in cultures of C3H10T1/2 cells treated with transforming growth factor-ß1 (TGF-ß1). Spheroid formation was observed in micromass cultures treated with either TGF-ß1 (**B**) or bovine bone extract (**C**), while untreated micromass (**A**) and treated monolayer cultures (**D**) did not form spheroids. Cultures were viewed using Hoffman modulation contrast optics. Bars = 100 µm (in A, B and C), and 67 µm (in D). From Denker *et al*. (1995a), with permission.

bone, kidney, liver, spleen and lung) and species (human, murine, porcine, chicken, *Drosophila*, *Xenopus*). A more extensive list of the TGF-ß gene superfamily and its subfamilies is shown in Figure 2.

Several members of the TGF-ß subfamily, in particular TGF-ß1, have been implicated in bone and cartilage development (Centrella *et al*., 1991, 1994). TGF-ß1, TGF-ß2, and TGF-ß3 are present in humans, with the highest levels of TGF-ß1 found in cartilage and bone (Seyedin *et al*., 1986; Jakowlew, 1993). TGF-ß1 was first purified from human platelets, human placenta, and bovine kidney (Assoian *et al*., 1983; Frolik *et al*., 1983; Roberts *et al*., 1983). TGF-ß2 was later purified from several sources, including porcine platelets, bovine bone, and simian kidney cells (Seyedin *et al*., 1985; Cheifetz *et al*., 1987; Hanks *et al*., 1988), while TGF-ß3 through -ß5 were identified by cDNA library screening (Jakowlew *et al*., 1988a,b; ten Dijke *et al*., 1988; Kondaiah *et al*., 1990). Numerous researchers have shown that TGF-ß subfamily members exhibit osteo-chondrogenic bioactivity *in vivo*. For example, TGF-ß1 has been localized in the mouse embryo in areas undergoing endochondral and intramembranous bone formation (Heine *et al*., 1987), and in sites of fracture repair such as the fracture hematoma, hard callus, and soft callus (Joyce *et al*., 1990a). In addition, further *in vivo* experiments by Bolander and colleagues demonstrated that subperiosteal injection of TGF-ß1 and TGF-ß2 into the rat femur induced proliferation, chondrogenesis and endochondral ossification (Joyce *et al*., 1990b).

Together with the various TGF-ß isoforms, members of the bone morphogenetic protein (BMP) subfamily have been shown to play a critical role in the development of mesodermal tissues such as bone and cartilage (Rosen and Thies, 1992). The presence of factors in bone matrix capable of stimulating bone and cartilage formation was first suggested by Marshall Urist through experiments in which ectopic bone formation resulted from intramuscular implantation of decalcified bone matrix (Urist, 1965). These factors, referred to as bone morphogenetic protein (Urist and Strates, 1971), were first purified from bovine long bones using hydroxyapatite chromatography (Urist *et al*., 1984). Multiple subfamily members were later isolated and cloned from bovine bone matrix (BMP-2 through BMP-7) (Wozney, 1988; Celeste *et al*., 1990), while still others were identified by cDNA library screening (BMP-8, BMP-9) (Ozkaynak *et al*., 1992; Celeste *et al*., 1994). *In vivo*, many BMPs have been implicated in skeletal development and repair, and have been shown to promote cartilage and bone formation both ectopically and in bone defects. Specifically, the expression of BMP-2, -4, and -6 has been described in the mouse embryo in regions undergoing cartilage and bone formation (Lyons *et al*., 1989, 1990), and BMP-2 and BMP-4 expression has been localized during fracture healing processes in rodents (Nakase *et al*., 1994; Bostrom *et al*., 1995). Wang and associates have shown that BMP-2, BMP-3 and BMP-4 stimulate ectopic bone and cartilage formation when implanted into rodents (Wang *et al*., 1988, 1990; Wozney, 1993), while BMP-7 induces bone formation in segmental bone defects in rabbits (Cook *et al*., 1994).

Given the *in vivo* evidence for both the localization of TGF-ß family members in skeletal tissues and their

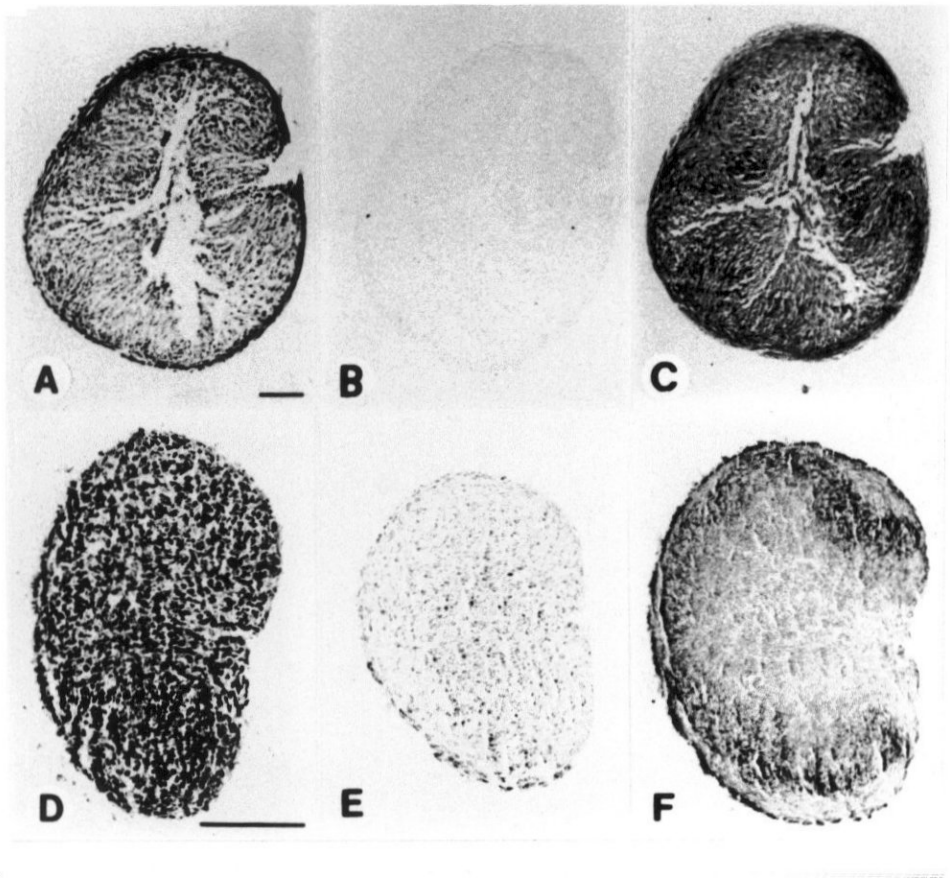


Figure 4. Immunohistochemical detection of cartilage matrix components in the C3H10T1/2 spheroids. Paraffin embedded spheroid sections were immunostained with monoclonal antibody CIIC1 to type II collagen (**A**), nonimmune serum (**B**), monoclonal antibody 8-A-4 to cartilage proteoglycan link protein (**D**), or nonimmune IgG (**E**), and visualized with horseradish peroxidase histochemistry. Serial sections were stained with Alcian blue (**C**, **F**). The presence of type II collagen (A), link protein (D), and Alcian blue positive staining material (C, F) in the spheroid is indicated. Sections were viewed with bright-field optics. Bars = 100 µm. Similar magnifications within (A-C) and (D-F). From Denker *et al*. (1995a), with permission.

putative involvement in bone and cartilage formation, it has been suggested that these factors act on undifferentiated mesenchymal cells to induce their differentiation along an osteogenic or chondrogenic pathway (Urist *et al*., 1983). Not surprisingly, an extensive body of recent work suggests that several members of the TGF-ß gene superfamily are intimately involved in the commitment and differentiation of primary mesenchymal stem cells and clonal cell lines into bone and cartilage-forming cells. A selection of these studies is shown in Table 1. In particular, two studies undertaken in our laboratory showed that 10T1/2 cells cultured at high cell density in the presence of TGF-ß1 and BMP-2 result exclusively in the expression of the chondrocyte-like phenotype (Denker *et al*., 1995a,b). This is noteworthy, since as mentioned earlier, the chondrocyte phenotype is the most infrequently expressed in this multipotential cell line (Taylor and Jones, 1979; Konieczny and Emerson, 1984).

In these studies, 10T1/2 cells were seeded at a high cell density (2 x 10^7 cells/ml) in a 10-20 µl drop, i.e., the micromass culture described by Ahrens *et al*. (1977) and San Antonio and Tuan (1986), and treated with 5 ng/ml recombinant human TGF-ß1. These cultures formed three-dimensional cellular aggregates or spheroids that exhibited cartilage nodule-like properties (Denker *et al*., 1995a). This was confirmed by several methods including histological and immunohistochemical detection of cartilage matrix components, as well as western immunoblot analysis and metabolic labeling experiments. A typical spheroid resulting from TGF-ß1 treatment of micromass cultures of 10T1/2 cells is shown in Figure 3, in addition to untreated micromass and treated monolayer cultures, which did not form spheroids. Sections of paraffin embedded spheroids immunostained with monoclonal antibodies to the cartilage matrix proteins, collagen type II and proteoglycan link protein, stained positively in contrast to non-immune control sections (Fig. 4). Similarly, positive staining was observed in serial sections stained with 1% Alcian blue, pH 1.0, confirming the presence of sulfated proteoglycans in the spheroid matrix. Western analysis of collagen synthesis by 10T1/2 cells indicated that TGF-ß1 treatment increased collagen synthesis in both micromass and monolayer cultures, but synthesis of type II collagen was detected only in TGF-ß1-treated micromass cultures in which spheroids had formed (not shown). TGF-ß1 was also shown to significantly affect the synthesis of sulfated glycosaminoglycans by 10T1/2 cells cultured in micromass. From 36-60 hours, the time corresponding to spheroid

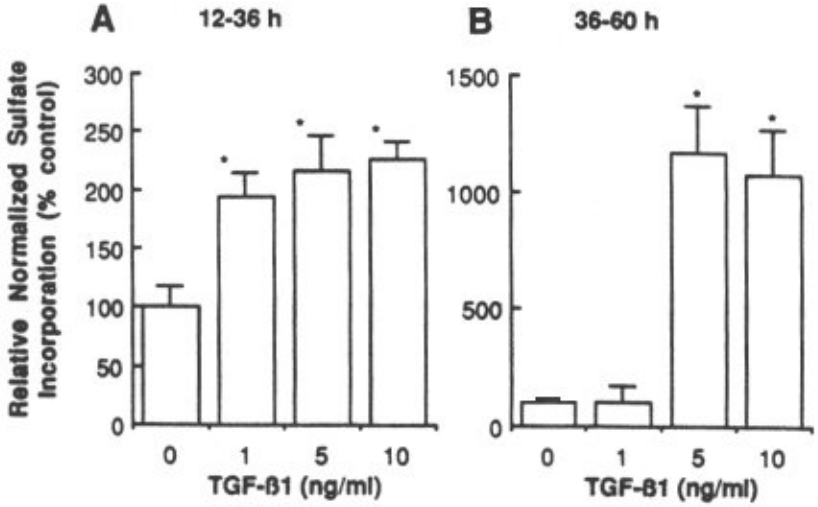


Figure 5. Effect of TGF-ß1 treatment on sulfated glycosaminoglycan synthesis in micromass cultures of C3H10T1/2 cells. Cells were metabolically labeled with [35-S]-sulfate (2.5 µCi/ml) and [3-H]-thymidine (2 µCi/ml) from 12-36 hours (**A**) and 36-60 hours (**B**). A dose-dependent increase in sulfate incorporation was observed in micromass cultures treated with TGF-ß1 from 36-60 hours, the time that coincides with spheroid formation. Values represent the mean (± standard deviation, S.D.) of normalized sulfate incorporation (ratio of [35-S]-sulfate to [3-H]-thymidine) (n = 5-6) expressed as a percentage of that in untreated control cultures. Asterisks indicates statistically a significant difference ($p < 0.001$) compared to corresponding untreated control. From Denker *et al*. (1995a), with permission.

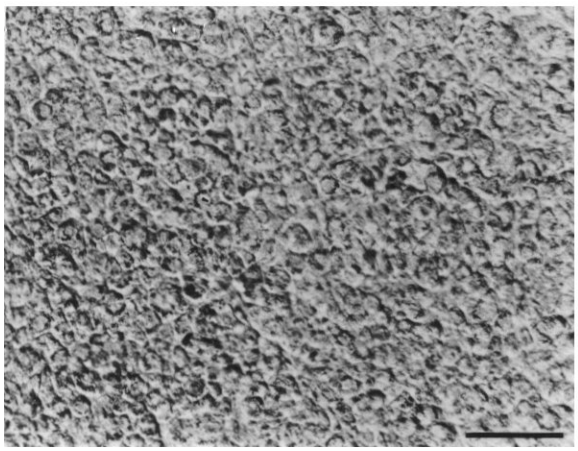
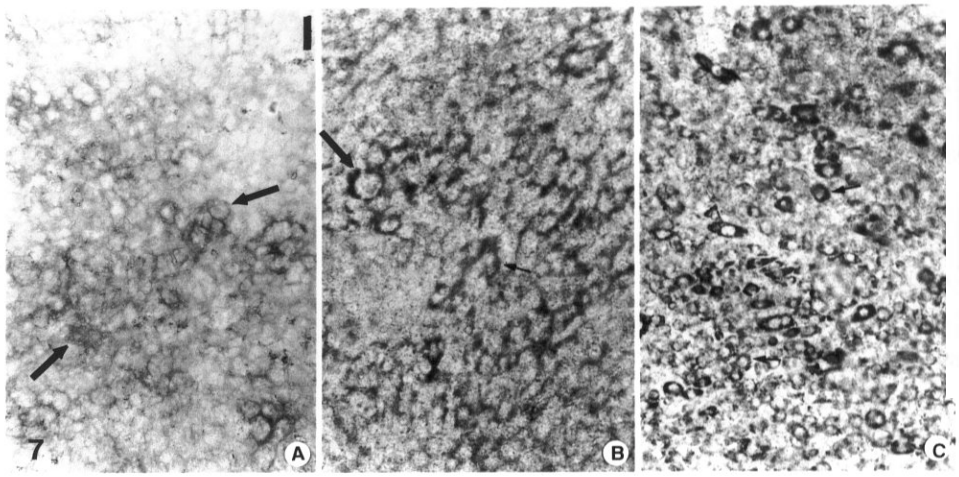


Figure 6. Appearance of chondrocyte-like morphology in cultures of C3H10T1/2 cells treated with BMP-2. Rounded cells with metachromatic borders were observed at six days. Viewed with Hoffman modulation contrast optics. Bar = 50 µm.

formation, a dose-dependent increase in relative [35-S]-sulfate incorporation (normalized to DNA synthesis with [3-H]-thymidine) was seen in micromass cultures treated with TGF-ß1 (0-10 ng/ml) (Fig. 5).

The treatment of micromass cultures of 10T1/2 cells with BMP-2 resulted in the formation of chondrocyte-like cells which elaborated an extracellular matrix exhibiting markers specific for cartilage (Denker *et al*., 1994a,b, 1995b). 10T1/2 cells cultured in micromass in the presence of BMP-2 (100 ng/ml) displayed a rounded, cobblestone-shaped morphology characteristic of chondrocytes by six days (Fig. 6), which was not observed in untreated monolayer and micromass cultures, or treated monolayer cultures (not shown). These cells stained positively with Alcian blue and with monoclonal antibodies to type II collagen and link protein (Fig. 7). The presence of sulfated proteoglycan aggregates in the extracellular matrix, considered an important marker of chondrogenesis, was confirmed by the pericellular staining pattern of Alcian blue dye and link protein antibodies. Type II collagen antibody staining was seen in both the cytoplasmic and extracellular region, suggesting the active synthesis and secretion of type II collagen into the matrix. A dose-dependent increase in relative [35-S]-sulfate incorporation (normalized to that of [3-H]-leucine) was also seen in micromass cultures of 10T1/2 cells treated with BMP-2 (Denker *et al*., 1995b).

The above findings clearly demonstrate that both TGF-ß1 and BMP-2 are capable of inducing cellular differentiation towards chondrogenesis in high density micromass cultures of 10T1/2 cells *in vitro*. Moreover, the ability of such growth factors to convert undifferentiated mesenchymal cells to functional connective tissue cells (as shown by our work and that of other investigators) may be of great clinical significance to the repair of connective



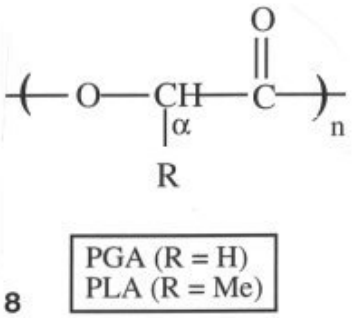


Figure 7 (left). Detection of cartilage matrix components in BMP-2 treated micromass cultures of C3H10T1/2 cells. After 12 days, cultures were fixed and stained with Alcian blue (**A**), and immunostained with monoclonal antibodies 8-A-4 and CIIC1 to link protein (**B**) and type II collagen (**C**), respectively. Alcian blue and link protein exhibited a pericellular staining pattern, indicating the presence of aggrecan complexes in the extracellular matrix of treated cultures. Type II collagen was detected in both the extracellular and cytoplasmic regions (arrows). Bar = 50 μm.

Figure 8 (right). Structure of the poly(α-hydroxy acids), poly(glycolic acid) and poly(lactic acid).

tissues like bone and cartilage. However, direct transplantation of mesenchymal-derived cells may not allow for proper localization and organization of the repair tissue. The use of an appropriate substrate for cell seeding and guided tissue regeneration, fundamental aspects of tissue engineering, are discussed in the following sections.

Tissue Engineering: An Alternative Approach To Connective Tissue Repair

Recent advances in the fields of biotechnology, cell and molecular biology, and biomaterials have led to the emergence of tissue engineering, an exciting new area of research focusing on the repair and replacement of functional mammalian tissues and organs (Langer and Vacanti, 1993). Tissue engineering was first defined at a workshop held in Lake Tahoe, California upon the recommendation of the National Science Foundation. The working definition as formulated is:

"The application of principles and methods of life sciences toward fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve tissue functions."

— Skalak *et al.* (1988)

Tissue engineering represents a promising therapy for the repair of connective tissues such as bone and cartilage, as current methods which involve primarily the use of tissue grafts are far from ideal. Autogenous grafts harvested from a patient's own tissue are biocompatible, but their use is limited due to a lack of tissue supply, and because of pain and morbidity which often develop at the donor site (Lane and Sandhu, 1987; Kenley *et al.*, 1993). Allogeneic grafts from donors other than the host are more plentiful, but present the risk of disease transmission, and may trigger an immune response to alloantigens, resulting in host rejection (Lane and Sandhu, 1987; Kenley *et al.*, 1993). Equally problematic is the fact that, regardless of origin, it is difficult to form three-dimensional constructs from existing tissue (Langer and Vacanti, 1993). A method currently being tested clinically is the grafting of autogenous chondrocytes which are expanded *in vitro* following harvest (Brittberg *et al.*, 1994, 1996). The long-term results of this procedure have yet to be fully described (Breinan *et al.*, 1997).

Biodegradable Polymer Scaffolds in Tissue Engineering

As a result of the inherent difficulties associated with tissue grafts, several biodegradable polymeric systems have been used as materials for the engineering of load-bearing biological tissues. These include polyesters (Elgendy *et al.*, 1993; Thomson *et al.*, 1995; Lo *et al.*, 1996), polyanhydrides (Lucas *et al.*, 1990), poly(orthoesters) (Solheim *et al.*, 1992a,b), polyurethanes (Nielsen *et al.*, 1992),

Table 2. Orthopaedic applications of PLA and PGA polymers {adapted from Athanasiou *et al*. (1996)}.

Material	Application	Results	Reference
PLA	Sutures in primate mandible	Minimal inflammatory response	Cutright *et al*., 1971
PLA	Fracture fixation of canine mandible	Tissue repair with no adverse host response	Getter *et al*., 1972
PGA-PLA	Bone repair of rat tibia	Little foreign body reaction	Nelson *et al*., 1977
PLA	Bone repair of sheep femur	Satisfactory tissue compatibility	Christel *et al*., 1983
PGA-PLA	Bone repair of rat tibia	No adverse host tissue responses	Hollinger, 1983
L-PLA	Fracture fixation in dogs and sheep	Well tolerated, increased cellular activity	Leenslag *et al*., 1987
PGA-PLA	Bone repair of rabbit calvarium	No adverse host tissue responses	Schmitz and Hollinger, 1988
PGA	Cytological apiration from human ankle fracture	PGA is an immunologically inert implant material	Santavirta *et al*., 1990
PGA	Fracture fixation of human pediatric elbow	No infection or foreign body reaction	Hope *et al*., 1991
L-PLA/DL-PLA	Bone fixation in rats	No inflammation or foreign body reaction	Majola *et al*., 1991
PLA	Articular defects in rabbits	Well tolerated, minimal inflammatory response	von Schroeder *et al*., 1991
PGA-PLA	Articular defects in rabbits	Good long-term compatibility	Athanasiou *et al*., 1992
PGA	Fracture fixation of rabbit femur	No contraindications for clinical application of PGA	Böstman *et al*., 1992
PGA	Fracture fixation of human hand	No allergic reactions	Kumta *et al*., 1992
L-PLA	Bone repair of rabbit femur	No inflammation or foreign body reaction	Matsusue *et al*., 1992
L-PLA	Fracture fixation of rabbit femur	No disturbance of bone growth	Miettinen *et al*., 1992
PGA-PLA	Fracture fixation of rabbit femur	Mild inflammatory response	Päivärinta *et al*., 1993
PGA	Fracture fixation of human pediatric elbow	No adverse clinical effects	Böstman *et al*., 1994
PLA	Fracture fixation of human ankle	Safe and effective, no complications	Bucholz *et al*., 1994
PGA	Bone repair of rat femur	No infection or adverse reactions	Ashammakhi *et al*., 1995
PGA-PLA	Fixation of calvarial bone grafts in rabbits	No adverse local inflammatory reactions	Eppley and Sadove, 1995

PGA: polyglycolic acid; PLA: polylactic acid; L-PLA: poly(L-lactic acid); DL-PLA: poly(DL-lactic acid).

and polycarbonates (Ertel *et al*., 1995) among others (Kimura, 1993). The most frequently investigated biodegradable implant materials are the poly(α-hydroxy acids), poly(glycolic acid) (PGA) and poly(lactic acid) (PLA) (Fig. 8). Also referred to as alpha polyesters, PGA and PLA degrade by hydrolytic scission into biological metabolites (i.e., glycolic acid and lactic acid, respectively) and have been approved for human use by the Food and Drug Administration (Hollinger and Battistone, 1986; Athanasiou *et al*., 1996). Initially used as resorbable sutures (Frazza and Schmitt, 1971; Kulkarni *et al*., 1971), both PGA and PLA offer several advantages over other materials with respect to design flexibility. For example, they can be easily processed into a variety of shapes and three-dimensional structures that more closely mimic the native extracellular environment (Hollinger and Battistone, 1986). Porosity can also be achieved using certain fabrication methods, allowing for essential nutrient transport between cells and for a high

Table 3. Application of PLA/PGA scaffolds for bone and cartilage cell seeding.

Materials	*Experimental Model*	*Results*	*Reference*
Bovine chondrocytes seeded onto PLGA fiber matrices	Cultured *in vitro* and implanted subcutaneously in nude mice	Cartilage formation in > 90% of implants indicated by gross examination and matrix components (PG and Type II collagen)	Cima *et al*., 1991
Bovine articular chondrocytes seeded onto polyglactin fibers	Cultured *in vitro* and implanted subcutaneously in nude mice	Cartilage formation indicated by gross examination and matrix components (PG and Type II collagen)	Vacanti *et al*., 1991
Bovine/human articular/costal chondrocytes seeded onto PGA & L-PLA matrices	Cultured *in vitro* and implanted subcutaneously in nude mice	*IN VITRO* - PGA exhibited chondrocyte-like phenotype and matrix (PG and Type II collagen); L-PLA - spindle shaped cells with little matrix *IN VIVO* - both PGA & L-PLA formed cartilage	Freed *et al*., 1993
Bovine periosteum-derived osteoblastic cells seeded onto PGA meshes	Cultured *in vitro* and implanted subcutaneously in nude mice	Early cartilage formation replaced later by bone tissue; rate of morphogenesis related to vascularity of implant site	Vacanti *et al*., 1993
Lapine articular chondrocytes seeded onto PGA scaffolds	Cultured *in vitro* and implanted in full-thickness defects in rabbit knee joints	Hyaline and fibrocartilage formation indicated by gross examination and matrix components (PG and Type II collagen)	Freed *et al*., 1994
Rodent osteoblasts seeded onto L-PLA, PLGA, and PGA films	Cultured *in vitro* for two weeks	Cells proliferated on polymer films and maintained osteoblastic phenotype as indicated by ALP activity	Ishaug *et al*., 1994
Bovine articular chondrocytes seeded onto PGA meshes	Cultured *in vitro* and implanted cranial defects in nude rats	Cartilage formation in 80% of experimental bony defects	Kim *et al*., 1994
Rodent calvarial osteoblasts seeded onto PLGA/HA composites	Cultured *in vitro* short-term	Cells adhered and migrated on composite substrates and maintained osteoblastic phenotype as detected by OC symbols	Attawia *et al*., 1995
Lapine perichondrocytes seeded onto D,D-L, PLA scaffolds	Cultured *in vitro* and implanted in osteochondral defects in femoral condyles of rabbits	Cartilage formation in 96% of implants indicated by gross examination and matrix components (PG and Type II collagen)	Chu *et al*., 1995
Lapine articular chondrocytes seeded onto PGA scaffolds	Cultured *in vitro* statically a closed bioreactor system system for 4 weeks	Cartilage formation indicated by gross examination and matrix components (PG and Type II collagen) - enhanced in closed bioreactor culture system	Dunkelman *et al*., 1995
Bovine articular chondrocytes seeded onto PGA and Vicryl (90% PGA: 10% PLA) meshwork	Cultured *in vitro* under both static and closed loop recirculation conditions for 5 weeks	Cells directly attached to polymer fibers assumed a fibroblast morphology while unattached cells displayed a rounded chondrocyte shape. Early increase in PG synthesis in closed loop vs. static culture system	Grande *et al*., 1997

PGA: polyglycolic acid; PLA: polylactic acid; L-PLA: poly(L-lactic acid); DL-PLA: poly(DL-lactic acid); PLGA: poly(D,L-lactide-co-glycolide); HA: hydroxyapatite; ALP: alkaline phosphatase; OC: osteocalcin; PG: cartilaginous proteoglycan.

Table 4. Application of PLA/PGA scaffolds for delivery of osteo-chondro-inductive factors.

Materials	*Experimental Model*	*Results*	*Reference*
Purified bovine BMP incorporated into	Implanted in cranial defects in monkeys	Bone formation at 16 weeks with unresorbed PLGA segments present between bone deposits	Ferguson *et al*., 1987
Purified human BMP incorporated into PLGA	Composites placed as onlay over femoral nonunion fracture gap	Solid fracture union in 100% of subjects	Johnson *et al*., 1988
Bovine BMP incorporated into PLA strips	Augmentation of spinal fusion in dogs	BMP/PLA implants associated with higher levels of bone mass production with unresorbed PLA fragments as late as 6 months	Lovell *et al*., 1989
PLA (MW 160 Da - 105 kDa) combined with semipurified BMP (PLA160/BMP - PLA105,000/BMP)	PLA/BMP composites implanted intramusculary in mice	Only PLA/650/BMP composites adsorbed and replaced by bone (after 3 weeks)	Miyamoto *et al*., 1992
Rabbit DBM powder sandwiched between PLGA disks	Implanted in calvarial defects in rabbits	Mature bone formation observed by 6 weeks	Kleinschmidt *et al*., 1993
Bone matrix extract derived from bovine cortical bone combined with PLGA	PLGA/BBE composites cultured *in vitro* (5 weeks) to assay growth factor release	60-75% biological activity released within 1 week	Meikle *et al*., 1993
PLGA combined with DBM and TGF-β1	Cultured *in vitro* to assay TGF-β1 release	Protein released from devices for > 600 hrs.; 80-90% TGF-β1 released retained activity	Gombotz *et al*., 1993
PLA (650 Da) and PLA-PEG copolymer combined with semi-purified BMP	PLA/BMP and PLA-PEG/BMP composites implanted intramuscularly in mice (3 weeks)	PLA-PEG/BMP composites fully absorbed and induced twice as much bone as PLA/BMP as PLA/BMP composites	Miyamoto *et al*., 1993
PLGA combined with DBM and TGF-β1 active TGF-β1	PLGA/DBM/TGF-β1 materials assayed for TGF-β1 release *in vitro* and implanted in rat calvarial defects	Biologically active TGF-β1 released *in vitro* for >300 hrs. but minimal bone formation observed *in vivo*	Gombotz *et al*., 1994
PLGA combined with rhBMP-2	PLGA/BMP-2 composites implanted in rat calvarial defects	Significant resorption of PLGA particles coupled with mature bone formation by 3 weeks	Kenley *et al*., 1994
PLGA combined with rhBMP-2	PLGA/BMP-2 composites implanted in segmental defects in rat femurs	PLGA/BMP-2 composites induced bone formation; higher doses of BMP-2 and smaller PLGA particle size result in greater stiffness and strength of new tissue	Lee *et al*., 1994
PLGA combined with bovine bone matrix extract	PLGA/BBE composites implanted in rabbit calvarial defects	Significant resorption of PLGA by 4 weeks with little osseous repair due to immune response	Meikle *et al*., 1994
PLA combined with DBM	PLA/DBM materials implanted subcutaneously in	Cartilage formation at 2 weeks later replaced by bone (4 weeks)	Saitoh *et al*., 1994
BMP encapsulated in PLGA	PLGA/BMP capsules assayed for BMP release *in vitro* and implanted subcutaneously in rats	80% of the BMP released *in vitro* by 1 week and ectopic bone formation at 3 weeks	Isobe *et al*., 1996

polyethylene glycol; DBM: demineralized bone matrix; BBE: bovine bone extract; rhBMP-2: recombinant human BMP-2; MW: molecular weight.

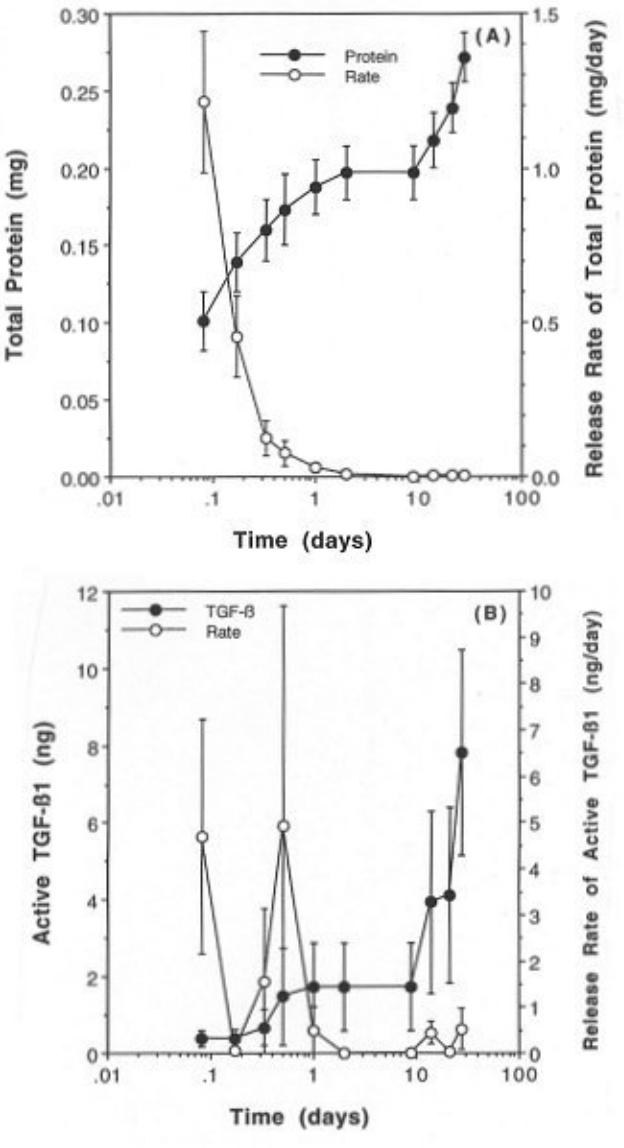


Figure 9. Release kinetics of total protein and active TGF-ß1 from PLLA scaffolds loaded with recombinant human TGF-ß1 (in bovine serum albumin, BSA, as a carrier protein) upon immersion in physiological saline at 37°C, with 5% CO_2. Total protein release was measured spectrophotometrically, while TGF-ß1 activity was assessed using a standard epithelial cell growth inhibition bioassay. An initial burst of total protein was followed by a gradual release over a four week period (**A**). Biologically active TGF-ß1 was released in a more sporadic fashion, characterized by an initial, rapid release superseded by a fluctuating pattern marked by apparently random bursts (**B**). Results represent the mean (± standard deviation, S.D.) of triplicate samples at each time point. From Nicoll *et al.* (1995), with permission.

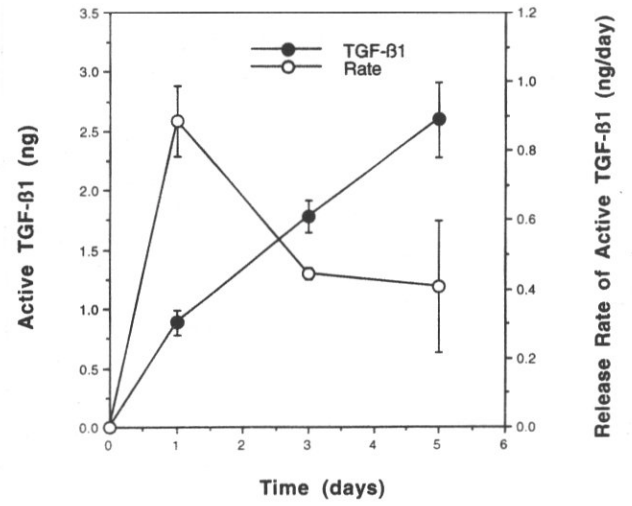


Figure 10. Release kinetics of the active TGF-ß1 from PLLA scaffolds upon immersion in serum-containing culture medium at 37°C, 5% CO_2 for 5 days. The release of TGF-ß1 in serum-containing medium was on the same order of magnitude as that measured in physiological saline (see Fig. 9), but following an initial rapid burst, a more controlled and stable release profile was observed. Results represent the mean (± S.D.) of triplicate samples at each time point. Adapted from Nicoll *et al.* (1995), with permission.

surface area to volume ratio to facilitate cell-polymer interactions (Cima *et al.*, 1991). Finally, the resorption rate may be controlled by altering the polymer constituents, as low molecular weight polymers and less crystalline, racemic forms (i.e., DL versus L) degrade more rapidly than their counterparts (Hollinger and Battistone, 1986). Several *in vivo* studies have shown PGA and PLA (and copolymers of the two polyesters) to be biocompatible in applications related to bone and cartilage tissue repair (i.e., fracture fixation and the repair of osseous or osteochondral defects). A chronological list of prominent *in vivo* studies examining the biocompatibility of PGA/PLA materials used in orthopaedic applications is shown in Table 2. Although there is a large body of work which suggests PGA and PLA to be biocompatible, some studies have shown these polyesters to produce toxic byproducts *in vitro* (Daniels *et al.*, 1992; Taylor *et al.*, 1994). Nevertheless, such *in vitro* experimental models do not take into account the hydrodynamic clearance of degradation products and the effects of physiological buffering (Athanasiou *et al.*, 1996).

Despite their demonstrated biocompatibility, PGA and PLA are not inherently chondrogenic and/or osteogenic. As such, many investigators have seeded primary, differentiated cells (i.e., chondrocytes and osteoblasts) isolated from existing tissue onto PGA/PLA scaffolds to

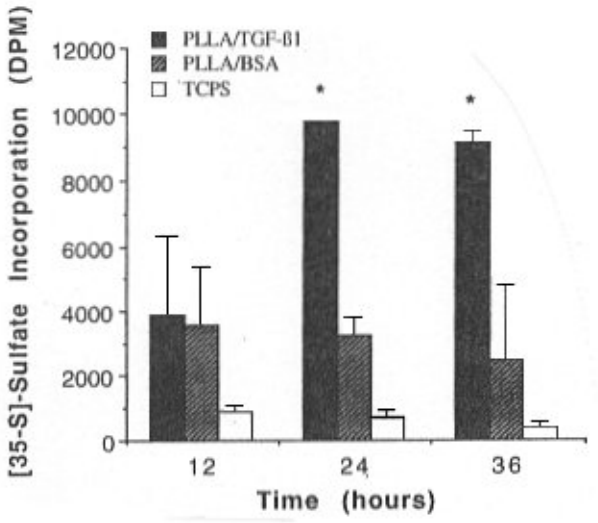


Figure 11. Estimation of sulfated glycosaminoglycan synthesis by C3H10T1/2 cells cultured on polymer substrates and metabolically labeled with [35-S]-sulfate (5 µCi/ml). Sulfated glycosaminoglycan synthesis by 10T1/2 cells was enhanced in PLLA disks loaded with TGF-ß1, with levels of sulfate incorporation three times greater than in PLLA/BSA disks, and thirteen times greater than in controls cultured on tissue culture polystyrene (TCPS). Results represent the mean (± S.D.) of triplicate samples at each time point. Asterisks denote significantly more incorporation ($p < 0.05$) than corresponding TCPS controls. From Nicoll *et al*. (1995), with permission.

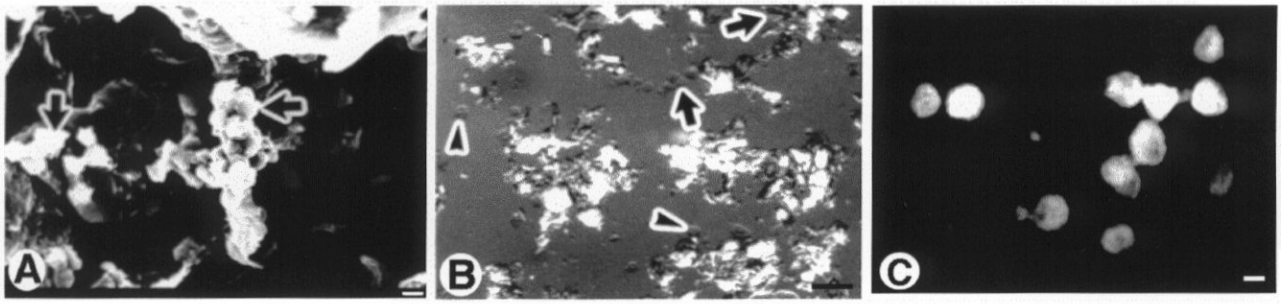


Figure 12. Morphology and chondrocytic nature of C3H10T1/2 cells cultured on PLLA disks loaded with TGF-ß1. (**A**) As observed by scanning electron microscopy at 3 days of culture, the seeded cells displayed a small, rounded morphology, resembling that of a chondrocyte-like phenotype. The cells proliferated throughout the porous PLLA network, and were observed to form discrete cellular clusters (arrows). Bar = 10 µm. From Nicoll *et al*. (1995), with permission. (**B**) Paraffin embedded sections viewed by Nomarski differential interference optics on day 7 of culture showed the presence of rounded, chondrocyte-like cells (arrows and arrowheads) distinct from the refractile PLLA polymeric network. Bar = 50 µm. From Nicoll *et al*. (1995), with permission. (**C**) Upon immunofluorescence staining for collagen type II (CIICI monoclonal antibody; see Denker *et al*., 1995), the seeded cells stained positively, with a pericellular pattern. Bar = 10 µm.

promote osteo-chondrogenic tissue formation and repair. A review of some of the more salient *in vitro* and *in vivo* studies is presented in Table 3. The pioneering studies by Robert Langer, Charles and Joseph Vacanti, and their colleagues were the first to demonstrate the potential use of resorbable poly(α-hydroxy acid) matrices as templates specifically for cartilage tissue regeneration (Cima *et al*., 1991; Vacanti *et al*., 1991; Freed *et al*., 1993, 1994; see Table 3). The basic experimental model involved harvesting primary chondrocytes from allogeneic or xenogeneic tissues, culturing these cells on polymeric scaffolds for a period of 2-3 weeks *in vitro*, and subsequently implanting the cell-polymer composites into athymic, nude mice or other host animals. After several weeks, cartilage tissue had formed, although in some cases, the neocartilage consisted of a mixture of both hyaline and fibrocartilage (Freed *et al*., 1994). More recently, improved viability and greater matrix deposition by seeded chondrocytes has been demonstrated using closed bioreactor systems (Dunkelman *et al*., 1995; Grande *et al*., 1997).

One of the more challenging problems encountered with such cell-polymer composites is inconsistent and incomplete bonding between the repair tissue generated from the scaffolds and the adjacent, endogenous tissue (Mow *et al*., 1991). In a clinical setting, the lack of integration with host tissue may ultimately compromise the success of the composite implant. To counter this difficulty, bioactive factors have been incorporated into PGA/PLA scaffolds to promote ingrowth with the surrounding tissue (Hollinger and Leong, 1996). Of these factors, demineralized

bone matrix, TGF-ß1, and forms of BMP are the most widely utilized. A partial listing of notable studies in which these bioactive factors were employed together with PGA/PLA carrier materials is shown in Table 4. The first experiments of this kind were conducted by Marshall Urist and associates, in which purified BMPs were incorporated into poly(D,L-lactide-co-glycolide) (PLGA) and PLA scaffolds and used to repair a variety of bone tissue anomalies including cranial defects, fracture non-unions, and spinal fusions (Ferguson *et al*., 1987; Johnson *et al*., 1988; Lovell *et al*., 1989). Enhanced bone induction was observed, although in some cases, unresorbed polymer fragments were still present at the implantation site. Later studies by Gombotz *et al*. (1993, 1994) showed that TGF-ß1 could be released in a biologically active form from comparable PLGA materials, while others demonstrated findings similar to those of Urist and co-workers using recombinant isoforms of BMP (Kenley *et al*., 1994; Lee *et al*., 1994). Although the release characteristics may vary greatly depending on the composition of the resorbable carrier, the release of BMPs from PLGA capsules has been reported to be as high as 80% of the total incorporated BMP after just one week (Isobe *et al*., 1996). Such rapid release kinetics may not be ideally suited for the long-term repair processes often associated with damaged connective tissues. A more controlled release afforded by surface eroding polymers such as polyanhydrides (Langer, 1990; Ron *et al*., 1993) may be more effective for this application, provided that the materials are fabricated in a porous array to facilitate cellular attachment and ingrowth. It should be noted that, with respect to bone tissue repair, Urist and co-workers have suggested that more rapidly degrading polymer carriers may be better suited for growth factor delivery (Ferguson *et al*., 1987; Lovell *et al*., 1989).

A recent *in vitro* study conducted in our laboratory employed both of the approaches discussed above. Biodegradable polymer scaffolds were used as substrates for cell seeding and as delivery vehicles for osteo-chondroinductive factors. Specifically, porous poly(L-lactic acid) (PLLA) matrices prepared by a solvent-casting particulate-leaching technique were loaded by direct adsorption with recombinant human TGF-ß1 (16.7 ng/mg PLLA) and seeded at high cell density with C3H10T1/2 cells (Nicoll *et al*., 1995). 10T1/2 cells were used in this study as a model for other, more clinically relevant mesenchymal stem cell populations (i.e., bone marrow or periosteum-derived cells), as a potential alternative to harvesting primary, differentiated cells from host tissue. TGF-ß1 was administered by adsorption loading onto the polymer scaffolds to facilitate its continuous release, as our previous studies on two-dimensional polystyrene with both TGF-ß1 and BMP-2 (described earlier) indicated that continual exposure to these growth factors was most optimal for maintaining the desired chondrogenic phenotype of 10T1/2 cells in culture. The periodic addition of TGF-ß1 to the culture medium or pretreatment prior to cell seeding were less effective. Moreover, we were also interested in assessing the ability of the matrices to deliver the growth factor not only to the cells seeded directly onto the polymers, but also to cells that might be in the surrounding environment as a model for delivery in an *in vivo* setting. TGF-ß1 released from the polymer scaffold was assayed for biological activity and for its ability to induce chondrogenesis in seeded 10T1/2 cells, as was previously observed in micromass cultures of 10T1/2 cells (Denker *et al*., 1995a). A sustained yet sporadic release of active TGF-ß1 was observed in polymer matrices immersed in physiological saline (Fig. 9), while a more controlled release was detected in serum-containing culture medium (Fig. 10). [35-S]-sulfate incorporation by 10T1/2 cells seeded onto PLLA/TGF-ß1 disks was significantly greater than in PLLA control disks loaded with bovine serum albumin or on tissue culture polystyrene (Fig. 11). Scanning electron microscopic analysis of 10T1/2 cells cultured on PLLA/TGF-ß1 disks revealed a small, rounded morphology, typical of chondrocytes by three days (Fig. 12A). Histological sections of 10T1/2 cells seeded onto PLLA/TGF-ß1 constructs showed similar cell clusters (Fig. 12B), which immunostained positively for collagen type II, characteristic of chondrocytes (Fig. 12C). These observations provide sufficient evidence for both the effective delivery of active TGF-ß1 from the PLLA carrier materials, and for the subsequent induction of the chondrocyte-like phenotype in seeded 10T1/2 cells.

Conclusions and Future Directions

The intensive research efforts conducted over the past several years strongly suggest that osteo-chondroinductive agents, namely members of the TGF-ß family of growth factors, regulate the differentiated phenotype of mesenchymal stem cells. These pluripotent cells represent a promising source of cellular material for the repair of bone and cartilage lesions. In combination with suitable biocompatible substrates such as resorbable polymers (e.g., PGA and PLA), biohybrid composites may be generated that offer several advantages over existing replacement therapies. Bioactive growth factors may also be incorporated into the cell-polymer constructs to promote the commitment and differentiation of seeded mesenchymal stem cells and to enhance the integration of composite implants with adjacent host tissue. As the precise function of growth factors is elucidated and as advances in polymer engineering give rise to novel polymeric systems, the repair of load-bearing skeletal tissues may one day involve the use of mesenchymal stem cells on a routine basis, given the likely shortage of viable donor tissue in the near future.

Acknowledgments

This work was supported in part by NIH HD 15822, HD 29937, ES 07005 and DE 11327. The authors wish to thank Mr. David S. Kreitzer for excellent assistance with scanning electron microscopy and photographic preparation, Mrs. Jeannine Fitzpatrick and Dr. Fang-Ju Lin for immunohistochemical analysis, and Ms. Lynn Stierle for assistance with manuscript preparation.

References

Aikawa T, Shirasuna K, Iwamoto M, Watatani K, Nakamura T, Ohura M, Yoshioka H, Matsuya T (1996) Establishment of bone morphogenetic protein 2 responsive chondrogenic cell line. J. Bone Min. Res. **11**: 544-553.

Ahrens PB, Solursh M, Reiter RS (1977) Stage-related capacity for limb chondrogenesis in cell culture. Dev. Biol. **60**: 69-82.

Asahina I, Sampath TK, Hauschka PV (1996) Human osteogenic protein-1 induces chondroblastic, osteoblastic, and/or adipocytic differentiation of clonal murine target cells. Exp. Cell Res. **222**: 38-47.

Ashammakhi N, Mäkelä EA, Vihtonen K, Rokkanen P, Törmälä P (1995) Effect of self-reinforced polyglycolide membranes on cortical bone: An experimental study in rats. J. Biomed. Mater. Res. **29**: 687-694.

Ashton BA, Allen TD, Howlett CR, Eaglesom CC, Hattori A, Owen M (1980) Formation of bone and cartilage by marrow stromal cells in diffusion chambers *in vivo*. Clin. Orthop. Rel. Res. **151**: 294-307.

Ashton BA, Eagelsom CC, Bab I, Owen ME (1984) Distribution of fibroblastic colony-forming cells in rabbit bone marrow and assay of their osteogenic potential by an *in vivo* diffusion chamber method. Calcif. Tiss. Int. **36**: 83-86.

Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB (1983) Transforming growth factor-ß in human platelets. J. Biol. Chem. **258**: 7155-7160.

Athanasiou KA, Schenck RC, Constantinides G, Sylvia V, Aufdemorte T, Boyan BD (1992) The use of biodegradable implants for repairing large articular cartilage defects in the rabbit. Trans. Orthop. Res. Soc. **17**: 172 (abstract).

Athanasiou KA, Niederauer GG, Agrawal CM (1996) Sterilization, toxicity, biocompatibility and clinical applications of polylactic/polyglycolic acid copolymers. Biomaterials **17**: 93-102.

Attawia MA, Herbert KM, Laurencin CT (1995) Osteoblast-like cell adherence and migration through 3-dimensional porous polymer matrices. Biochem. Biophys. Res. Commun. **213**: 639-644.

Aubin JE, Turksen K, Heersche JMN (1993) Osteoblastic cell lineage. In: Cellular and Molecular Biology of Bone. Noda M (ed.). Academic Press, San Diego, CA. pp. 1-45.

Bab I, Ashton BA, Gazit D, Marx G, Williamson MC, Owen ME (1986) Kinetics and diffusion of marrow stromal cells in diffusion chambers *in vivo*. J. Cell Sci. **84**: 139-151.

Bostrom MP, Lane JM, Berberian WS, Missri AA, Tomin E, Weiland A, Doty SB, Glaser D, Rosen VM (1995) Immunolocalization and expression of bone morphogenetic proteins 2 and 4 in fracture healing. J. Orthop. Res. **13**: 357-367.

Böstman O, Päivärinta U, Partio E, Manninen M, Vasenius J Majola A, Rokkanen P (1992) The tissue-implant interface during degradation of absorbable polyglycolide fracture fixation screws in the rabbit femur. Clin. Orthop. Rel. Res. **285**: 263-272.

Böstman O, Mäkelä EA, Södergard J, Hirvensalo E, Törmälä P, Rokkanen P (1994) Absorbable polyglycolide pins in internal fixation of fractures in children. J. Ped. Orthop. **13**: 242-245.

Breinan HA, Minas T, Hsu-H-P, Nehrer S, Sledge CB, Spector M (1997) Effect of cultured autologous chondrocytes on repair of chondral defects in a canine model. J. Bone Joint Surg. **79A**: 1439-1451.

Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L (1994) Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N. Engl. J. Med. **331**: 889-895.

Brittberg M, Nilsson A, Lindahl A, Ohlsson C, Peterson L (1996) Rabbit articular cartilage defects treated with autologous cultured chondrocytes. Clin. Orthop. Rel. Res. **326**: 270-283.

Bruder SB, Fink DJ, Caplan AI (1994) Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy. J. Cell. Biochem. **56**: 283-294.

Bucholz RW, Henry S, Henley MB (1994) Fixation with bioabsorbable screws for the treatment of fractures of the ankle. J. Bone Joint Surg. **76A**: 319-324.

Caplan AI (1970) Effects of the nicotinamide-sensitive teratogen 3-acetylpyridine on chick limb cells in culture. Exp. Cell Res. **62**: 341-355.

Caplan AI (1977) Muscle, cartilage and bone development and differentiation from chick limb mesenchymal cells. In: Vertebrate Limb and Somite Morphogenesis. Ede DA, Hinchliffe JR, Balls M (eds.). Cambridge University Press, New York. pp. 199-213.

Caplan AI (1991) Mesenchymal stem cells. J. Orthop. Res. **9**: 641-650.

Caplan AI, Koutroupas S (1973) The control of muscle and cartilage development in the chick limb: The role of differential vascularization. J. Embryol. Exp. Morph. **29**: 571-583.

Carrington JL, Chen P, Yanagishita M, Reddi AH (1991) Osteogenin (bone morphogenetic protein-3) stimulates cartilage formation by chick limb bud cells *in vitro*. Dev. Biol. **146**: 406-415.

Celeste AJ, Iannazzi JA, Taylor RC, Hewick RM, Rosen V, Wang EA, Wozney JM (1990) Identification of transforming growth factor ß family members present in bone-inductive protein purified from bovine bone. Proc. Natl. Acad. Sci. U.S.A. **87**: 9843-9847.

Celeste AJ, Song JJ, Cox K, Rosen V, Wozney JM (1994) Bone morphogenetic protein-9, a new member of the TGF-ß superfamily. J. Bone Min. Res. (Suppl.) **9**: S136.

Centrella M, McCarthy TL, Canalis E (1991) Transforming growth factor-ß and remodeling of bone. J. Bone Joint Surg. **73A**: 1418-1428.

Centrella M, Horowitz MC, Wozney JM, McCarthy TL (1994) Transforming growth factor-ß gene family members and bone. Endocrine Rev. **15**: 27-39.

Cheifetz S, Weatherbee JA, Tsang MLS, Anderson JK, Mole JE, Lucas R, Massague J (1987) The transforming growth factor system, a complex pattern of cross-reactive ligands and receptors. Cell **48**: 409-415.

Chen P, Carrington JL, Hammonds RG, Reddi AH (1991) Stimulation of chondrogenesis in limb bud mesoderm cells by recombinant human bone morphogenetic protein 2B (BMP-2B) and modulation by transforming growth factor ß1 and ß2. Exp. Cell Res. **195**: 509-515.

Chimal-Monroy J, De Leon LD (1997) Differential effects of transforming growth factors beta-1, beta-2, beta-3 and beta-5 on chondrogenesis in mouse limb bud mesenchymal cells. Internat. J. Dev. Biol. **41**: 91-102.

Christel PS, Vert M, Chabot F, Abols Y, Leary JL (1983) Polylactic acid for intramedullary plugging. In: Biomaterials and Biomechanics. Ducheyne P, Van der Perre G, Aubert AE (eds.). Elsevier Science Publishers, Amsterdam. pp. 1-6.

Chu CR, Coutts RD, Yoshioka M, Harwood FL, Monosov AZ, Amiel D (1995) Articular cartilage repair using allogeneic perichondrocyte-seeded biodegradable porous polylactic acid (PLA): A tissue-engineering study. J. Biomed. Mater. Res. **29**: 1147-1154.

Cima LG, Vacanti JP, Vacanti C, Ingber D, Mooney D, Langer R (1991) Tissue engineering by cell transplantation using degradable polymer substrates. J. Biomech. Eng. **113**: 143-151.

Cohen J, Lacroix P (1955) Bone and cartilage formation by periosteum. J. Bone Joint Surg. **37A**: 717-730.

Constantinides PG, Jones PA, Gevers W (1977) Functional striated muscle cells from non-myoblast precursors following 5-azacytidine treatment. Nature **267**: 364-366.

Cook SD, Baffes GC, Wolfe MW, Sampath TK, Rueger DC, Whitecloud TS (1994) The effect of recombinant human osteogenic protein-1 on healing of large segmental bone defects. J. Bone Joint Surg. **76A**: 827-838.

Cutright DE, Hunsuck EE, Beasley JD (1971) Fracture reduction using a biodegradable material, polylactic acid. J. Oral Surg. **29**: 393-397.

Daniels AE, Taylor MS, Andriano KP, Heller J (1992) Toxicity of absorbable polymers proposed for fracture fixation devices. Trans. Orthop. Res. Soc. **17**: 88.

Denker AE, Nicoll SB, Tuan RS (1994a) Induction of chondrogenesis by high density micromass culture of C3H10T1/2 cells using TGF-ß1 and BMP-2 treatment. Mol. Biol. Cell **5**: 314a (Abstract).

Denker AE, Nicoll SB, Tuan RS (1994b) Induction and characterization of chondrogenesis in multipotential mesenchymal cells. Matrix Biol. **14**: 373 (Abstract).

Denker AE, Nicoll SB, Tuan RS (1995a) Formation of cartilage-like spheroids by micromass cultures of murine C3H10T1/2 cells upon treatment with transforming growth factor-ß1. Differentiation **59**: 25-34.

Denker AE, Nicoll SB, Tuan RS (1995b) Induction of chondrogenesis in multipotential mesenchymal cells by high density micromass culture and BMP-2 treatment. Trans Orthop. Res. **21**: 465 (Abstract).

Dunkelman NS, Zimber MP, LeBaron RG, Pavelec R, Kwan M, Purchio AF (1995) Cartilage production by rabbit articular chondrocytes on polyglycolic acid scaffolds in a closed bioreactor system. Biotech. Bioeng. **46**: 299-305.

Duprez DM, Coltey M, Amthor H, Brickell PM, Tickle C (1996) Bone morphogenetic protein-2 (BMP-2) inhibits muscle development and promotes cartilage formation in chick limb bud cultures. Dev. Biol. **174**: 448-452.

Elgendy HM, Norman ME, Keaton AR, Laurencin CT (1993) Osteoblast-like cell (MC3T3-E1) proliferation on bioerodible polymers: An approach towards the development of a bone-bioerodible polymer composite material. Biomaterials **14**: 263-269.

Eppley BL, Sadove AM (1995) A comparison of resorbable and metallic fixation in healing of calvarial bone grafts. Plast. Reconstr. Surg. **96**: 316-322.

Erlebacher A, Filvaroff EH, Gitelman SE, Derynck R (1995) Toward a molecular understanding of skeletal development. Cell **80**: 371-378.

Ertel SI, Kohn J, Zimmerman MC, Parsons JR (1995) Evaluation of poly(DTH carbonate), a tyrosine-derived degradable polymer, for orthopaedic applications. J. Biomed. Mater. Res. **29**: 1337-1348.

Fell HB (1925) The histogenesis of cartilage and bone in the long bones of the embryonic fowl. J. Morphol. **40**: 417-459.

Fell HB (1932) The osteogenic capacity *in vitro* of periosteum and endosteum isolated from the limb skeleton of fowl embryos and young chicks. J. Anat. **66**: 157-180.

Ferguson D, Davis WL, Urist MR, Hurt WC, Allen EP (1987) Bovine bone morphogenetic protein (bBMP)

fraction-induced repair of craniotomy defects in the rhesus monkey (*Macaca speciosa*) Clin. Orthop. Rel. Res. **219**: 251-258.

Frazza EJ, Schmitt EE (1971) A new absorbable suture. J. Biomed. Mater. Res. Symp. **1**: 43-58.

Freed LE, Marquis JC, Nohria A, Emmanual J, Mikos AG, Langer R (1993) Neocartilage formation *in vitro* and *in vivo* using cells cultured on synthetic biodegradable polymers. J. Biomed. Mater. Res. **27**: 11-23.

Freed LE, Grande DA, Lingbin Z, Emmanual J, Marquis JC, Langer R (1994) Joint resurfacing using allograft chondrocytes and synthetic biodegradable polymer scaffolds. J. Biomed. Mater. Res. **28**: 891-899.

Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP (1968) Heterotopic transplants of bone marrow. Transplant. **6**: 230-247.

Frolik CA, Dart LL, Meyers CA, Smith DM, Sporn MB (1983) Purification and initial characterization of type beta transforming growth factor from human placenta. Proc. Natl. Acad. Sci. U.S.A. **80**: 3676-3680.

Gehris AL, Oberlender SA, Shepley KJ, Tuan RS, Bennett VD (1996) Fibronectin mRNA alternative splicing is temporally and spatially regulated during chondrogenesis *in vivo* and *in vitro*. Dev. Dynam. **206**: 219-230.

Gehris AL, Stringa E, Spina J, Desmond ME, Tuan RS, and Bennett VD (1997) The region encoded by the alternatively spliced exon IIIA in mesenchymal fibronectin appears essential for chondrogenesis at the level of cellular condensation. Dev. Biol. **190**: 191-205.

Getter L, Cutright DE, Bhashar SN, Augsburg JK (1972) A biodegradable intraosseous appliance in the treatment of mandibular fractures. J Oral Surg. **30**: 344-348.

Gilbert SF (1997) Developmental Biology, 5th Edn. Sinauer Associates, Sunderland, MA. Chapter 9, pp. 341-388.

Gitelman SE, Kirk M, Ye J-Q, Filvaroff EH, Kahn AJ, Derynck R (1995) Vgr-1/BMP-6 induces osteoblastic differentiation of pluripotential mesenchymal cells. Cell Growth Differ. **6**: 827-836.

Gombotz WR, Pankey SC, Bouchard LS, Ranchalis J, Puolakkainen PA (1993) Controlled release of TGF-beta 1 from a biodegradable matrix for bone regeneration. J. Biomater. Sci., Polymer Ed. **5**: 49-63.

Gombotz WR, Pankey SC, Bouchard LS, Phan DK, Puolakkainen PA (1994) Stimulation of bone healing by transforming growth factor-beta 1 released from polymeric or ceramic implants. J. Appl. Biomater. **5**: 141-150.

Grande DA, Halberstadt C, Naughton G, Schwartz R, Manji R (1997) Evaluation of matrix scaffolds for tissue engineering of articular cartilage grafts. J. Biomed. Mater. Res. **34**: 211-220.

Grigoriadis AE, Heersche JNM, Aubin JE (1988) Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. J. Cell Biol. **106**: 2139-2151.

Grigoriadis AE, Heersche JNM, Aubin JE (1990) Continuously growing bipotential and monopotential myogenic, adipogenic, and chondrogenic subclones isolated from the multipotential RCJ 3.1 clonal cell line. Dev. Biol. **142**: 313-318.

Hanks SK, Armour R, Baldwin JH, Maldonado F, Spiess J, Holley RW (1988) Amino acid sequence of the BSC-1 cell growth inhibitor (polyergin) deduced from the nucleotide sequence of the cDNA. Proc. Natl. Acad. Sci. U.S.A. **85**: 79-82.

Hay ED (1981) Collagen and embryonic development. In: Cell Biology of Extracellular Matrix. Plenum Press, New York. pp. 379-409.

Heine UI, Munoz EF, Flanders KC, Ellingsworth LR, Lam H-YP, Thompson NL, Roberts AB, Sporn MB (1987) Role of transforming growth factor-beta in the development of the mouse embryo. J. Cell Biol. **105**: 2861-2876.

Hollinger JO (1983) Preliminary report on the osteogenic potential of a biodegradable copolymer of polylactide (PLA) and polyglycolide (PGA). J. Biomed. Mater. Res. **17**: 71-82.

Hollinger JO, Battistone GC (1986) Biodegradable bone repair materials. Synthetic polymers and ceramic. Clin. Orthop. Rel. Res. **207**: 290-305.

Hollinger JO, Leong K (1996) Poly(α-hydroxy acids): Carriers for bone morphogenetic proteins. Biomaterials **17**: 187-194.

Hope PG, Williamson DM, Coates CJ, Cole WG (1991) Biodegradable pin fixation of elbow fractures in children. J. Bone Joint Surg. **73B**: 965-968.

Howlett CR, Cavé J, Williamson M, Farmer J, Ali SY, Bab I, Owen ME (1986) Mineralization in *vitro* cultures of rabbit marrow stromal cells. Clin. Orthop. Rel. Res. **213**: 251-263.

Ishaug SL, Yaszemski MJ, Bizios R, Mikos AG (1994) Osteoblast function on synthetic biodegradable polymers. J. Biomed. Mater. Res. **28**: 1445-1453.

Isobe M, Yamazaki Y, Oida S, Ishihara K, Nakabayashi N, Amagasa T (1996) Bone morphogenetic protein encapsulated with a biodegradable and biocompatible polymer. J. Biomed. Mater. Res. **32**: 433-438.

Iwasaki M, Nakata K, Nakahara H, Nakase T, Kimura T, Kimata K, Caplan AI, Ono K (1993) Transforming growth factor-ß1 stimulates chondrogenesis and inhibits osteogenesis in high density culture of periosteum-derived cells. Endocrinology **132**: 1603-1608.

Izumi T, Scully SP, Heydemann A, Bolander ME (1992) Transforming growth factor ß1 stimulates type II collagen expression in cultured periosteum-derived cells. J. Bone Miner. Res. **7**: 115-121.

Jakowlew SB (1993) Structural organization of the multiple TGF-ß genes. In: Growth Factors, Peptides, Receptors. Moody TW (ed.). Plenum Press, New York. pp. 103-113.

Jakowlew SB, Dillard PJ, Kondaiah P, Sporn MB, Roberts AB (1988a) Complementary deoxyribonucleic acid cloning of a novel transforming growth factor-ß messenger ribonucleic acid from chick embryo chondrocytes. Mol. Endocrinol. **2**: 747-755.

Jakowlew SB, Dillard PJ, Kondaiah P, Sporn MB, Roberts AB (1988b) Complementary deoxyribonucleic acid cloning of an mRNA encoding transforming growth factor-beta 4 from chick embryo chondrocytes. Mol. Endocrinol. **2**: 1186-1195.

Johnson EE, Urist MR, Fineraman GA (1988) Bone morphogenetic protein augmentation grafting of resistant femoral nonunions. Clin. Orthop. Rel. Res. **230**: 257-265.

Joyce ME, Jingushi S, Bolander ME (1990a) Transforming growth factor-ß in the regulation of fracture repair. Orthop. Clin. North. Amer. **21**: 199-209.

Joyce ME, Roberts AB, Sporn MB, Bolander ME (1990b) Transforming growth factor-ß1 and the initiation of chondrogenesis and osteogenesis in the rat femur. J. Cell Biol. **110**: 2195-2207.

Katagiri T, Yamaguchi A, Ikeda T, Yoshiki S, Wozney JM, Rosen V, Wang EA, Tanaka H, Omura S, Suda T (1990) The non-osteogenic pluripotent cell line, C3H10T1/2, is induced to differentiate into osteoblastic cells by recombinant human morphogenetic protein. Biochem. Biophys. Res. Commun. **172**: 295-299.

Kenley RA, Yim K, Abrams J, Ron E, Turek T, Marden LJ, Hollinger JO (1993) Biotechnology and bone graft substitutes. Pharm. Res. **10**: 1393-1401.

Kenley R, Marden L, Turek T, Jin L, Ron E, Hollinger JO (1994) Osseous regeneration in the rat calvarium using novel delivery systems for recombinant human bone morphogenetic protein-2 (rhBMP-2). J. Biomed. Mater. Res. **28**: 1139-1147.

Kim S-J, Ballock RT (1993) Cellular and molecular biology of transforming growth factor ß. In: Cellular and Molecular Biology of Bone. Noda M (ed.). Academic Press, San Diego, CA. pp. 97-129.

Kim WS, Vacanti CA, Upton J, Vacanti JP (1994) Bone defect repair with tissue-engineered cartilage. Plast. Reconstr. Surg. **94**: 580-584.

Kimura Y (1993) Biocompatible polymers. In: Biomedical Applications of Polymeric Materials. Tsurata T, Hayashi T, Kataoki K, Ishihara K, Kimura Y (eds.). CRC Press, Boca Raton, FL. pp. 163-189.

Kingsley D (1994) The TGF-ß superfamily: New members, new receptors, and new genetic tests of function in different organisms. Genes Dev. **8**: 133-146.

Kleinschmidt JC, Marden LJ, Kent D, Quigley N, Hollinger JO (1993) A multiphase system bone implant for regenerating the calvaria. Plast. Reconstr. Surg. **91**: 581-588.

Kondaiah P, Sands MJ, Smith JM, Fields A, Roberts AB, Sporn MB, Melton DA (1990) Identification of a novel transforming growth factor-ß mRNA in *Xenopus laevis*. J. Biol. Chem. **265**: 1089-1093.

Konieczny SF, Emerson Jr. CP (1984) 5-azacytidine induction of stable mesodermal stem cell lineages from 10T1/2 cells: Evidence for regulatory genes controlling determination. Cell **38**: 791-800.

Kosher RA, Church RL (1975) Stimulation of *in vitro* chondrogenesis by procollagen and collagen. Nature **258**: 327-330.

Kulkarni RK, Moore EG, Hegyeli AF, Leonard F (1971) Biodegradable poly(lactic acid) polymers. J. Biomed. Mater. Res. **5**: 169-181.

Kulyk WM, Rodgers BJ, Greer K, Kosher RA (1989) Promotion of embryonic limb cartilage differentiation by transforming growth factor-ß. Dev. Biol. **135**: 424-430.

Kumta SM, Spinner R, Leung PC (1992) Absorbable intramedullary implants for hand fractures. J. Bone Joint Surg. **74B**: 563-566.

Lane JM, Sandhu HS (1987) Current approaches to experimental bone grafting. Orthop. Clin. North Amer. **18**: 213-225.

Langer R (1990) New methods of drug delivery. Science **249**: 1527-1533.

Langer R, Vacanti JP (1993) Tissue engineering. Science **260**: 920-926.

Lash JW, Vasan NS (1978) Somite chondrogenesis *in vitro* stimulation by exogenous matrix components. Dev. Biol. **66**: 151-171.

Lee SC, Shea M, Battle MA, Kozitza K, Ron E, Turek T, Schaub RG, Hayes WC (1994) Healing of large segmental defects in rat femurs is aided by RhBMP-2 in PLGA matrix. J. Biomed. Mater. Res. **28**: 1149-1156.

Leenslag JW, Pennings AJ, Bos RRM, Rozema FR, Boering G (1987) Resorbable materials of poly(L-lactide): VI. Plates and screws for internal fracture fixation. Biomaterials **8**: 70-73.

Leonard CM, Fuld HM, Frenz DA, Downie SA, Massague J, Newman SA (1991) Role of transforming growth factor-ß in chondrogenic pattern formation in the embryonic limb: Stimulation of mesenchymal condensation and fibronectin gene expression by exogenous TGF-ß and evidence for endogenous TGF-ß-like activity. Dev. Biol. **145**: 99-109.

Lo H, Kadiyala S, Guggino SE, Leong KW (1996) Poly(L-lactic acid) foams with cell seeding and controlled-release capacity. J. Biomed. Mater. Res. **30**: 475-484.

Lovell TP, Dawson EG, Nilson OS, Urist MR (1989) Augmentation of spinal fusion with bone morphogenetic

protein in dogs. Clin. Orthop. Rel. Res. **243**: 266-274.

Lu Y, Raptis L, Anderson S, Corbley MJ, Zhou YC, Pross H, Haliotis T (1992) Ras modulates commitment and maturation of 10T1/2 fibroblasts to adipocytes. Biochem. Cell Biol. **70**: 1249-1257.

Lucas PA, Laurencin C, Syftestad GT, Domb A, Goldberg VM, Caplan AI, Langer RS (1990) Ectopic induction of cartilage and bone by water-soluble proteins from bovine bone using a polyanhydride delivery vehicle. J. Biomed. Mater. Res. **24**: 901-911.

Lyons KM, Pelton RW, Hogan BLM (1989) Patterns of expression of murine Vgr-1 and BMP-2a RNA suggest that transforming growth factor-ß-like genes coordinately regulate aspects of embryonic development. Genes Dev. **3**: 1657-1668.

Lyons KM, Pelton RW, Hogan BLM (1990) Organogenesis and pattern formation in the mouse: RNA distribution patterns suggest a role for bone morphogenetic protein-2A (BMP-2A). Development **109**: 833-844.

Majola A, Vainionpää S, Vihtonen K, Mero M, Vasenius J, Törmälä P, Rokkanen P (1991) Absorption, biocompatibility and fixation properties of polylactic acid in bone tissue: An experimental study in rats. Clin. Orthop. Rel. Res. **268**: 260-269.

Maniatopoulos C, Sodek J, Melcher AH (1988) Bone formation *in vitro* by stromal cells obtained from bone marrow of young adult rats. Cell Tiss. Res. **254**: 317-330.

Massague J (1990) The transforming growth factor beta family. Annu. Rev. Cell Biol. **6**: 597-641.

Matsusue Y, Yamamuro T, Oka M, Shikinami Y, Hyon S-H, Ikada Y (1992) *In vitro* and *in vivo* studies on bioabsorbable ultra-high-strength poly(L-lactide) rods. J. Biomed. Mater. Res. **26**: 1553-1567.

Meikle MC, Mak W-Y, Papaioannou S, Davies EH, Mordan N, Reynolds JJ (1993) Bone-derived growth factor release from poly(α-hydroxy acid) implants *in vitro*. Biomaterials **14**: 177-183.

Meikle MC, Papaioannou S, Ratledge TJ, Speight PM, Watt-Smith SR, Hill PA, Reynolds JJ (1994) Effect of poly DL-lactide-co-glycolide implants and xenogeneic bone matrix-derived growth factors on calvarial bone repair in the rabbit. Biomaterials **15**: 513-521.

Miettinen H, Makela EA, Vainio J, Rokkanen P, Tormala P (1992) The effect of an intramedullary self-reinforced poly-L-lactide (SR-PLLA) implant on growing bone with special reference to fixation properties: An experimental study on growing rabbits. J. Biomater. Sci. Polymer Edn. **3**: 443-450.

Miyamoto S, Takaoka K, Okada T, Yoshikawa H, Hashimoto J, Suzuki S, Ono K (1992) Evaluation of polylactic acid homopolymers as carriers for bone morphogenetic protein. Clin. Orthop. Rel. Res. **278**: 274-285.

Miyamoto S, Takaoka K, Okada T, Yoshikawa H, Hashimoto J, Suzuki S, Ono K (1993) Polylactic acid-polyethylene glycol block copolymer: A new biodegradable synthetic carrier for bone morphogenetic protein. Clin. Orthop. Rel. Res. **294**: 333-343.

Mow VC, Ratcliffe A, Rosewasser MP, Buckwalter JA (1991) Experimental studies on repair of large osteochondral defects at a high weight bearing area of the knee joint: A tissue engineering study. J. Biomech. Eng. **113**: 198-207.

Nakahara H, Bruder SP, Goldberg VM, Caplan AI (1990a) *In vivo* osteogenic potential of cultured cells derived from the periosteum. Clin. Orthop. Rel. Res. **259**: 223-232.

Nakahara H, Bruder SP, Haynesworth SE, Holecek JJ, Babar MA, Goldberg VM, Caplan AI (1990b) Bone and cartilage formation in diffusion chambers by subcultured cells derived from periosteum. Bone **11**: 181-188.

Nakase T, Nomura S, Yoshikawa H, Hashimoto J, Hirota S, Kitamura Y, Oikawa S, Ono K, Takaoka K (1994) Transient and localized expression of bone morphogenetic protein 4 messenger RNA during fracture healing. J. Bone Miner. Res. **9**: 651-659.

Nelson JF, Standford HG, Cutright DE (1977) Evaluation and comparisons of biodegradable substances as osteogenic agents. J Oral Surg. **43**: 836-843.

Nicoll SB, Denker AE, Tuan RS (1995) *In vitro* characterization of transforming growth factor-ß1-loaded composites of biodegradable polymer and mesenchymal cells. Cells Mater. **5**: 231-244.

Nielsen FF, Karring T, Gogolewski S (1992) Biodegradable guide for bone regeneration: Polyurethane membranes tested in rabbit radius defects. Acta Orthop. Scand. **63**: 66-69.

Nogami H, Urist MR (1970) A substratum of bone matrix for differentiation of mesenchymal cells into chondro-osseous tissues *in vitro*. Exp. Cell. Res. **63**: 404-410.

Nogami H, Urist MR (1974) Substrata prepared from bone matrix for chondrogenesis in tissue culture. J. Cell Biol. **62**: 510-519.

Oberlender SA, Tuan RS (1994) Expression and functional involvement of N-cadherin in embryonic limb chondrogenesis. Development **120**: 177-187.

Osdoby P, Caplan AI (1979) Osteogenesis in cultures of limb mesenchymal cells. Dev. Biol. **73**: 84-102.

Owen M (1988) Marrow stromal stem cells. J. Cell Sci. Suppl. **10**: 63-76.

Owens EM, Solursh M (1981) *In vitro* histogenic capacities of limb mesenchyme from various stage mouse embryos. Dev. Biol. **88**: 297-311.

Ozkaynak E, Schnegelsberg PNJ, Jin DF, Clifford GM, Warren FD, Drier EA, Oppermann H (1992) Osteogenic protein-2. J. Biol. Chem. **267**: 25220-25227.

Päivärinta U, Böstman O, Majola A, Toivonen T, Törmälä P, Rokkanen P (1993) Intraosseous cellular response

to biodegradable fracture fixation screws made of polyglycolide polylactide. Arch. Orthop. Trauma Surg. **112**: 71-74.

Prockop DJ (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. Science **276**: 71-74.

Reddi AH (1981) Cell biology and biochemistry of endochondral bone development. Coll. Rel. Res. **1**: 209-226.

Reddi AH, Huggins C (1972) Biochemical sequences in the transformation of normal fibroblasts in adolescent rats. Proc. Natl. Acad. Sci. U.S.A. **69**: 1601-1605.

Reznikoff CA, Brankow DW, Heidelberger C (1973) Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to post confluence inhibition of division. Cancer Res. **33**: 3231-3238.

Rickard DJ, Sullivan TA, Shenker BJ, Leboy PS, Kazhdan I (1994) Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2. Dev. Biol. **161**: 218-228.

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

Roberts AB, Anzano MA, Meyers CA, Widerman J, Blacher R, Pan Y-C, Stein S, Lehrman SR, Smith JM, Lamb LC, Sporn MB (1983) Purification and properties of type beta transforming growth factor from bovine kidney. Biochemistry **22**: 5692-5698.

Ron E, Turek T, Mathiowitz E, Chasin M, Hageman M, Langer R (1993) Controlled release of polypeptides from polyanhydrides. Proc. Natl. Acad. Sci. U.S.A. **90**: 4176-4180.

Rosen V, Thies RS (1992) The BMP proteins in bone formation and repair. Trends Genet. **8**: 97-102.

Rosen V, Nove J, Song JJ, Thies RS, Cox K, Wozney JM (1994) Responsiveness of clonal limb bud cell lines to bone morphogenic protein 2 reveals a sequential relationship between cartilage and bone cell phenotypes. J. Bone Miner. Res. **9**: 1759-1768.

Rosin A, Freiberg H, Zajicek G (1963) The fate of rat bone marrow, spleen and periosteum cultivated *in vivo* in the diffusion chamber, with special reference to bone formation. Exp. Cell Res. **29**: 176-187.

Saitoh H, Takata T, Nikai H, Shintani S-H, Ikada Y (1994) Effect of polylactic acid on osteoinduction of demineralized bone: Preliminary study of the usefulness of polylactic acid as a carrier of bone morphogenetic protein. J. Oral Rehab. **21**: 431-438.

Sampath TK, Nathanson MA, Reddi AH (1984) *In vitro* transformation of mesenchymal cells derived from embryonic muscle into cartilage in response to extracellular matrix components of bone. Proc. Natl. Acad. Sci. U.S.A. **81**: 3419-3423.

San Antonio JD, Tuan RS (1986) Chondrogenesis of limb bud mesenchyme *in vitro*: Stimulation by cations. Dev. Biol. **115**: 313-324.

Santavirta S, Konttinen YT, Saito T, Gronblad M, Partio E, Kemppinen P, Rokkanen P (1990) Immune response to polyglycolic acid implants. J. Bone Joint Surg. **72B**: 597-600.

Schmitz JP, Hollinger JO (1988) A preliminary study of the osteogenic potential of a biodegradable alloplastic-osteoinductive alloimplant. Clin. Orthop. Rel. Res. **237**: 245-255.

Searls RL (1965) An autoradiographic study of the uptake of S-35 sulfate during the differentiation of limb bud cartilage. Dev. Biol. **11**: 155-168.

Searls RL, Janners MY (1969) The stabilization of cartilage properties in the cartilage-forming mesenchyme of the embryonic chick limb. J. Expt. Zool. **175**: 365-376.

Sevitt S (1981) Bone Repair and Fracture Healing in Man. Churchhill Livingstone, Edinburgh, U.K. pp. 25-88.

Seyedin SM, Thomas TC, Thompson AY, Rosen DM, Piez KA (1985) Purification and characterization of two cartilage-inducing factors from bovine demineralized bone. Proc. Natl. Acad. Sci. U.S.A. **82**: 2267-2271.

Seyedin SM, Thompson AY, Bentz H, Rosen DM, McPherson JM, Conti A, Siegel NR, Galluppi GR, Piez KA (1986) Cartilage-inducing factor-A. Apparent indentity to transforming growth factor-beta. J. Biol. Chem. **261**: 5693-5695.

Skalak R, Fox CF, Fung YC (1988) Preface. In: Tissue Engineering. Skalak R, Fox CF (eds.). Alan R. Liss, New York. pp. xix-xxi.

Solheim E, Pinholt EM, Andersen R, Bang G, Sudmann E (1992a) The effect of a composite of polyorthoester and demineralized bone on the healing of large segmental defects of the radius in rats. J. Bone Joint Surg. **74A**: 1456-1463.

Solheim E, Pinholt EM, Bang G, Sudmann E (1992b) Regeneration of calvarial defects by a composite of bioerodible polyorthoester and demineralized bone in rats. J. Neurosurg. **76**: 275-279.

Stringa E, Tuan RS (1996) Chondrogenic cell subpopulation of chick embryonic calvarium: Isolation by peanut agglutinin affinity chromatography and *in vitro* characterization. Anat. Embryol. **194**: 427-437.

Taylor SM, Jones PA (1979) Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell **17**: 771-779.

Taylor MS, Daniels AU, Andriano KP, Heller J (1994) Six bioabsorbable polymers: *in vitro* toxicity of accumulated degradation products. J. Appl. Biomater. **5**: 151-157.

ten Dijke P, Hanson P, Iwata KK, Pieler C, Foulkes JG (1988) Identification of a new member of the transforming growth factor-ß gene family. Proc. Natl. Acad. Sci. U.S.A. **85**: 4715-4719.

Tenenbaum HC, Heersche JMN (1982) Differentia-

tion of osteoblasts and formation of mineralized bone *in vitro*. Calcif. Tiss. Int. **34**: 76-79.

Tenenbaum HC, Heersche JMN (1986) Differentiation of osteoid-producing cells *in vitro*: Possible evidence for the requirement of a micro environment. Calcif. Tiss. Int. **38**: 262-267.

Terashima Y, Urist MR (1977) Chondrogenesis in outgrowths of muscle tissue onto modified bone matrix in tissue culture. Clin. Orthop. Rel. Res. **127**: 248-256.

Thies RS, Bauduy M, Ashton BA, Kurtzberg L, Wozney JM, Rosen V (1992) Recombinant human bone morphogenetic protein-2 induces osteoblastic differentiation in W-20-17 stromal cells. Endocrinology **130**: 1318-1324.

Thomson RC, Yaszemski MJ, Powers JM, Mikos AG (1995) Fabrication of biodegradable polymer scaffolds to engineer trabecular bone. J. Biomater. Sci. Polymer Edn. **7**: 23-38.

Tuan RS, Turchi DM, Kreitzer DS (1991) Polylysine stimulation of ectopic cartilage formation. Cells Mater. **1**: 157-170.

Tuan RS, McBride S, Stringa E (1996) Identification and isolation of cells with chondrogenic potential from chicken embryonic muscle. Trans. Orthop. Res. Soc. **21**: 578 (abstract).

Tyndall WA, Tuan RS (1994) Involvement of N-cadherin mediated cell adhesion in TGF-ß1/BMP-2 stimulation of limb mesenchymal chondrogenesis. Mol. Biol. Cell **5**: 103A (abstract).

Tyndall WA, Tuan RS (1996) Effect of TGF-ß1 and BMP-2 on limb mesenchyme chondrogenesis *in vitro*: Modulation of N-cadherin and catenin association. Trans. Orthop. Res. Soc. **21**: 179 (abstract).

Urist MR (1965) Bone: Formation by autoinduction. Science **150**: 893-899.

Urist MR (1984) Purification of bovine bone morphogenetic protein by hydroxyapatite chromatography. Proc. Natl. Acad. Sci. U.S.A. **81**: 371-375.

Urist MR, McLean FC (1952) Osteogenic potency and new bone formation by induction in transplants to the anterior chamber of the eye. J. Bone Joint Surg. **34A**: 443-476.

Urist MR, Strates BS (1971) Bone morphogenetic protein. J. Dent. Res. Suppl. **50**: 1392-1406.

Vacanti CA, Langer R, Schloo B, Vacanti JP (1991) Synthetic polymers seeded with chondrocytes provide a template for new cartilage formation. Plast. Reconstr. Surg. **88**: 753-759.

Vacanti CA, Kim WS, Upton J, Vacanti MP, Mooney D, Schloo B, Vacanti JP (1993) Tissue-engineered growth of bone and cartilage. Transplant. Proc. **25**: 1019-1021.

von Schroeder HP, Kwan M, Amiel D, Coutts RD (1991) The use of polylactic acid matrix and periosteal grafts for the reconstruction of rabbit knee articular defects. J. Biomed. Mater. Res. **25**: 329-339.

Vukicevic S, Luyten FP, Reddi AH (1989) Stimulation of the expression of osteogenic and chondrogenic phenotypes *in vitro* by osteogenin. Proc. Natl. Acad. Sci. U.S.A. **86**: 8793-8797.

Wang EA, Rosen V, Cordes P, Hewick RM, Kriz MJ, Luxenberg DP, Sibley BS, Wozney JM (1988) Purification and characterization of other distinct bone-inducing factors. Proc. Natl. Acad. Sci. U.S.A. **85**: 9484-9488.

Wang EA, Rosen V, D'Alessandro JS, Bauduy M, Cordes P, Harada T, Israel DI, Hewick RM, Kerns KM, LaPan P, Luxenberg DP, McQuaid D, Moutsatsos IK, Nove J, Wozney JM (1990) Recombinant human bone morphogenetic protein induces bone formation. Proc. Natl. Acad. Sci. U.S.A. **87**: 2220-2224.

Wang EA, Israel DI, Kelly S, Luxenberg DP (1993) Bone morphogenetic protein-2 causes commitment and differentiation in C3H10T1/2 and 3T3 cells. Growth Factors **9**: 57-71.

Wozney JM (1993) Bone morphogenetic proteins and their gene expression. In: Cellular and Molecular Biology of Bone. Noda M (ed.). Academic Press, San Diego, CA. pp. 131-167.

Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Hriz RW, Hewick RM, Wang EA (1988) Novel regulators of bone formation: Molecular clones and activities. Science **242**: 1528-1534.

Yamaguchi A (1995) Regulation of differentiation pathway of skeletal mesenchymal cells in cell lines by transforming growth factor-ß superfamily. Sem. Cell Biol. **6**: 165-173.

Yamaguchi A, Kahn AJ (1991) Clonal osteogenic cell lines express myogenic and adipocytic developmental potential. Calcif. Tiss. Int. **49**: 221-225.

Yamaguchi A, Katagiri T, Ikeda T, Wozney JM, Rosen V, Wang EA, Kahn AJ, Suda T, Yoskiki S (1991) Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation *in vitro*. J. Cell Biol. **113**: 681-687.

Discussion with Reviewers

R. Langer: The authors used both the terms of "mesenchymal stem cells" and "mesenchymal cells." Is the second term meant to be synonymous with the first?

Authors: Mesenchymal cells refer to cells which, during tissue morphogenesis, act in an unconnected manner and are migratory. Operationally, these do not need to be "stem" cells, i.e., capable of giving rise to multiple tissue types. On the other hand, there exists one or more mesenchymal populations that persist into adult life of the organism and possess multipotential differentiation characteristics, and

are operationally referred to as “mesenchymal stem cells.” The appropriate usage of these terms has been followed in the manuscript.

R. Langer: The authors have presented their study of the effects of TGF-ß1 incorporation into a PLLA polymer support on 10T1/2 cells. How is the “incorporation”/ ”loading” done? Please clarify the proposed benefits of this system over periodically adding TGF-ß1 directly to the cell culture medium or first using the growth factor to differentiate the 10T1/2 cells to chondrocyte-like cells in traditional cell culture prior to seeding the polymer support? Were these benefits realized? Also, please comment on the desirability of the rapid release of incorporated BMP and TGF-ß1 for the proposed application, and consider presenting the release data for TGF-ß1 into serum-containing culture medium.

Authors: In previous studies (e.g., Denker *et al*., 1995a), we have observed that continuous exposure of 10T1/2 cells to TGF-ß1 is most optimal for chondro-induction. The periodic addition of TGF-ß1 to the culture medium or pretreatment prior to cell seeding were less effective. For this reason, TGF-ß1 was administered by direct adsorption loading onto the polymer scaffolds to facilitate its continuous release. Moreover, we were also interested in assessing the ability of the matrices to deliver the growth factor not only to the cells seeded directly onto the polymers, but also to cells that might be in the surrounding environment as a model for delivery in an *in vivo* setting. Our initial findings indeed support the induction of the chondrogenic phenotype in these cultures. In the presence of physiological saline, growth factors were released rapidly; however, the release became more sustained in the presence of serum-containing culture medium (Fig. 10), suggesting that such a release profile, although by no means ideal, is capable of inducing chondrogenic differentiation. Future studies aim to further optimize growth factor release with respect to chondrogenesis.