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Tissue Engineering of Bone

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1. BACKGROUND

With the progressive aging of the population, the need for functional tissue substitutes is increasing. Organ transplantation and mechanical devices have revolutionized medical practice but have limitations. Skeletal tissue loss due to congenital defects, disease, and injury is currently treated by autologous tissue grafting, a method limited by the availability of the host tissue, harvesting difficulties, donor site morbidity, and the clinician's ability to contour delicate 3D shapes [Gross, 2003]. However, autologous grafts are ideal implants as they provide an (i) osteo*con*ductive scaffold (i.e., an environment in which cells can thrive), (ii) osteo*in*ductive growth factors, which are stored in the graft and released during osteoclastic resorption and kick-start the osteogenic process, and (iii) autologous cells, a viable component capable to (re)generate bone tissue and facilitate tissue integration at the implantation site. The generation of autologous bone grafts in vitro, avoiding the harvest of autologous tissue at a second anatomic location, is the ultimate goal in bone tissue engineering. Consequently, scientific strategies utilize and integrate all three components, (i) the scaffold, (ii) the osteoinductive factors, and (iii) the cells, to meet the gold standard for implants, the autologous graft.

Osteoconductive scaffolds facilitate cell attachment and tissue development, and they biodegrade in parallel with the accumulation of tissue components. Therefore, they initially provide a structural and logistical template and degrade matching the rate of bone deposition. The osteoconductive environment is essential for promoting orderly tissue regeneration and in particular benefits from appropriate geometry [Abe et al., 1982; Chu et al., 1995; Caplan et al., 1997; Schaefer et al., 2000; Hunziker, 2002]. Scaffold structure determines the transport of nutrients, metabolites, and regulatory molecules to and from the cells, and pore sizes and interconnectivity influence the geometry of the engineered tissue. These constraints directly impact the uniform distribution of cells throughout the scaffold. Optimized interconnected porosity facilitates cell colonization throughout the construct and is the requirement for fully viable implants. The importance of scaffold geometry has been demonstrated for some porous biodegradable polymers, where new tissue formation has been restricted to a superficial and only 200-μm-thick layer of calcified tissue [Crane et al., 1995; Ishaug et al., 1997].

2. 3D SILK SCAFFOLDS

2.1. Silk as a Biomaterial

The chemical nature of the biomaterial critically affects tissue formation. Physiologically, collagen type I makes up to 90% of the total protein content as present in bone and—following a biomimetic strategy—this biomaterial should be considered the ideal material. A problem in using natural collagen type I is the inadequate biodegradation of this fibrous polymer, resulting in insufficient mechanical properties for bone tissue engineering and a collapse of the engineered tissue resulting

in unconnected calcified and unorganized bone clusters [Meinel et al., 2004b]. The biodegradation of collagen type I can be adapted through cross-linkage of the molecules, thereby matching the requirements for bone implants. However, the tissue reaction to cross-linked collagen scaffolds was quite unpredictable and resulted in spontaneous calcification, cytotoxic effects, and scar formation around the implant [van Luyn et al., 1992]. Spontaneous calcification might in particular result in problems for the engineering of osteochondral plugs, when the formation of pure cartilaginous tissue is impaired by the uncontrolled, and not cell-mediated, mineralization. These remarkable differences in cytotoxicity were connected to residual agents as a consequence of processing and the cross-linking agents used [van Luyn et al., 1992, van Wachem et al., 2001].

Among the available natural fibers, silks do have the strongest mechanical properties and even rival the best synthetic high-performance fibers, such as Kevlar, in overall performance (energy absorbed to break) [Cunniff et al., 1994]. The mechanical and thermal properties of dragline silk from the spider *Nephila claviceps* have previously been characterized with reference to silkworm silk [Cunniff et al., 1994]. The best properties of the native fibers collected and tested at quasi-static rates were 60 GPa and 2.9 GPa for initial modulus and ultimate tensile strength, respectively. Based on microscopic evaluations of knotted single fibers, no evidence of kink-band failure on the compressive side of a knot curve was observed [Cunniff et al., 1994]. Synthetic high-performance fibers fail by this mode even at relatively low stress levels; this is a major limitation with synthetic fibers in many applications. Furthermore, silks are mechanically stable up to almost 200 °C in dynamic mechanical evaluations [Cunniff et al., 1994]. In terms of material properties, silks also provide a range of mechanical features that suggest future applications in many different biomaterials needs. Table 13.1 provides some comparisons of the mechanical properties of silks (spider dragline and silkworm), collagen, cross-linked collagen scaffolds, and scaffolds prepared from a synthetic polymer, polylactic acid.

Table 13.1. Comparative mechanical properties of some key fibrous biomaterials for scaffolds.

Materials	Ultimate tensile Str. (mPa)	% Strain at failure	Modulus (MPa)	Reference
Collagen[1]	0.9–7.4	24.1–68.0	1.8–46.0	Pins et al. (1997)
Collagen (cross-linked)[2]	46.8–71.5	11.2–15.6	383–767	Pins et al. (1997)
Collagen[3]	—	—	1820–11,900	Cusack and Miller (1979)
Dragline silk[4]	200–2900	9–39	2000–60,000	Cunniff et al. (1994)
Silkworm silk[5]	600	15–35	5000	Cunniff et al. (1994)
L-PLA[6]	28–50	2.0–6.0	1200–3000	Engelberg and Kohn (1991)

[1] Collagen—tested after stretching from 0 up to 50%.
[2] Collagen—cross-linked and tested after stretching from 0 up to 50%.
[3] Collagen—rat tail: properties determined by light scattering
[4] Dragline silk—from *Nephila claviceps* spider
[5] Silkworm silk—from *Bombyx mori* silkworm cocoon
[6] L-PLA—molecular weights from 50,000 to 300,000, properties for D,L-PLA were in similar ranges

Silks are natural fibers predominantly harvested from the domesticated silkworm, *Bombyx mori*, and have been used traditionally in the form of threads in textiles and sutures for biomedical needs for thousands of years [Halsted, 1892; Lange, 1903, 1907; Ludloff, 1927; Perez-Rigueiro et al., 1998; Sofia et al., 2001]. Recently, silks have also been explored for an extended variety of biomedical applications including osteoblast and fibroblast cell support matrices and for ligament tissue engineering [Sofia et al., 2001; Altman et al., 2002; Meinel et al., 2004b,c].

Aside from existing natural sources of silk (*B. mori* silkworm silk from sericulture), future options from the availability of reasonable quantities of genetically engineered silk variants (such as from Polymer Technologies, Nexia Biotechnologies, and others) would expand the set of structures available for use in vivo.

Silkworm silk contains a fibrous protein termed fibroin (both heavy and light chains) that forms the thread core, which is encased in a sericin coat. Sericins are a family of gluelike proteins that hold the individual fibroin fibers together to form the composite fibers of the cocoon case to protect the metamorphosing worm. Fibroin is a protein up to 90% of which consists of the amino acids glycine, alanine, and serine that form water insoluble crystalline β-sheets on shearing, drawing, heating, spinning, or exposure in an electric field or to polar solvents such as methanol [Guhrs et al., 2000].

Silks from silkworm have been extensively characterized for biocompatibility, because they have been used for decades as sutures in vivo. Initially, adverse immunological reactions were found, and this prompted the replacement of silkworm silk sutures with nylons approximately 20 years ago [Soong and Kenyon, 1984]. However, it was clearly shown that adverse reactions to silks were due to the presence of residual sericin and not the fibroin itself [Soong and Kenyon, 1984]. The biocompatibility was also evaluated for mesenchymal stem cells. The inflammatory reaction was significantly lower in cells seeded on silk films as compared to collagen and polylactide (PLA) films. Furthermore, the accumulation of inflammatory cells around films implanted intramuscularly was again lower for silk as compared to collagen and PLA [Meinel et al., 2005; Meinel et al., 2003]. These data, both in vitro [Santin et al., 1999], and in vivo [Soong and Kenyon, 1984], illustrate that biocompatible silkworm silk rivals other biomaterials in use today.

A misconception with silk revolves around its degradation in vivo. The U.S. Pharmacopoeia defines an absorbable material as one that loses “most of its tensile strength within 60” days in vivo. By this definition, silk is correctly classified as nondegradable. However, according to the literature, silk is degradable over longer time frames as a function of proteolytic degradation and matrix mechanical fatigue [Soong and Kenyon, 1984; Rossitch, 1987; Bagi et al., 1995; Altman et al., 2003]. Several studies detail silk degradation in vivo with variable rates dependent on the animal model and implantation site [Postlethwait, 1970; Salthouse et al., 1977; Greenwald et al., 1994; Lam et al., 1995; Bucknall et al., 1983]. In general, silks lost the majority of their tensile strength within 1 year in vivo, and failed to be recognized in the implantation site within 2 years.

Taken together, silk protein-based polymers combine several advantages in particular as they potentially address the needs for bone tissues regrown in vitro for subsequent implantation.

- The *natural role* of structural/fibrous proteins in tissue remodeling, including collagens in the extracellular matrix (ECM).
- *Biocompatibility* with *degradability* properties for ingrowth and reintegration into native tissues, the need for materials that can be degraded or resorbed that will have minimal negative impact on surrounding tissues. Silks have been used as sutures for decades and been shown to be biocompatible and degradable [Meinel et al., 2003].
- The need for materials with robust *mechanical integrity* until new tissue is regenerated. Silks are unique in this feature, exhibiting strength, flexibility, and compression properties that exceed those of all other natural fibers. This is particularly important for in situ pairs where a matrix will need to be formed and retain mechanical integrity during osseoreintegration.
- The need for *matrices that can be functionalized* with cell growth factors, with control over placement and density of these factors using chemistries that are nontoxic and biocompatible. This approach has already been used successfully with silks in our prior studies [Sofia et al., 2001].
- The ability to *self-assemble to establish conformal fill-ins* in vivo *during tissue regeneration*, thus avoiding gaps leading to fibrous encapsulations and scar tissue. This feature would be required if the scaffolds were to be used in vivo as temporary matrices to fill in defects during osseointegration, and this is a characteristic of silks.

2.2. Silk Purification

It is apparent from the above that silk protein-based polymers are logical choices for biocompatible scaffolding for the formation of advanced matrices to induce bone tissue repair because of their mechanical properties as well as a wide range of other advantages.

Protocol 13.1. Purification of Silk for Bone Tissue Scaffolds

Reagents and Materials

- Silk cocoons from *Bombyx mori*
- Sodium carbonate
- Lithium bromide
- UPW
- Glass beakers, 2 L
- Falcon tubes, 50 ml
- Glass bottle, 100 ml

- ❑ Syringe 18G, 20 ml, and needle
- ❑ Millex-SV Syringe-driven filter unit, pore size 5 μm
- ❑ Slide-a-Lyzer® Dialysis Cassette 3–12 ml
- ❑ Oven (set at 55 °C)
- ❑ Magnetic heating/stirring plate with magnet
- ❑ Freezer at −70 °C to −80 °C
- ❑ Lyophilizer

Protocol

(a) Heat 0.75 L UPW in a glass beaker until it boils.
(b) Dissolve 1.59 g Na_2CO_3 in the 0.75 L UPW, up to a final molarity of 0.02 M.
(c) Cut 3 cocoons in 8 parts each, clean from larvae and debris.
(d) Transfer the cocoons into the boiling solution and boil under stirring for 1 h. Replace evaporated water.
(e) Rinse the silk with 1 L hot UPW.
(f) Rinse 10 times with 1 L cold UPW.
(g) Dry the silk overnight in a fume hood and note the dry weight.
(h) Prepare a 9 M solution (781.6 mg/ml) of LiBr in UPW.
(i) Prepare a 10% (w/v) solution of dried silk in 9 M LiBr in a glass bottle and leave silk at 55 °C for 4–5 h, until it is completely dissolved (some debris may be left).
(j) Filter the solution through a 5-μm pore size syringe filter.
(k) With a syringe, insert 6–8 ml of the filtered solution into a dialysis cassette and dialyze against 1 L of UPW (per cassette) on a magnetic stirrer plate.
(l) Replace UPW after 1 h, after 3 h, after 12 h, after 24 h and after 36 h (a total of 5 changes).
(m) Pipette the dialyzed solution into 25-ml aliquots in 50-ml Falcon tubes.
(n) Freeze the solution for 2 h at −70 °C to −80 °C.
(o) Lyophilize until silk has completely dried. This takes up to 3 days.
(p) Lyophilized silk can be stored at room temperature.

2.3. Preparation of Silk-RGD

Protocol 13.2 is for an efficient two-step coupling of proteins in solution using EDC and Sulfo-NHS. The procedure allows for sequential coupling of two proteins without exposing the second protein to EDC and thus affecting carboxyls on the second protein. This procedure quenches the first reaction with a thiol compound.

Protocol 13.2. Coupling of RGD Motif to Silk

Reagents and Materials

- ❑ Silk from Protocol 13.1 Step (k)
- ❑ Modified MES buffer

For a volume of 500 mL, pour 1 package of BupH™ MES buffered saline in a beaker and rinse empty package once with UPW. Add 10.11 g sodium chloride. Fill up with UPW to 450 ml. Titrate pH with a highly concentrated sodium hydroxide solution to pH 6. Add UPW to a final volume of 500 ml.

- ❑ GRGDS
- ❑ Sulfo-NHS
- ❑ EDC
- ❑ 2-Mercaptoethanol
- ❑ Hydroxylamine HCl
- ❑ UPW
- ❑ Glass beakers, 2 L
- ❑ Falcon tubes, 50 ml
- ❑ Syringe, 20 ml, and needle 18G
- ❑ Slide-a-Lyzer® Dialysis Cassette, 3–12 ml
- ❑ Magnetic heating/stirring plate with magnet
- ❑ Freezer at −70 °C to −80 °C
- ❑ Lyophilizer
- ❑ Glass bottle, 100 ml

Protocol

(a) Follow Protocol 13.1 until Step (k).
(b) Change UPW after 1 h, after 3 h, and the next morning.
(c) The next evening, discard the water and add 1 L modified MES buffer per dialysis cassette.
(d) Change modified MES buffer after 12 h.
(e) The next evening, carefully transfer the solution from the dialysis cassette into a glass bottle with a syringe.
(f) Per mg of dry silk protein—as measured in Protocol 13.1, step (g)—add 0.4 mg EDC and 1.1 mg Sulfo-NHS. Allow reaction to take place at room temperature for 15 min.
(g) In a fume hood: Per ml of solution, add 1.4 μl 2-mercaptoethanol per ml solution to quench the EDC.
(h) For 1.1 g of dry silk—as measured in Protocol 13.1, step (g)—add 7.5 mg GRGDS protein to the reaction mixture.
(i) Allow reaction at room temperature for 2 h.
(j) Add hydroxylamine HCl powder to a final concentration of 10 mM. This method of quenching hydrolyzes any unreacted NHS present on the surface of the silk and results in regeneration of the original carboxyls.
(k) Transfer 6–8 ml of the silk-RGD solution into a dialysis cassette and dialyze against 1 L UPW (per cassette) on a magnetic stirrer plate.
(l) Change UPW after 1 h, after 3 h, after 12 h, after 24 h and after 36 h (total of 5 changes).
(m) Transfer the dialyzed solution into 25-ml aliquots in 50-ml Falcon tubes.
(n) Freeze the solution for 2 h at −70 °C to −80 °C.

(o) Lyophilize until silk-RGD has completely dried. This may take up to 3 days.
(p) Lyophilized silk-RGD can be stored at room temperature.

Protocol 13.3. Preparation of Silk Scaffolds

Reagents and Materials

- ❑ Lyophilized silk (or silk-RGD) from Protocol 13.1 (or 13.2)
- ❑ HFIP

Note: HFIP is volatile, store bottle at 2–8 °C for 30 min prior to use. Precool pipette tips to 2–8 °C. Work under fume hood with HFIP. Close vessels immediately after use and wrap with Parafilm. Avoid skin contact or inhalation of HFIP. Read security sheets before working with HFIP.

- ❑ Sodium chloride USP, granular
- ❑ Methanol
- ❑ UPW
- ❑ Glass container, 20 ml, with snap cap
- ❑ Teflon container with snap cap
- ❑ Glass beaker, 1 L
- ❑ Parafilm
- ❑ Razor blade

Protocol

(a) Prepare a 17% (w/v) solution of lyophilized silk in HFIP in a glass container. Close the container firmly and seal with Parafilm. The dissolution procedure may take up to 1 day at room temperature.
(b) Add 3.4 g porogen (e.g., NaCl) into a Teflon container.
(c) Add 1 ml dissolved silk on the porogen and immediately cover the Teflon container (minimize evaporation of HFIP). The general ratio of NaCl to silk is 20:1 (w/w).
(d) Leave container closed for 6 h.
(e) Then open the Teflon container and allow evaporation of the solvent only in a fume hood. This may take up to 4 days.
(f) Carefully remove the salt-silk composite from the container by tapping it upside down on the bench. If the scaffold doesn't come out easily, let it dry for another 1 or 2 days.
(g) Immerse the salt-silk composite in 90% methanol in H_2O (v/v) for 30 min. Polar solvents such as methanol induce a conformational change of the water-soluble silk I into the water-insoluble silk II conformation.
(h) Remove the scaffold from the methanol solution and dry overnight in a fume hood.
(i) Transfer the dry scaffold into a 1-L beaker with UPW for at least 24 h for salt leaching. Change the water at least 5 times.

(j) Air dry scaffold. This may take up to 3 days.
(k) To cut the scaffold into the desired shape, completely soak scaffolds in UPW for 10 min. Cut scaffold with a sharp razor blade.

3. PREPARATION OF REAGENTS AND MEDIA

For the engineering of bone from mesenchymal stem cells, medium composition guides differentiation along the osteogenic lineage. This differentiation process is accompanied by the expression/activity of transcription factors, regulating lineage restriction, commitment, and/or differentiation within some of the mesenchymal lineages including osteoblasts [Ducy et al., 1997; Ducy, 2000; Liu et al., 2001]. A master regulatory transcription factor for osteogenic differentiation is cbfa1. Cbfa1 gene deletion results in complete absence of bone formation and mature osteoblasts [Komori et al., 1997; Otto et al., 1997]. This demonstrates the importance of this master transcription factor for osteoblast differentiation and bone formation. Notably, cbfa1 overexpression blocks osteoblast maturation [Liu et al., 2001]. In analogy to the consequences of overexpression or deletion of cbfa1, many reports document a stimulatory effect of BMPs on osteoblasts, and a few show inhibitory effects. A possible explanation may be the dependence of the actions of growth factors on the relative stage of differentiation of the target cells.

BMP-2 is generally used at high concentrations of 1 μg/ml, but significant effects on total mineralization were observed at concentrations as low as 40 ng/ml compared to medium without supplementation of BMP-2 [Meinel et al., 2004a]. Another approach, avoiding cost-intensive use of recombinant growth factors, is the use of adenoviral transfection. However, the transduction of MSCs with the adenoviral vector carrying the BMP-2 gene resulted in significantly lower mineralization compared to untransfected MSCs cultured in the presence of BMP-2 concentrations as measured for the transduced cells. A possible explanation is that the dual role of the transduced cells—BMP-2 production and mineralization—prevents them from performing equally well as untransduced cells exposed to BMP-2-supplemented medium.

Glucocorticoids such as dexamethasone, added routinely in assays of osteoprogenitors (CFU-O), also have both inhibitory and stimulatory effects on skeletal cells, and an emerging view is that this reflects opposite effects on precursors versus more mature cells in the lineages [Gronthos et al., 1994; Aubin and Liu, 1996; Aubin and Triffitt, 2002]. It has also been suggested that even a transient exposure of stem cells to dexamethasone may be effective in inducing and maintaining the osteoblastic phenotype [Jaiswal et al., 1997]. Glycerophosphate has been found to have a significant effect to induce osteogenic differentiation and increases alkaline phosphatase activity and osteocalcin production. L-Ascorbic acid (AA, vitamin C) increases cell viability and is a cofactor in the hydroxylation of proline and lysine residues and is therefore necessary for the production of collagen. AA has also been demonstrated to increase alkaline phosphatase activity [Choong et al., 1993]. Together with β-glycerophosphate, AA was found to be a prerequisite for

the formation and mineralization of the extracellular matrix [Maniatopoulos et al., 1988]. The composition of the medium directly affects the osteogenic differentiation process. The protocols suggest the use of a metabolically stable variant of ascorbic acid, ascorbic acid 2-phosphate.

Prepare culture media aseptically. Store at 4 °C; preheat to 37 °C prior to use.

3.1. Control Medium

Dulbecco's modified Eagle's medium (DMEM)
Fetal bovine serum (FBS), 10%
Penicillin, 100 U/ml, streptomycin, 100 μg/ml
Fungizone, 0.5 μg/ml

Can be stored at 4 °C for up to 1 week.

3.2. Expansion Medium

Dulbecco's modified Eagle's medium (DMEM)
Fetal bovine serum (FBS), 10%
Nonessential amino acids solution, 1%
Penicillin, 100 U/ml, streptomycin, 100 μg/ml
Fungizone, 0.5 μg/ml
bFGF (human, recombinant), 1 ng/ml

Can be stored at 4 °C for up to 1 week.

3.3. Osteogenic Medium

Dulbecco's modified Eagle's medium (DMEM)
Fetal bovine serum (FBS), 10%
Penicillin, 100 U/ml, streptomycin, 100 μg/ml
Fungizone, 0.5 μg/ml
Ascorbic acid 2-phosphate, 50 μg/ml
Dexamethasone, 10 nM
β-Glycerophosphate, 10 mM
BMP-2, 1 μg/ml

Use medium immediately; do not store or reuse.

3.4. Osteogenic Medium Double Concentration

Dulbecco's modified Eagle's medium (DMEM)
fetal bovine serum (FBS), 10%
Penicillin, 100 U/ml, streptomycin, 100 μg/ml
Fungizone, 0.5 μg/ml
Ascorbic acid 2-phosphate, 100 μg/ml
Dexamethasone, 20 nM

β-Glycerophosphate, 20 mM
BMP-2, 2 μg/ml

Use medium immediately; do not store or reuse.

3.5. Cartilage Medium (for Cell Characterization)

Dulbecco's modified Eagle's medium (DMEM)
Fetal bovine serum (FBS), 10%
Penicillin, 100 U/ml, streptomycin, 100 μg/ml
Fungizone, 0.5 μg/ml
Ascorbic acid 2-phosphate, 50 μg/mL
Dexamethasone, 10 nM
Nonessential amino acids solution, 1%
Insulin, 5 μg/ml
TGF-β_1, 5 ng/ml

Use medium immediately; do not store or reuse.

3.6. Cartilage Medium Double Concentration

Dulbecco's modified Eagle's medium (DMEM)
Fetal bovine serum (FBS), 10%
Penicillin, 100 U/ml, streptomycin, 100 μg/ml
Fungizone, 0.5 μg/ml
Ascorbic acid 2-phosphate, 100 μg/ml
Dexamethasone, 20 nM
Nonessential amino acids solution, 2%
Insulin, 10 μg/ml
TGF-β_1, 10 ng/ml

Use medium immediately; do not store or reuse.

3.7. Preparation of bFGF

Lyophilized samples should be reconstituted with sterile 10 mM Tris pH 7.6 containing 1% BSA to a final bFGF concentration of 0.1 mg/ml. For longer term storage, aliquot and store in polypropylene vials at −20 °C. Avoid repeated freeze-thaw cycles. In applications requiring long-term use of this growth factor in cell cultures, refilter material after dilution in protein (BSA or FBS)-containing buffer, through a 0.22-μm low-protein-binding filter.

3.8. Preparation of BMP-2

Prepare a 1 mg/ml BMP-2 stock solution in a buffer containing 0.5% sucrose, 2.5% glycine, 5.0 mM glutamic acid, 5.0 mM sodium chloride, 0.01% Tween 80, pH 4.5. Store in single-use aliquots at −80 °C or −20 °C. Avoid repeated freeze-thawing. To avoid loss of protein due to adherence to surfaces, BMP-2 should

always be added to culture medium after the addition of carrier protein (0.1% BSA or 1–10% appropriate serum).

3.9. Preparation of TGF-β_1

Purified recombinant human TGF-β_1 is an extremely hydrophobic protein that adheres strongly to surfaces. To ensure recovery, lyophilized samples should be reconstituted with sterile 4 mM HCl containing 1 mg/ml BSA to a final TGF-β_1 concentration of no less than 1 µg/ml. Upon reconstitution, this cytokine can be stored under sterile conditions at 2–8 °C for 1 month or at −20 °C to −80 °C in a manual defrost freezer for 3 months without detectable loss of activity. Avoid repeated freeze-thaw cycles.

4. ISOLATION AND CULTURE METHODOLOGY OF HMSC

Bone marrow contains at least two distinct populations of stem cells, one hematopoietic and the other nonhematopoietic mesenchymal. Hematopoietic stem cells in the adult give rise to all components of the immune and blood system [Lagasse et al., 2000], whereas mesenchymal stem cells (MSC) can differentiate into bone, cartilage, or adipose tissue [Pittenger et al., 1999]. However, MSCs are present within adult bone marrow at an exceedingly low frequency of <1 MSC per 10^6 bone marrow cells [Bruder et al., 1994]. The low frequency of mesenchymal cells within the marrow compartment has led investigators to develop a variety of techniques for stem cell isolation and culture. The described isolation procedure is based on Histopaque gradient centrifugation isolating mononuclear cells with a density of 1.077 g/cm^3, the cells' ability to adhere to the tissue culture plate surface, and the ability of the cells to undergo chondrogenic and osteogenic differentiation. A thorough characterization of the isolated cells is essential for comparison with other experiments performed with cells harvested from other volunteers. A routine assessment can involve the analysis of the cell morphology, as mesenchymal cells are typically adherent marrow cells, generally considered to be spindle shaped. Further analysis involves the surface antigen expression by flow cytometry and their ability to differentiate along skeletal lineages [Pittenger et al., 1999; Meinel et al., 2004b]. The analysis of cells in different passages can provide information about the number of expansion cycles possible without a significant reduction in differentiation capacity. We generally observe stable differentiation capacity up to 4 passages, using the techniques as described in Protocols 13.5, 13.6, 13.8 and Fig 13.1.

To date, an antigen surface determinant considered specific for mesenchymal stem cells has not been found. The so-called SH2 antibodies originally developed by Caplan and colleagues recognize CD105, which is also known as endoglin and is a regulatory component of the TGF-β receptor complex. Many investigators have explored CD105 as an important antigenic determinant in the identification of mesenchymal stem cells. However, it is a matter of debate whether mesenchymal stem cells can be identified solely by flow cytometry and, thereby, clearly distinguished

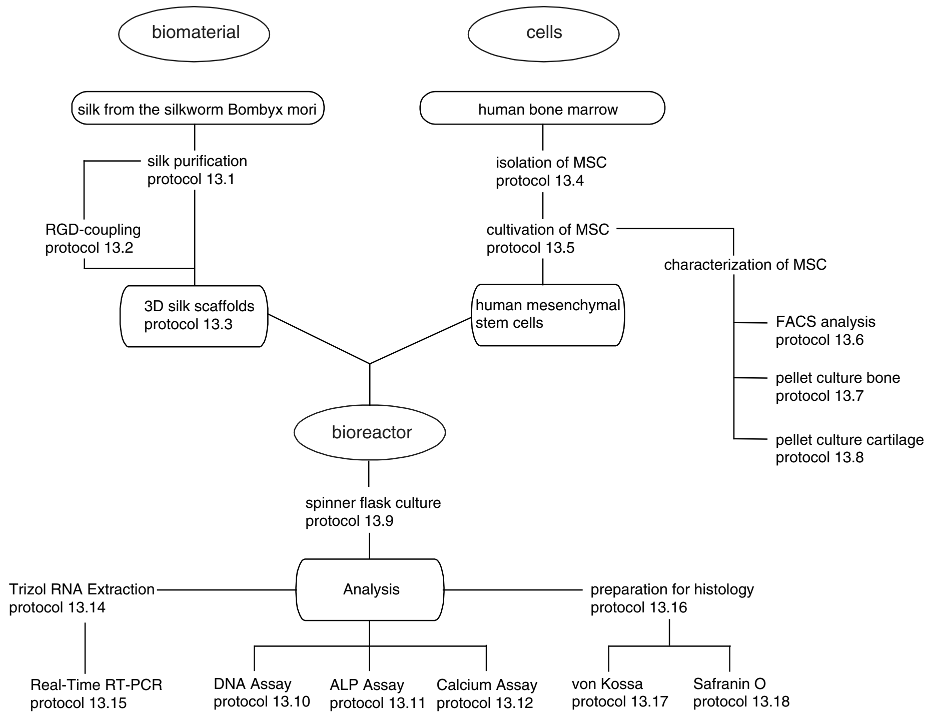

Figure 13.1. Schematic overview of experimental approach.

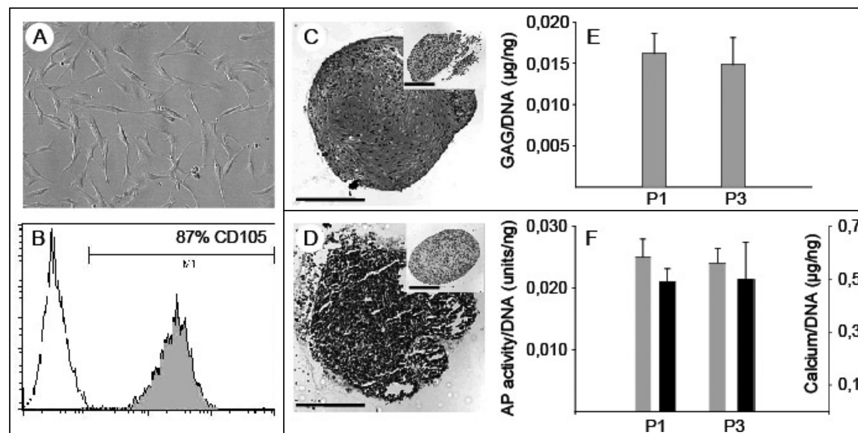

Figure 13.2. Characterization of MSCs. A) phase-contrast photomicrographs of putative passage 2 MSCs at approximately 40% confluence at an original magnification of ×20. B) Surface CD105 expression of passage 2 MSCs. C) Characterization of chondrocyte differentiation of MSCs in pellet culture, treated either with chondrogenic medium or with control medium (insert). Pellet diameter is approximately 2 mm, stained with safranin O. D) Characterization of osteoblast differentiation of MSCs in pellet culture, treated either with osteogenic medium or with control medium (insert). Pellet diameter is approximately 2 mm, stained according to von Kossa. E) Sulfated GAG/DNA (µg/ng) deposition of passage 1 and 3 MSC pellet culture after 4 weeks. F) Calcium deposition/DNA (µg/ng) and AP activity/DNA (units/ng) of passage 1 and 3 MSC pellet culture. Reprinted with the permission of the Biomedical Research Society. (See Color Plate 8.)

from other adherent mesenchymal cells solely by antigen expression [Prockop et al., 2001, 2003]. Therefore, we suggest evaluating the absence of adherent and nonadherent cells, in particular of hematopoietic (CD34) or endothelial origin (CD31) [Spangrude et al., 1988; DeLisser et al., 1993; Negrin et al., 2000]. The protocols also recommend the analysis of CD71, the transferrin receptor, to assess the proliferative state of the isolated cells.

The ability of hMSCs to undergo selective differentiation in response to environmental factors is documented by chondrogenic or osteogenic differentiation in chondrogenic or osteogenic culture medium, respectively, and the lack of differentiation in control medium. Similar per-cell amounts of glycosaminoglycan (GAG) and calcium measured in cells of higher passages (e.g., P_1, P_3, and P_5) are also important as they enable the use of small initial bone marrow aspirates to obtain sufficient amounts of cells for seeding clinically sized scaffolds. Together, the capacity for expansion in the undifferentiated state and the maintained ability for subsequent chondrogenic or osteogenic differentiation establish the feasibility of using hMSCs for bone tissue engineering (Fig. 13.2, See Color Plate 8) [Meinel et al., 2004a].

4.1. Isolation of Mesenchymal Stem Cells

Protocol 13.4. Isolation of hMSCs from Bone Marrow

Reagents and Materials

Sterile

- ❑ Fresh bone marrow, EDTA- or heparin treated; keep on ice
- ❑ Expansion medium (See Section 3.2)
- ❑ Control medium (See Section 3.1)
- ❑ RPMI 1640 medium supplemented with 5% FBS
- ❑ Histopaque®-1077
- ❑ PBSA
- ❑ Trypan Blue
- ❑ Trypsin-EDTA
- ❑ FBS supplemented with 10% DMSO
- ❑ Culture flasks, 150 cm^2
- ❑ Accuspin™ tube
- ❑ Polypropylene Falcon tubes, 50 ml
- ❑ Cryovials, 2 ml

Nonsterile

- ❑ Hemocytometer
- ❑ Nalgene™ Cryo 1 °C Freezing Container
- ❑ Centrifuge
- ❑ Incubator at 37 °C, 5% CO_2

Protocol

(a) Add 15 ml Histopaque®-1077 (room temperature) to the upper chamber of the Accuspin™ tube.

(b) Centrifuge at 800 *g* for 30 s at room temperature. Histopaque®-1077 is now in the lower chamber of the tube.
(c) Add 10 ml RPMI-FBS solution to 5-ml bone marrow aliquots and gently mix by pipetting.
(d) Pipette suspension carefully to the upper chamber of the prepared Accuspin™ tube.
(e) Centrifuge at 800 *g* for 15 min at room temperature.
(f) Carefully transfer the opaque interface, which contains the mononuclear cells, into a clean Falcon tube with a pipette.
(g) Pellet cells by centrifugation at 300 *g* for 10 min and 4 °C, aspirate supernate, and resuspend cells in RPMI-FBS medium.
(h) Count an aliquot of the cell suspension using a hemocytometer and determine viability by dye exclusion with Trypan Blue.
(i) Centrifuge cells at 300 *g* for 10 min and 4 °C, aspirate supernate, and resuspend cells in expansion medium.
(j) Plate suspension at a density of 5×10^3 cells/cm^2 in expansion medium in a cell culture flask (40 ml per flask).
(k) Incubate the cells in a humidified 37 °C, 5% CO_2 incubator.
(l) At days 3 and 5: add 20 ml expansion medium to each flask.
(m) When cells reach 60% confluence: wash the cells 2 times with PBSA and add fresh expansion medium.
(n) Change medium twice a week until cells reach 80% confluence (days 12–22), then aspirate expansion medium and wash cells twice with PBSA at 37 °C.
(o) Add 8 ml trypsin-EDTA to each flask and distribute the solution evenly.
(p) Incubate for 5 min or until cells start to detach at 37 °C.
(q) Inactivate the trypsin by adding 8 ml control medium cooled to 4 °C, mix, and transfer into a clean 50-ml Falcon tube. Use cold serum-containing medium at this step to block trypsin activity.
(r) Wash the flask once again by adding 8 ml control medium cooled to 4 °C and combine with the mix from Step (q) in the Falcon tube.
(s) Centrifuge at 300 *g* for 10 min at 4 °C.
(t) Aspirate the supernate and resuspend the cells in 10 ml control medium.
(u) Combine all cell suspensions in one Falcon tube, mix well by pipetting.
(v) Count an aliquot of the cell suspension using a hemocytometer and determine cell viability by dye exclusion with Trypan Blue. Count unstained cells.
(w) Centrifuge cells at 300 *g* for 10 min at 4 °C, aspirate the supernate, and resuspend cells at a concentration of 5×10^6 in FBS-DMSO solution. Work rapidly as unfrozen cells should be exposed to DMSO for the shortest time possible.
(x) Freeze 1 ml per cryovial at a rate of 1 °C/min in a freezing container for 6 h at −80 °C.
(y) Transfer cells into liquid nitrogen freezer.

4.2. Thawing and Maintenance of hMSC Cell Culture

Bone marrow mononuclear cells are obtained by Histopaque density gradient centrifugation. hMSCs, or possibly a specific subpopulation, can be expanded in vitro as undifferentiated cells responsive to environmental cues inducing differentiation toward mesenchymal lineages as well as other lineages [Cancedda et al., 2003a,b]. bFGF supplementation of the culture medium promotes hMSC proliferation and maintains their multilineage potential during expansion [Bruder et al., 1997; Freed et al., 1997; Meinel et al., 2004b,c].

Protocol 13.5. Expansion of Human Mesenchymal Stem Cells

Reagents and Materials

Sterile

- ❑ Expansion medium (See Section 3.2)
- ❑ Control medium (See Section 3.1)
- ❑ Trypan blue
- ❑ PBSA
- ❑ Trypsin-EDTA
- ❑ Polypropylene Falcon tubes, 50 ml
- ❑ Triple flasks (Nalgene Nunc)

Unless noted otherwise, reagent temperatures are 37 °C.

Nonsterile

- ❑ Hemocytometer
- ❑ Centrifuge
- ❑ Water bath, 37 °C
- ❑ Incubator at 37 °C, 5% CO_2

Protocol

(a) To recover the frozen cells, thaw rapidly at 37 °C (water bath). Unfrozen cells should be exposed to DMSO for the shortest time possible.
(b) Transfer the cells to a 50-ml Falcon tube containing 30 ml DMEM at 4 °C.
(c) Centrifuge at 300 *g* for 10 min at 4 °C, aspirate the supernate, and resuspend the cells in expansion medium.
(d) Count an aliquot of the cell suspension using a hemacytometer and determine cell viability through dye exclusion with Trypan Blue. Count unstained cells only.
(e) Plate cells at a density of 5×10^3 cells/cm^2 in expansion medium in a Triple flask (100 ml).
(f) At day 3, add 50 ml expansion medium per Triple flask.
(g) At day 5, replace medium with 100 ml fresh expansion medium.
(h) When cells reach confluence (around day 7), aspirate expansion medium and wash cells twice with PBSA at 37 °C.

(i) Add 15 ml of Trypsin-EDTA to each flask and distribute the solution evenly.
(j) Incubate for 5 min or until cells start to detach at 37 °C.
(k) Inactivate the trypsin by adding 15 ml control medium at 4 °C, mix, and pour into a clean 50-ml Falcon tube. As the antitryptic activity of serum-free medium may be insufficient to quench the proteolytic activity of the trypsin, it is important to use serum-containing medium at this step.
(l) Wash the flask once again by adding 15 ml of control medium at 4 °C and combine with the mix from Step (j) in the Falcon tube.
(m) Centrifuge at 300 *g* for 10 min at 4 °C.
(n) Aspirate the supernate and resuspend the cells in 10 ml control medium.
(o) Combine all cell suspensions in one Falcon tube; mix well by pipetting.
(p) Count an aliquot of the cell suspension using a hemocytometer and determine viability through dye exclusion with Trypan Blue. Count unstained cells.
(q) Centrifuge cells at 300 *g* for 10 min at 4 °C, aspirate the supernate, and resuspend cells at the desired concentration.

5. CHARACTERIZATION OF HMSCS

Bone marrow is a complex tissue comprised of hematopoietic precursors, their differentiated progeny, and a connective tissue network referred to as stroma. The stroma itself is a heterogenous mixture of cells including adipocytes, reticulocytes, endothelial cells, and fibroblastic cells that are in direct contact with the hematopoietic elements. Since it was well established that the stroma contains cells that differentiate into bone, cartilage, fat, and a connective tissue that supports the differentiation of hematopoietic stem cells, identification of the progenitor cells for these mesenchymal tissues has been an area of investigation [Bruder et al., 1997].

hMSCs identified with established isolation techniques (plastic adhesion, flow cytometry) are highly heterogeneous and have a variable potential for mesenchymal tissue development. Recently identified markers of mesenchymal and bone [Gronthos et al., 2003] progenitors have been used to address this problem. Consequently, we characterize our isolated and expanded hMSCs by their surface antigen pattern with fluorescence-activated cell sorting (FACS) and their capacity to differentiate into bone or cartilage depending on the number of culture passages before their use.

Purified hMSCs have been extensively characterized with respect to their complement of cell surface and extracellular matrix molecules, as well as their secretory cytokine profile in control and experimental conditions [Haynesworth et al., 1992].

The low frequency of mesenchymal stem cells within the marrow compartment (less than 1 MSC per 10^6 bone marrow mononuclear cells [Caplan, 1994]) has led investigators to the development of a variety of methods for stem cell isolation and culture. It is important to show that the cells are indeed hMSCs.

5.1. Authentication of Surface Antigen Analysis

Protocol 13.6. FACS Analysis of Human Mesenchymal Stem Cells

Reagents and Materials

Sterile

- ❑ RPMI 1640 medium supplemented with 5% FBS
- ❑ Centrifuge tubes, 50 ml (Falcon)
- ❑ Polystyrene tubes, 5 ml (Falcon)
- ❑ Antibodies: CD14-FITC, CD31-PE, CD34-APC, CD44-FITC, CD45-APC, CD71-APC, mouse anti-human CD105, goat anti-mouse IgG-FITC

Nonsterile

- ❑ Formalin 2% in PBSA (prepare fresh, keep at 4 °C)
- ❑ Incubator at 37 °C, 5% CO_2
- ❑ Hemocytometer
- ❑ Vortex mixer

Protocol

(a) Prepare cells according to Protocol 13.5 until step (m).
(b) Wash cells twice in RPMI-FBS medium.
(c) Count an aliquot of the cell suspension using a hemocytometer and determine cell viability through dye exclusion with Trypan Blue. Count unstained cells.
(d) Centrifuge cells at 300 *g* for 10 min at 4 °C, aspirate supernate, and resuspend cells in RPMI-FBS at a concentration of 1×10^7 cells/ml in a 50-ml Falcon tube.
(e) Add 50 μl of this cell suspension (500,000 cells) in each 5-ml Falcon tube.
(f) Add 2 μl of each desired CD stock solution (CD45-APC, CD44-FITC, CD31-PE, CD34-APC, CD14-FITC, CD71-APC, CD105; concentration of each: 0.5 μg/μl) into each corresponding 5-ml Falcon tube.
(g) Incubate for 30 min on ice in the dark.
(h) Centrifuge at 300 *g* for 5 min at 4 °C and aspirate supernate.
(i) Wash in 1 ml RPMI-FBS medium.
(j) Centrifuge at 300 *g* for 5 min at 4 °C.
(k) Aspirate medium.
(l) Add 1 ml RPMI-FBS medium and resuspend cells gently.
(m) Centrifuge at 300 *g* for 5 min at 4 °C.
(n) Carefully aspirate supernate.
(o) Resuspend cells in 1 ml RPMI-FBS medium; place on ice, except for tubes designated CD105 (go to Step (p) for non-labelled antibodies; here for CD105; and for others continue at (u)).
(p) All tubes designated CD105: add 50 μl RPMI-FBS and 2 μl secondary antibody (polyclonal goat anti-mouse IgG-FITC); incubate on ice for 30 min in the dark.

(q) All tubes designated CD105: Centrifuge at 300 *g* for 5 min at 4 °C, aspirate supernate.
(r) All tubes designated CD105: Wash in 1 ml RPMI-FBS medium.
(s) All tubes designated CD105: Centrifuge at 300 *g* for 5 min at 4 °C, aspirate supernate.
(t) All tubes designated CD105: Resuspend cells in 1 ml RPMI-FBS medium, place on ice.
(u) Centrifuge all tubes at 300 *g* for 5 min at 4 °C.
(v) Aspirate supernate and resuspend in 1 ml PBSA.
(w) Centrifuge all tubes at 300 *g* for 5 min at 4 °C.
(x) Carefully aspirate supernate.
(y) All tubes: Add 100 μl Formalin 2% in PBSA.
(z) All tubes: Store at 4 °C in the dark (aluminum foil) and measure within a day.

5.2. Pellet Culture of Bone

Protocol 13.7. Osteogenic Differentiation in Pellet Culture

Reagents and Materials

Sterile or aseptically prepared

- ❑ hMSCs, prepared according to Protocol 13.5
- ❑ Osteogenic medium
- ❑ Screw cap microcentrifuge tubes, 2 ml

Nonsterile

- ❑ Incubator at 37 °C, 5% CO_2

Protocol

(a) Prepare cells according to Protocol 13.5.
(b) Dilute cells to 2×10^5 cells/ml in osteogenic medium.
(c) Pipette 1 ml of the cell suspension (200,000 cells) into each screw cap tube. Make sure cells are well resuspended, to get the same number of cells per tube.
(d) Centrifuge at 300 *g* for 10 min at 4 °C.
(e) Unscrew the cap to allow ventilation and prevent contamination.
(f) Incubate cells at 37 °C, 5% CO_2.
(g) Change medium 3 times per week by carefully aspirating 0.5 ml medium with a pipette and adding 0.5 ml osteogenic medium double concentration (See Section 3.4).

Note: For analysis of the osteogenic differentiation of the cell pellet use Protocols 13.10, 13.11, 13.12, 13.14, 13.15, 13.16 and 13.17.

5.3. Pellet Culture of Cartilage

Protocol 13.8. Chondrogenic Differentiation in Pellet Culture

Reagents and Materials

Sterile or aseptically prepared

- ❑ hMSCs, prepared according to Protocol 13.5
- ❑ Cartilage medium
- ❑ Screw cap microcentrifuge tubes, 2 ml

Nonsterile

- ❑ Incubator at 37 °C, 5.0% CO_2

Protocol

(a) Prepare cells according to Protocol 13.5.
(b) Dilute cells to 2×10^5 cells/ml in cartilage medium.
(c) Pipette 1 ml of the cell suspension (200,000 cells) into each screw cap tube. Make sure cells are well resuspended, to get the same number of cells per tube.
(d) Centrifuge at 300 *g* for 10 min at 4 °C.
(e) Unscrew the cap to allow ventilation and prevent contamination.
(f) Incubate cells at 37 °C, 5% CO_2.
(g) Change medium 3 times per week by carefully aspirating 0.5 ml medium with a pipette and adding 0.5 ml Cartilage medium double concentration (See Section 3.6).

Note: For analysis of the differentiation of the cell pellet into cartilage use Protocols 13.10, 13.13, 13.14, 13.15, 13.16 and 13.18.

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6. TISSUE ENGINEERING OF BONE

Mechanically active environments generally result in better engineered tissue composition, morphology, and mechanical properties than static environments. Most likely, this is due to enhanced mass transport at tissue surfaces [Freed and Vunjak-Novakovic, 2000]. Transport limitations are a significant problem in the engineering of bone, a highly vascularized tissue, which could be cultured only to thicknesses of 250–500 μm in the best case in static culture [Ishaug et al., 1997; Martin et al., 2001].

The mechanical environment is modulated through the use of bioreactors, including spinner flasks, with tissue constructs fixed in place and cultured either statically or in well-mixed medium [Vunjak-Novakovic et al., 1996]. Other bioreactors are rotating vessels, in which the tissue constructs are suspended in dynamic laminar flow [Freed et al., 1997], and perfused chambers with flow of medium around [Glowacki et al., 1998] or through the constructs [Pazzano et al., 2000; Meinel et al., 2004b]. The presented protocols describe the use of spinner flasks, which are easy to set up and lead to higher mineralization rates than static cultures

[Pazzano et al., 2000; Meinel et al., 2004b,c]. The protocols suggest the use of scaffolds with a diameter of 12 mm, and diameter can be chosen between 3 and 20 mm with the same protocol. The thickness of the construct is the limiting factor. Generally, silk scaffolds prepared according to Protocol 13.3 and cut to a final thickness between 0.5 and 3 mm result in a homogenous mineralization throughout the scaffold, compared to a less dense mineralized zone in the center for scaffolds exceeding a thickness of 5 mm.

In addition to biological stimuli, bioreactors provide physiologically relevant physical signals (e.g., interstitial fluid flow, shear, pressure, mechanical compression). A recent study details the geometry and extent of mineralized tissue under laminar and dynamic flow conditions [Meinel et al., 2004b].

6.1. Preparation of Spinner Flask

Protocol 13.9. Culture of hMSCs on Silk-RGD Scaffolds in Spinner Flasks

Reagents and Materials

Sterile

- ❑ Silk or Silk-RGD scaffolds prepared according to Protocol 13.3
- ❑ Cells prepared according to Protocol 13.5
- ❑ PBSA
- ❑ Matrigel
- ❑ Ethanol 70%
- ❑ Ethanol pads
- ❑ Control medium (See Section 3.1)
- ❑ Osteogenic medium (See Section 3.3)
- ❑ Osteogenic medium double concentration (See Section 3.4)
- ❑ Autoclaved dermal puncher (diameter 3 mm–1 cm)
- ❑ Razor blades
- ❑ Tube for stoppers
- ❑ Spinner flasks
- ❑ Tweezers
- ❑ Petri dish
- ❑ Plate, 6 well
- ❑ Snap cap centrifuge tubes, 1.5 or 2 ml
- ❑ Syringe, 5 ml
- ❑ Gloves
- ❑ Tubing (for stoppers)
- ❑ Autoclaved towel (as an underlay)

Nonsterile

- ❑ Centrifuge
- ❑ Magnetic stirrer, placed in the incubator
- ❑ Incubator 37 °C, 5% CO_2

Protocol

A. Preparation of silk scaffolds

(a) Soak the scaffolds in PBSA for 10 min (e.g., in a Petri dish), until they are completely soaked.
(b) Cut scaffolds into 1- to 2-mm-thick disks with razor blades.
(c) Punch out scaffolds with diameters of 3–10 mm.
(d) Transfer scaffolds into a Petri dish and air dry overnight.
(e) Autoclave the scaffolds (121 °C, 15 min).

Note: From now on work under aseptic conditions.

(f) Transfer the scaffolds with sterile tweezers into a 6-well plate; add DMEM and transfer to incubator.

B. Preparation of spinner flasks

(a) Clean and assemble the spinner flask.
(b) Check if 20G needle can be slipped over the wire of the spinner flask.
(c) Mark 75 and 150 ml levels.
(d) Close spinner flask, wrap in aluminum foil, and autoclave.

C. Preparation of stoppers

(a) Cut tubing into 0.5-mm-thick disks.
(b) Cut disks into 4 pieces (2 mm long and 2 mm wide).
(c) Autoclave stoppers.

D. Preparation of cells

Note: Matrigel must be kept on ice to prevent from gelling. Defrost the Matrigel on ice in a 2–8 °C refrigerator (takes about 2 h)

(a) Resuspend cells from Protocol 13.5 in control medium at a concentration of 5×10^6 cells/ml.
(b) Pipette 1 ml of the cell suspension (5×10^6 cells) into each snap cap tube. Make sure you always resuspend the cells well to get the same number of cells per well.
(c) Centrifuge tubes at 300 *g* for 10 min at 4 °C.
(d) Aspirate supernate.
(e) Add 20 μl Matrigel to each cell pellet; keep suspension on ice.
(f) Take 6-well plate with scaffolds out of incubator.
(g) Aspirate the medium completely.
(h) Carefully resuspend the cells in the Matrigel with the pipette tip.
(i) Transfer cells from one tube onto one scaffold.

(j) After completing one 6-well plate, put it into the incubator for 15 min without medium to allow gel formation.
(k) Add 5 ml control medium per well.

E. Preparation of spinner flask culture

Note: Work with sterile gloves and under aseptic conditions. Work with an assistant.

(a) Let the assistant unwrap the autoclaved towel without touching it; take the towel, unwrap and use as an underlay.
(b) Same procedure with the wrapped spinner flask, tweezers, the 5-ml syringe, and needles. Place everything on the towel.
(c) Open the spinner flask; assemble syringe and needle.
(d) The assistant gets the cells seeded on the scaffolds from the incubator and opens the 6-well plate in the laminar flow hood. Everything that has been touched by the assistant is now under the hood, just next to the sterile towel ("your" aseptic area).
(e) With syringe and needle, thread one stopper; fix it with the tweezers in the middle of the needle.
(f) Use tweezers for the first scaffold to get it from the 6-well plate. Thread the cell-seeded scaffold onto the needle. Make sure the cells are on the top side!
(g) Thread a second and a third stopper on the same needle, then the second scaffold, then the fourth stopper.
(h) Slip the needle over the needle of the spinner flask and position the tip of it at the position where the upper scaffold should be. Use tweezers to slide over the first scaffold plus upper and lower stopper on the flask's needle (scaffold must be below the 150 ml mark).
(i) Repeat for the second cell-seeded scaffold.
(j) Repeat Steps (f)–(i) 3 times, so you have a total of 8 scaffolds per spinner flask thread on 4 wires.
(k) Carefully close the spinner flask.
(l) Add 150 ml medium through one sidearm.
(m) Transfer the spinner flask on the magnetic stirrer plate in the incubator.
(n) Unscrew the screw cap on both sides a little for ventilation. Do not open completely, to avoid contamination.
(o) Stir at 60 rpm.
(p) Change medium 3 times a week.

F. Medium change
(a) Close screw caps and turn off the magnetic stirrer.
(b) Take the spinner flask from the incubator into the hood; spray well with ethanol 70%.
(c) Clean sidearms with an ethanol pad and open one sidearm.

(d) With a 2-ml sterile pipette, take out a medium sample, e.g., for determination of secreted proteins.
(e) With a Pasteur pipette, aspirate medium until the liquid level equals the 75 ml mark. Avoid touching the scaffolds with the pipette.
(f) Add 75 ml fresh medium—either control medium or osteogenic medium with a double concentration—using a 25-ml pipette.
(g) Close the screw cap.
(h) Put the spinner flask back into the incubator.
(i) Unscrew the screw cap for aspiration; do not open completely to avoid contamination.
(j) Stir at 60 rpm.

7. ANALYTICAL ASSAYS

A thorough analysis of mineralization and the progression of differentiation relies on biochemical assays and quantification of gene expression, respectively.

Routine assessments involve the quantification of total calcium content and the activity of alkaline phosphatase (ALP), a cell surface protein bound to the plasma membrane through phosphatidylinositol phospholipid complexes. High ALP activities are associated with active formation of mineralized matrix, and highest levels are found in the mineralization front in bone healing [Bruder and Caplan, 1990].

Particularly important in defining the phenotype of the differentiating stem cells is an understanding of bone tissue development in relation to gene expression of the cells.

The mRNA expression of the genes encoding important proteins during osteogenic differentiation of hMSC in vitro has not yet been systematically investigated, and results from different studies are often contradictory. Identification of mRNA markers characterizing the progression of hMSC toward the osteogenic lineage is further complicated by the known variability of cells from different individuals or due to differences in the isolation protocols [Phinney et al., 1999] or limitations inherent to the techniques used (i.e., semiquantitative techniques like Northern blots or conventional RT-PCR) [Frank et al., 2002].

Recently established real-time quantitative RT-PCR technology has made mRNA analysis more reproducible, precise, and sensitive than conventional RT-PCR, because it allows (i) measurement of the amount of amplified product with a quantitative laser-based method and (ii) data collection in the early exponential phase of the PCR reaction, when none of the reagents is rate-limiting [Gibson et al., 1996].

We developed real-time quantitative RT-PCR assays for genes encoding for (i) osteoblast-related membrane and extracellular matrix molecules (i.e., alkaline phosphatase (ALP), aggrecan (Agg), bone sialoprotein (BSP), osteopontin (OP), type 1 collagen (Col1)); (ii) bone morphogenetic protein 2 (BMP-2), an important growth factor determining cell mesenchymal precursors; and (iii) cbfa1 (a genetic

regulator of osteoblast function and also known as Osf2 or RUNX2), a transcription factor related to osteogenesis.

BMP-2 is a molecule attracting MSCs, and it induces proliferation and differentiation of mesenchymal progenitor cells, producing bone tissue even at ectopic sites [Yamagiwa et al., 2001].

BSP has been proposed as the main nucleator of hydroxyapatite crystal formation and correlates with the initial phase of matrix mineralization [Ganss et al., 1999]. BMP-2 overexpression, as found in newly formed osteoblasts, is regulated through hormones and cytokines that promote bone formation [Wozney, 1989]. Osteocalcin comprises 10–20% of the noncollagenous proteins in bone, depending on age and species. Levels of ostecalcin are low at early stages and increase with increasing age. The function of ostecalcin may be to inhibit calcification until the appropriate temporal and spatial conditions are met. This was supported in vitro because ostecalcin inhibits hydroxyapatite crystal growth in solution [Luo et al., 1995].

Osteopontin is one of the most abundant noncollagenous proteins in bone; it binds to various extracellular molecules, including type I collagen, fibronectin, and osteocalcin, and may add physical strength to extracellular matrices [Denhardt and Noda, 1998].

7.1. DNA Assay

Protocol 13.10. DNA Assay of Cells Cultured on Scaffolds or as Pellets

Reagents and Materials

Nonsterile

- ❑ Cell lysis solution: Triton X-100, 0.1% (v/v) in UPW (if samples are also used for ALP Assay, use Triton X-100, 0.2% (v/v) + 5 mM Magnesium chloride in UPW)
- ❑ PicoGreen® dsDNA Quantitation Kit
- ❑ Paper towel
- ❑ Razor blade
- ❑ Microcentrifuge tubes with screw cap, 2 ml
- ❑ Steel balls
- ❑ Parafilm
- ❑ Analytical balance
- ❑ MinibeadBeater™
- ❑ Microcentrifuge
- ❑ Microtiter plate for fluorescence
- ❑ Fluorescence microplate reader

Protocol

A. Preparation of the scaffolds

(a) Grow MSCs on scaffolds in spinner flasks as described in Protocol 13.9.

(b) Dry scaffolds on a clean paper towel for 3 min on both sides.
(c) Measure wet weight of the scaffolds. Eventually cut the scaffolds into halves and measure the wet weight of each halves.
(d) Put each scaffold into a labeled microcentrifuge tube.
(e) Add 2 steel balls and 1 ml cell lysis solution per tube.
(f) Close tube firmly and wrap with Parafilm.
(g) Disintegrate scaffold by using a MinibeadBeater™ 3 times at 25,000 rpm for 10 s each time. Place on ice between cycles for cooling.
(h) Incubate at room temperature for 48 h.
(i) Transfer the content of the tube into a clean labeled tube without transferring the steel balls.
(j) Centrifuge at 3000 *g* for 10 min.
(k) Pipette the supernate into a clean, labeled tube without destroying the pellet.
(l) Proceed with the DNA Assay or store samples at −20 °C until measurement.

B. Preparation of cell pellets

(a) Grow MSCs in pellet culture according to Protocol 13.7 or 13.8.
(b) Centrifuge tube with cell pellet at 3000 *g* for 10 min at 4 °C.
(c) Aspirate supernate carefully without destroying the cell pellet.
(d) Add 300 μl cell lysis solution to each tube.
(e) Disintegrate the pellet with a pipette tip by pipetting 20 times up and down.
(f) Incubate at room temperature for 48 h.
(g) Centrifuge tube at 3000 *g* for 10 min.
(h) Transfer the supernate with a pipette into a clear microcentrifuge tube without destroying the cell pellet.
(i) Proceed with DNA Assay or store samples at −20 °C until measurement.

C. DNA Assay

(a) Thaw your samples if frozen and centrifuge at 3000 *g* for 10 min.
(b) Prepare a sample/standard setup table in your lab-book.
(c) Calculate the amount of reagents needed as follows:
provided in the kit:

Reagent A: PicoGreen dsDNA quantitation reagent (in DMSO—toxic)
Reagent B: 20× TE buffer
Reagent C: λ-DNA standard

To prepare:

Working A: Bring Reagent A to room temperature in the dark.
Amount (in ml) of Working A needed = (number of samples)/2 + 6.

Prepare a 200-fold dilution of Reagent A in Working B in a plastic container wrapped in aluminum foil.

Working B: Make a 1:20 dilution of Reagent B in DNase-free UPW. Working B is required to prepare solutions A and C and for the dilution of standards and samples. Prepare sufficient amounts according to the following equation: Amount (in ml) of Working B needed : $0.875 \times$ (number of samples) $+ 10$

Working C: Add 30 μl Reagent C to 1.47 ml Working B.

(d) Prepare the needed Working solutions.
(e) Prepare the standards according to the following table in Eppendorf tubes. Do not add Working A at this step:

DNA Concentration (ng/ml)	Working C (μl)	Working B (μl)	Working A (μl)
0 (zero standard)	0	500	500
10	5	495	500
50	25	475	500
250	125	375	500
1000	500	13050	500

(f) Add 125 μl of each sample to a new and labelled Eppendorf tube.
(g) Add 375 μl of Working B to each tube.
(h) Add 500 μl Working A into each standard or sample tube. From now on, minimize light exposure of your samples.
(i) Vortex tubes.
(j) Incubate tubes in the dark at room temperature for 3 min.
(k) Pipette 100 μl of each tube into the corresponding well of a microtiter plate.
(l) Read fluorescence with a fluorescence microplate reader set at excitation 480 nm, emission 520 nm.

△ *Safety note.* PicoGreen dsDNA quantitation reagent is toxic. Carefully read manufacturer's instructions.

D. Calculation of the results

(a) Average multiple readings for each standard or sample (if performed) and subtract the average zero standard fluorescence.
(b) Create a standard curve by reducing the data with computer software capable of generating a linear curve fit. Alternatively: plot mean fluorescence for each standard on the *y*-axis against the DNA concentration on the *x*-axis.
(c) As samples have been diluted, the concentration read from the standard curve must be multiplied by the chosen dilution factor.

7.2. ALP Assay

Protocol 13.11. Assay of Alkaline Phosphatase Activity in Bone Constructs

Reagents and Materials

Nonsterile

- ❑ Cell lysis solution: Triton X-100, 0.2% (v/v) + 5 mM magnesium chloride in UPW
- ❑ Alkaline phosphatase (ALP) Kit
- ❑ Sodium hydroxide solution, 0.2 M
- ❑ Paper towel
- ❑ Razor blade
- ❑ Eppendorf tubes
- ❑ Microcentrifuge tubes with screw cap, 2 ml
- ❑ Steel balls
- ❑ Parafilm
- ❑ Analytical balance
- ❑ MiniBeadBeater™
- ❑ Microcentrifuge
- ❑ Microtitration plate
- ❑ Microplate reader
- ❑ Waterbath

Protocol

Note: Work on ice all times unless otherwise stated.

A. Scaffolds

(a) Grow MSCs on scaffolds in spinner flasks as described in Protocol 13.9.
(b) Dry scaffolds on a clean paper towel for 3 min on both sides.
(c) Measure wet weight of the scaffolds. Eventually cut the scaffolds into halves and measure the wet weight of halves.
(d) Put each scaffold into a labeled microcentrifuge tube.
(e) Add 2 steel balls and 1 ml cell lysis solution per tube.
(f) Close tube firmly and wrap with Parafilm.
(g) Disintegrate scaffold by using a MinibeadBeater™ 3 times at 25,000 rpm for 10 s. Place on ice between cycles for cooling.
(h) Transfer the content of the tube into a clean labeled tube without transferring the steel balls.
(i) Centrifuge at 300 *g* for 10 min at 4 °C.
(j) Transfer the supernate into a clean, labeled tube. Avoid destruction of the pellet.
(k) Run ALP assay immediately.

B. Cell pellets

(a) Grow MSCs in pellet culture according to Protocol 13.7 or 13.8.

(b) Centrifuge tube with cell pellet at 300 *g* for 10 min at 4 °C.
(c) Aspirate supernate carefully without destroying the cell pellet.
(d) Add 300 μl cell lysis solution to each tube.
(e) Disintegrate the pellet with a pipette tip by pipetting 20 times up and down.
(f) Incubate at room temperature for 48 h.
(g) Centrifuge tube at 300 *g* for 10 min at 4 °C.
(h) Transfer the supernate into a clean, labeled tube. Avoid disruption of the pellet.
(i) Run ALP assay immediately.

Note: If you intend to perform the DNA Assay on the same samples, take the amount of sample needed for the ALP Assay (80 μl) out and incubate the rest of the sample for 48 h at room temperature. Proceed with Protocol 13.10A, step (i) for scaffolds and with Protocol 13.10B, step (g) for pellets.

C. ALP Assay

(a) Prepare a sample/standard setup table in your lab-book and dilute *p*-nitrophenol standards as follows.

1 mM p-Nitrophenol (ml) in Triton X-100 + $MgCl_2$	Triton X-100 0.2% (v/v) + 5 mM $MgCl_2$ (ml)	Final P-Nitrophenol Concentration (μg/ml)
0	1	0
0.05	0.95	6.955
0.2	0.8	27.82
0.6	0.4	83.46
0.9	0.1	125.19

(b) Standards can be stored at 2–8 °C for 2 months.
(c) Prepare reagents:

Working A: 0.75 M 2-Amino-2-methylpropanol (AMP):
Amount of Working A needed (in μl) :
(number of samples + number of standards + 2) × 40
Working B: 10 mM p-nitrophenylphosphate in 0.15 M AMP.
Amount of Working B needed (in μl): (number of samples + number of standards + 2) × 100

(d) Pipette 80 μl of samples or standards in labeled Eppendorf tubes at room temperature.
(e) Add 20 μl Working A to each tube.
(f) Add 100 μl of Working B to each tube.
(g) Incubate samples in a water bath at 37 °C until color develops and record time.
(h) As soon as a yellow color develops, add 100 μl of NaOH 0.2 M to stop reaction.
(i) According to the plate setup, pipette 100 μl from each tube into the corresponding well of the microtitration plate.

(j) Determine the optical density of each well with a microplate reader set to 405 nm.

D. Calculation of the results

(a) Average multiple readings for each standard and sample (if performed) and subtract the average zero standard optical density.
(b) Create a standard curve by reducing the data with computer software capable of generating a linear curve fit. Alternatively: plot mean fluorescence for each standard on the y-axis against the DNA concentration on the x-axis.
(c) As samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.
(d) Calculate the amount of p-nitrophenol produced per minute using the standard curve and the time required until color development. The incubation time chosen should fall within the linear portion of the OD versus time curve (See step (h) of Protocol C).

7.3. Calcium Assay

Protocol 13.12. Calcium Assay in Bone Constructs

Reagents and Materials

Nonsterile

- ❑ TCA 5%: trichloroacetic acid 5% (v/v) in UPW
- ❑ Calcium-phosphorus combined standard, 10 mg/dl calcium
- ❑ Calcium binding reagent
- ❑ Calcium buffer reagent
- ❑ Paper towel
- ❑ Razor blade
- ❑ Microcentrifuge tubes with screw cap, 2 ml
- ❑ Steel balls
- ❑ Parafilm
- ❑ Analytical balance
- ❑ MiniBeadBeater™
- ❑ Microcentrifuge
- ❑ Microtiter plate
- ❑ Microplate reader

Protocol

A. Scaffolds

(a) Grow MSCs on scaffolds in spinner flasks as described in Protocol 13.9.
(b) Dry scaffolds on a clean paper towel for 3 min on both sides.
(c) Measure wet weight of the scaffolds. Eventually cut the scaffolds into halves and measure the wet weight of halves.
(d) Transfer each scaffold into a labeled microcentrifuge tube.

(e) Add 2 steel balls and 1 ml TCA 5% per tube.
(f) Close tube firmly and wrap with Parafilm.
(g) Disintegrate scaffold by using a MinibeadBeater™ 3 times at 25,000 rpm for 10 s each time. Place on ice between cycles for cooling.
(h) Incubate the tubes at room temperature for 30 min.
(i) Centrifuge at 3000 *g* for 10 min.
(j) Pipette the supernate into a clean, labeled tube without disrupting the pellet.
(k) Add 1 ml of TCA 5% into the tube with the steel balls.
(l) Incubate the tubes at room temperature for 30 min.
(m) Combine the 2 corresponding TCA solutions.
(n) Centrifuge at 3000 *g* for 10 min.
(o) Transfer supernate into a clean, labeled tube without disrupting the pellet.

B. Cell pellets
(a) Grow MSCs in pellets according to Protocols 13.7 or 13.8.
(b) Centrifuge tube with cell pellet at 3000 *g* for 10 min at 4 °C.
(c) Aspirate supernate carefully without destroying the cell pellet.
(d) Add 250 μl of TCA 5% to each tube.
(e) Disintegrate the pellet with a pipette tip by pipetting 20 times up and down.
(f) Incubate the tubes at room temperature for 30 min.
(g) Centrifuge tube at 3000 *g* for 10 min at 4 °C.
(h) Transfer supernates in clear microcentrifuge tubes without destroying the cell pellet.
(i) Add 250 μl of TCA 5% to each tube containing the pelleted cell debris.
(j) Disintegrate the pellet with a pipette tip by pipetting 20 times up and down.
(k) Let the tube stand at room temperature for 30 min.
(l) Centrifuge tube at 3000 *g* for 10 min at 4 °C.
(m) Combine the supernate with the one previously collected at (h).

C. Calcium Assay
(a) Prepare a sample/standard setup table in your lab-book and dilute calcium standards as follows

Calcium Standard Sigma Ca^{2+} 10 mg/dl	100% TCA	UPW	Final Ca^{2+} concentration
1.6 ml	0.1 ml	0.3 ml	80 μg/ml
1.2 ml	0.1 ml	0.7 ml	60 μg/ml
0.8 ml	0.1 ml	1.1 ml	40 μg/ml
0.4 ml	0.1 ml	1.5 ml	20 μg/ml
0.2 ml	0.1 ml	1.7 ml	10 μg/ml
0 ml	0.1 ml	2.0 ml	0 μg/ml

(b) Standards can be stored at 2–8 °C.
(c) Pipet 10 μl of each standard or sample in duplicate into microtiter plate wells.

(d) Count total well number.
(e) Freshly prepare a 1:1 dilution of calcium binding reagent and calcium buffer reagent; 100 μl solution is required per well.
(f) Pipette 100 μl of this solution into each well.
(g) Incubate plate at room temperature in the dark for 5 min.
(h) Determine the optical density of each well with a microplate reader set to 575 nm.

Note: If absorption of sample exceeds the highest standard, dilute the sample in TCA 5% and read again.

D. Calculation of the results
(a) Average the duplicate readings for each standard and sample and subtract the average zero standard optical density.
(b) Create a standard curve by reducing the data with computer software capable of generating a linear curve fit. Alternatively: plot mean absorbance for each standard on the *y*-axis against total Ca^{2+} concentration on the *x*-axis.
(c) As samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

7.4. Glycosaminoglycan (GAG) Assay

Protocol 13.13. Assay of GAGs in Cartilage and Bone Constructs

Reagents and Materials
Nonsterile

- ❑ PBE buffer in UPW:
  0.1 M Disodium hydrogen phosphate ($M_r = 141.95$)
  0.01 M EDTA disodium salt ($M_r = 372.24$)
  Adjust pH to 6.5, filter sterilize, store at 4 °C; shelf life 6 months
- ❑ Chondroitin sulfate standard stock solution 50 mg/ml (CS standard) in PBE buffer
  50 mg/ml chondroitin sulfate
  Cysteine 14.4 mM ($M_r = 121.2$)
  Can be stored at −20 °C for 1 year. On the experiment day, dilute with PBE to a final concentration of 100 μg/ml
- ❑ Dimethylmethylene blue solution (DMMB) in UPW:
  40.5 mM Glycine ($M_r = 75.07$)
  Sodium chloride ($M_r = 58.45$)
  Dissolve 2.37 g sodium chloride and 3.04 g glycine in 905 ml UPW, then add 95 ml 0.1 M hydrochloric acid. While stirring, add 16 mg DMMB to the solution. Check pH = 3 and optical density at 525 nm = 0.31–0.34. Store at room temperature in the dark (aluminum foil).
- ❑ Papain solution (freshly prepared): 14.4 mM Cysteine ($M_r = 121.15$) in 20 ml PBE buffer and adjust to pH 6.3; filter through 0.22-μm filter. Add 0.1 ml sterile papain stock.

- ❑ Paper towel
- ❑ Razor blade
- ❑ Microcentrifuge tubes, 2 ml, with screw cap
- ❑ Needle, 20 G
- ❑ Water bath at 60 °C
- ❑ Parafilm
- ❑ Vortex mixer
- ❑ Photometer and cuvettes

Protocol

A. Scaffold

(a) Grow MSCs on scaffolds in spinner flasks as described in Protocol 13.9.
(b) Dry the scaffold on a clean paper towel for 3 min on both sides.
(c) Measure wet weight of the scaffolds. Eventually cut scaffolds into halves and note the wet weight of halves as well.
(d) Put each scaffold into a labeled microcentrifuge tube.
(e) Punch 10 little holes into the cap of a microcentrifuge tube.
(f) Freeze constructs at −80 °C overnight.
(g) Lyophilize for 2–3 days and measure the dry weight of each scaffold.
(h) Add 1 ml papain solution per tube.
(i) Close tube with a new cap; wrap with Parafilm.
(j) Incubate at 60 °C for 16 h, vortexing 5 times.
(k) Centrifuge at 3000 *g* for 10 min at 4 °C.
(l) Perform GAG assay with supernate.

B. Pellets

(a) Grow MSCs in pellets according to Protocols 13.7 or 13.8.
(b) Centrifuge tube with cell pellet at 3000 *g* for 10 min at 4 °C.
(c) Aspirate supernate.
(d) Punch 10 little holes into the cap of a microcentrifuge tube.
(e) Freeze constructs at −80 °C overnight.
(f) Lyophilize for 2–3 days.
(g) Add 0.5 ml of papain solution per tube.
(h) Close tube with a new cap; wrap with Parafilm.
(i) Incubate at 60 °C for 16 h, vortexing 5 times.
(j) Centrifuge at 3000 *g* for 10 min at 4 °C.
(k) Perform GAG assay with supernate.

C. GAG Assay

Note: DMMB solution must be checked before use. Keep indicated times constant.

(a) Set photometer to 525 nm.

(b) Pipet 0.1 ml standard solution (See table below) or sample into cuvette.
(c) Add 2.4 ml DMMB solution.
(d) Cover cuvette with Parafilm and vortex.
(e) Read absorbance against blank
(f) Standards:

STD No.	CS (100 μg/ml) (ml)	PBE (ml)	DMMB solution (ml)	CS (μg)
Blank	0	0.1	2.4	0
1	0.01	0.09	2.4	1
2	0.02	0.08	2.4	2
3	0.03	0.07	2.4	3
4	0.04	0.06	2.4	4
5	0.05	0.05	2.4	5
6	0.07	0.03	2.4	7
7	0.1	0	2.4	10

(g) Prepare all standards and samples in triplicates.

D. Calculation of the results

(a) Average the triplicate readings for each standard and sample and subtract the average blank optical density.
(b) Create a standard curve by reducing the data with computer software capable of generating a linear curve fit. Alternatively, plot mean absorbance for each standard on the *y*-axis against the CS concentration on the *x*-axis.
(c) As samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

7.5. TRIzol RNA Extraction

Protocol 13.14. RNA Extraction from Bone Constructs

Reagents and Materials

Nonsterile

- ❑ TRIzol
- ❑ Chloroform
- ❑ Ethanol, 70%
- ❑ RNAseZap®
- ❑ Qiagen RNeasy Mini Kit

△ *Safety notes.* TRIzol reagent is carcinogenic. Use gloves and eye protection. Avoid contact with skin or inhalation. Always work under a fume hood. Read manufacturer's safety instructions.

Chloroform is carcinogenic; wear safety glasses and gloves and always work under a fume hood. Read safety data instructions.

- ❑ Paper towel
- ❑ Microcentrifuge tubes, 2 ml, with screw cap
- ❑ Microcentrifuge

Protocol

Note: Contamination with RNA must be minimized. Please note: Human skin is the major source of contamination!

Precautions to prevent RNase contamination

- ♦ Always wear disposable gloves. Change gloves frequently.
- ♦ Use sterile RNAse-free, disposable plasticware and automatic pipettes reserved for RNA work.
- ♦ RNA is protected from RNase contamination in TRIzol reagent. Downstream sample handling requires RNase-free glassware or plasticware. Glass and metal items (tweezers, steel balls, beakers) can be exposed to 300 °C for 4 h, and plastic items (tubes) can be soaked for 10 min in 0.1 M NaOH + 1 mM EDTA, rinsed thoroughly with RNAse-free water, and autoclaved. RNAse-free containers are also commercially available.
- ♦ To prepare RNase-free water, 0.01% (v/v) diethylpyrocarbonate (DEPC) into UPW in RNase-free glass bottles. Incubate for 12 h at room temperature, then autoclave bottle (read instruction for autoclaving liquids).
- ♦ Clean all surfaces (for example, bench top) thoroughly with RNAseZap®, wipe with paper towel, rinse with water, and dry with clean paper towel.

A. Scaffolds

(a) Grow MSCs on scaffolds in spinner flasks according to Protocol 13.9.
(b) Dry scaffolds on a clean paper towel for 3 min on both sides.
(c) Measure wet weight of the scaffolds. Eventually cut the scaffolds into halves and measure the wet weight of halves.
(d) Transfer each scaffold into a labeled microcentrifuge tube.
(e) Add 2 steel balls and 1 ml TRIzol per tube.
(f) Close tube firmly and wrap with Parafilm.
(g) Disintegrate scaffolds by using a MinibeadBeater™ 6 times at 25,000 rpm for 10 s. Place on ice between cycles for cooling.
(h) Transfer the content of the tube into a clean labeled tube without transferring the steel balls.
(i) Centrifuge at 12,000 *g* for 10 min at 4 °C.
(j) Transfer supernate into a clean, labeled tube without destroying the pellet, and immediately perform RNA Extraction or freeze samples at −80 °C for later analysis.

B. Cell pellets

(a) Grow MSCs in pellets according to Protocols 13.7 or 13.8.
(b) Centrifuge tube with cell pellet at 3000 *g* for 10 min at 4 °C.
(c) Aspirate supernate carefully without destroying the cell pellet.
(d) Add 1 ml TRIzol to each tube.
(e) Disintegrate the pellet with a pipette tip by pipetting 20 times up and down.
(f) Centrifuge tube at 12,000 *g* for 10 min at 4 °C.
(g) Transfer supernate into a clear microcentrifuge tube without destroying the cell pellet, and immediately perform RNA Extraction or freeze samples at −80 °C for later analysis.

C. RNA Extraction

Before starting to work, clean all working surfaces with RNAse Zap® as above. Change gloves frequently. If not mentioned otherwise, work on ice.

(a) Incubate sample at room temperature for 5 min.
(b) Add 190 μl of chloroform to each tube. A clear layer should occur.
(c) Vortex tube vigorously for 15 s.
(d) Incubate sample at room temperature for 3 min.
(e) Centrifuge tube at 12,000 *g* for 15 min at 4 °C. An upper clear aqueous phase containing the RNA appears.
(f) Carefully transfer upper aqueous phase to a fresh tube (do not transfer the interface); estimate the transferred volume you can take out. It is important not to disturb the lower layers during extraction—this will contaminate your sample and may influence enzyme activity of later experiments.
(g) Add the same volume of 70% ethanol to the homogenized lysate (estimated in the previous step), and mix well by pipetting. Do not centrifuge.
(h) Apply up to 700 μl sample, including precipitate that may have formed, to an RNeasy mini spin column sitting in a 2-ml collection tube and centrifuge at 8000 *g* for 15 s. If the volume of your sample exceeds 700 μl, successively load aliquots onto the RNeasy column and centrifuge as above. Discard flow-through and reuse. Reuse the collection tube for the following step.
(i) Pipette 700 μl Buffer RW1 onto the RNeasy column, and centrifuge at 8000 *g* for 15 s.
(j) Transfer RNeasy column into a new 2-ml collection tube.
(k) Discard flow-through and old collection tube.
(l) Pipet 500 μl Buffer RPE onto the RNeasy column.
(m) Centrifuge at 8000 *g* for 15 s.
(n) Discard flow-through and reuse collection tube.
(o) Pipet 500 μl Buffer RPE onto the RNeasy column.
(p) Centrifuge at maximum speed for 2 min to dry the membrane.
(q) Transfer the RNeasy column into a new 1.5-ml Eppendorf tube and pipette 30 μl RNAse-free water directly onto the RNeasy membrane.

(r) Centrifuge at 8000 *g* for 1 min. The RNA is now in the collection tube.
(s) Close tube and immediately freeze at $-80\,^{\circ}C$.

7.6. Real-Time RT-PCR

Protocol 13.15. Gene Expression Analysis in Bone and Cartilage Constructs

Reagents and Materials

Nonsterile

- ❑ RNA from Protocol 13.14
- ❑ SuperScript™ First-Strand Synthesis System for RT-PCR
- ❑ Custom designed primers and probes or Assay on Demand (Applied Biosystems)
- ❑ TaqMan Universal PCR Mix
- ❑ Microcentrifuge tubes, 0.2 ml, or thin-walled PCR tubes, autoclaved
- ❑ PCR tubes or plates with caps
- ❑ Programmable thermal cycler
- ❑ Microcentrifuge
- ❑ ABI PRISM® 7000 Sequence Detection System
- ❑ Vortex mixer

Protocol

A. cDNA synthesis with Oligo(dT)

(a) Mix and briefly centrifuge each component prior to use.
(b) Prepare RNA-primer mixtures in a 0.2-ml PCR tube as follows:

RNA-primer mixture:	μl
RNA	8
dNTP mix	1
Oligo(dT)$_{12-18}$ (0.5 μg/μl)	1

(c) Incubate each sample at $65\,^{\circ}C$ for 5 min.
(d) Place samples on ice for at least 1 min.
(e) Prepare the following reaction mixture, adding each component in the indicated order. Prepare reaction mixture for total number of samples +3 reactions.

Reaction mixture	Per sample (μl)
10× RT buffer	2
25 mM $MgCl_2$	4
0.1 M DTT	2
RNaseOUT™	1

(f) Add 9 μl reaction mixture to each RNA-primer mixture, mix gently, and collect by brief centrifugation.

(g) Incubate at 42 °C for 2 min.
(h) Add 1 μl (50 units) of SuperScript™ II RT to each tube and mix by pipetting.
(i) Incubate at 42 °C for 50 min.
(j) Terminate the reactions at 70 °C for 15 min.
(k) Chill on ice.
(l) Add 1 μl RNase H to each tube and incubate at 37 °C for 20 min.
(m) Freeze samples at −80 °C or proceed directly with the PCR reaction protocol.

B. Real-time RT-PCR Reaction Protocol

Note: Keep all reagents on ice at all times; probes are light sensitive. Darken the room.

(a) Prepare Master Mix for each marker of interest in the order as outlined in the tables below. Prepare all mixes for your number of samples +3 reactions.
Custom designed Primers and Probes Master Mix:

Reagents	Volume (μl) per sample
H_2O	13
Forward primer	4.5
Reverse primer	4.5
Probe	2
2× TaqMan Universal Master Mix	25

Assay on Demand Master Mix:

Reagents	Volume (μl) per sample
H_2O	21.5
AoD Kit	2.5
2× TaqMan Universal Master Mix	25

(b) Add 1 μl cDNA to each reaction tube.
(c) Add 49 μl Master Mix to each tube and mix well by pipetting.
(d) Close wells tightly with cover strips.
(e) Perform RT-PCR according to the manufacturer's protocol.

Calculations:
(a) ct Gene of interest—ct GAPDH $= \Delta$ ct Gene of interest
(b) Δ ct zero point (control)—Δ ct Gene of interest $= \Delta\Delta$ ct
$2^{\Delta\Delta ct}$ = expression

Custom designed primers and probes (Applied Biosystems):

Forward GAPDH primer:	ATG GGG AAG GTG AAG GTC G
Reverse GAPDH primer:	TAA AAG CCC TGG TGA CC
GAPDH probe:	VIC CGC CCA ATA CGA CCA AAT CCG TTG AC TAMRA
Forward Collagen 1 primer:	CAG CCG CTT CAC CTA CAG C
Reverse Collagen 1 primer:	TTT TGT ATT CAA TCA CTG TCT TGC C
Collagen 1 probe:	6FAM CCG GTG TGA CTC GTG CAG CCA TC TAMRA
Forward Collagen 2 primer:	GGC AAT AGC AGG TTC ACG TAC A
Reverse Collagen 2 primer:	CGA TAA CAG TCT TGC CCC ACT T
Collagen 2 probe:	6FAM ATG GAA CAC GAT GCC TTT CAC CAC GA TAMRA

Dilute all of these in 1× TE buffer to the appropriate concentration (according to the table below).

Reference for all sequences: Eckstein et al. (2001).

Marker	Aliquoted Primers (μM)	Aliquoted Probe (μM)
GAPDH	10	6.25
Collagen 1	10	6.25
Collagen 2	10	6.25

Assay on Demand (Applied Biosystems):

Human aggrecan 1:	Hs00153936_m1
Human ALP:	Hs00240993_m1
Human BMP-2:	Hs00154192_m1
Human BSP:	Hs0000173720_m1
Human cbfa1 (RUNX2):	Hs00231692_m1
Human COX-2:	Hs00153133_m1
Human IGF-I:	Hs00153126_m1
Human IL-1β:	Hs00174097_m1
Human iNOS:	Hs00167248_m1
Human OP:	Hs00167093_m1
Human SRY-box-9:	Hs00165814_m1

Note: Always check updates on the homepage of Applied Biosystems: http://www.appliedbiosystems.com

8. HISTOLOGY

Protocol 13.16. Fixation and Paraffin Embedding of Bone and Cartilage Constructs

Reagents and Materials

Non-sterile

- ❑ 10% Neutral buffered formalin
- ❑ PBSA
- ❑ HistoGel™
- ❑ Ethanol
- ❑ Xylene (mixed xylene isomers)

△ *Safety note.* Xylenes and formalin is harmful, always work under fume hood. Read safety sheets carefully.

- ❑ Histology embedding cassettes
- ❑ Paraffin embedding station
- ❑ Lens paper
- ❑ Oven

Protocol

A. Scaffolds

(a) Grow MSCs on scaffolds in spinner flasks according to Protocol 13.9.
(b) Transfer each scaffold into a labeled histocassette.
(c) Incubate in 10% neutral buffered formalin at 4 °C for 24 h.
(d) Place in PBS at 4 °C until further processing.

B. Preparation of cell pellets

(a) Grow MSCs in pellets according to Protocol 13.7 or 13.8.
(b) Wrap each pellet into lens paper
(c) Transfer each package into a labeled histocassette
(d) Incubate in 10% neutral buffered formalin at 4 °C for 24 h.
(e) Place in PBS at 4 °C for at least 2 h.
(f) Encapsulate each pellet into HistoGel™ according to the manufacturer's instruction.
(g) Transfer to labeled histocassette and store in PBS at 4 °C until further processing.

C. Preparation for histology

(a) Dehydrate specimens in histocassettes with a series of alcohols: 70% ethanol for 24 h.
(b) 90% Ethanol for 1 h.

(c) 2 times 95% ethanol for 1 h each.
(d) 3 times 100% ethanol for 1 h each.
(e) 3 times xylene for 30 min each.
(f) Paraffin at 60 °C for 1 h.
(g) Paraffin at 60 °C overnight.
(h) Paraffin at 60 °C for 1 h.
(i) Embed specimens in paraffin blocks at an embedding station. If desired, cut scaffolds and show cross-sections by embedding accordingly.
(j) Cut 5-μm thin sections from the block with a microtome.
(k) Lift the cut sections onto a glass slide and air dry

Protocol 13.17. Von Kossa Staining of Sections of Bone Constructs

Note: Long exposure to strong light can lead to false positive stain.

Reagents and Materials

- ❑ Paraffin slides of specimens from Protocol 13.16
- ❑ Ethanol
- ❑ Xylene
- ❑ Freshly prepared 5% silver nitrate solution in UPW
- ❑ 5% Hypo solution (sodium thiosulfate) in UPW
- ❑ Nuclear-fast Red
- ❑ Cytoseal™ 60
- ❑ Oven
- ❑ Lamp, 60 W
- ❑ Aluminum foil
- ❑ Holder for histoslides
- ❑ Coverslips

Protocol

A. Deparaffinization and Hydration

(a) Dewax slides in oven at 55 °C for 30 min.
(b) 2 times xylene for 5 min each.
(c) 2 times 100% ethanol for 5 min each.
(d) 2 times 95% ethanol for 3 min each.
(e) 70% Ethanol for 3 min.
(f) UPW for 1 min.
(g) Proceed directly to staining.

B. Staining

(a) Incubate in 5% silver solution. Place in front of a 60 W lamp, place aluminum foil behind jar to reflect the light for 1 h or until calcium turns black.

(b) Rinse 3 times in UPW.
(c) 5% Hypo solution for 5 min.
(d) Wash in tap water.
(e) Rinse in UPW.
(f) Counterstain in Nuclear Fast Red for 5 min.
(g) Wash in UPW.
(h) Continue with dehydration and mounting step.

C. Dehydration and mounting
(a) Dehydrate slides with a series of alcohols: 2 times 5 dips in ethanol 95%.
(b) 2 times 5 dips in ethanol 100%.
(c) 2 times 3 min in xylene.
(d) Mount with Cytoseal™ 60 and cover with coverslip.

Protocol 13.18. Safranin-O Staining of Sections of Cartilage Constructs

Reagents and Materials

Non-sterile

- ❑ Paraffin slides of specimens from Protocol 13.16
- ❑ Ethanol
- ❑ Xylene (mixed xylene isomers)
- ❑ Harris hematoxylin
- ❑ 0.5% acetic acid in 70% ethanol
- ❑ 0.02% Fast Green in UPW
- ❑ 1% acetic acid in UPW
- ❑ 0.1% Safranin O in UPW
- ❑ Cytoseal™ 60
- ❑ Oven
- ❑ Holder for histoslides
- ❑ Coverslips

Protocol

(a) Deparaffinize and hydrate according to Protocol 13.17 A Steps (a) to (g).
(b) Incubate in Harris hematoxylin for 8 min.
(c) Rinse in UPW.
(d) Dip 2 times in 0.5% ethanolic acetic acid.
(e) Rinse with running water to enhance blue staining of nucleus for at least 5 min.
(f) 0.02% Aqueous Fast Green for 4 min.
(g) 3 Dips in 1% acetic acid.
(h) 0.1% Safranin O for 6 min.
(i) Continue with dehydration and mounting step from Protocol 13.17C, Steps (a) through (d).

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9. IN VITRO APPLICATIONS

A number of methods and techniques have been established to treat large bone defects after, for example, trauma or in the context of tumors. These methods do not restore major damage to a tissue or organ in a truly satisfactory way. At the same time, the need for bone substitutes increases with the progressive aging of the population. Routine techniques rely on the harvest of autologous bone, a method limited by the availability of transplant material, harvesting difficulties, donor site morbidity, additional pain, longer hospital stays, and higher treatment costs, with a direct impact on function, availability, and psychological and social well-being of patients. Novel methods currently being studied include conduction (by a scaffold) and induction (by bioactive molecules) of cell migration to repair relatively small defects and cell transplantation into the defect site (with or without biomaterial) to repair larger defects [Alsberg et al., 2001]. The tissue engineering approach as described in this chapter involves the use of mechanically robust and osteoconductive biomaterials seeded with mesenchymal stem cells, exposed to osteogenic stimuli [Meinel et al. 2004b,c]. Compared to the transplantation of cells alone, in vitro-grown tissue constructs offer the potential advantage of immediate functionality. The in vitro environment is indeed heavily simplified as compared to the complex physiological situation, and therefore tissue engineers search for simplifying principles that allow at least the recapitulation of some aspects of tissue morphogenesis and cellular assembly into tissue structures [Lauffenburger and Griffith, 2001]. The proposed approach uses silk as a biomaterial to provide an osteoconductive environment. The advantages are good biocompatibility, cell attachment, and cell differentiation along with unique mechanical properties. The sustained degradation of silk provides a robust template for the cells to deposit bone on the lattice surface, thereby resulting in a biomimetic and three-dimensional trabecular network.

We did not observe this orientation of the deposited bone in faster degrading collagen protein or on PLGA composite scaffolds. Therefore, silks can provide a blueprint for the desired bone geometry through the design of scaffold structures. In principle, this would allow the engineering of any trabecular geometry, but in reality it does not. We have shown that a network with pore sizes ranging between 200 and 700 μm can be engineered, and probably this can be extended to bigger pore sizes. Geometries less than 200 μm result in the formation of a dense and continuous bone plate, originating from a coalescing trabecular network (Fig. 13.3). This feature may be particularly interesting for the subchondral bone plate, present at the interface between the epiphysis and the overlying hyaline cartilage. Tissue engineering of cartilage with silk scaffolds has been demonstrated before [Meinel et al. 2004a], and therefore the presented results demonstrate all elements necessary to engineer ostechondral grafts. However, the engineering of complex composite tissues within a single scaffold requires the exposure of one cell source—mesenchymal stem cells—to different growth factors to drive the differentiation along the desired lineages. This requires a spatially restricted functionalization of the scaffold with growth factors and, consequently, functional

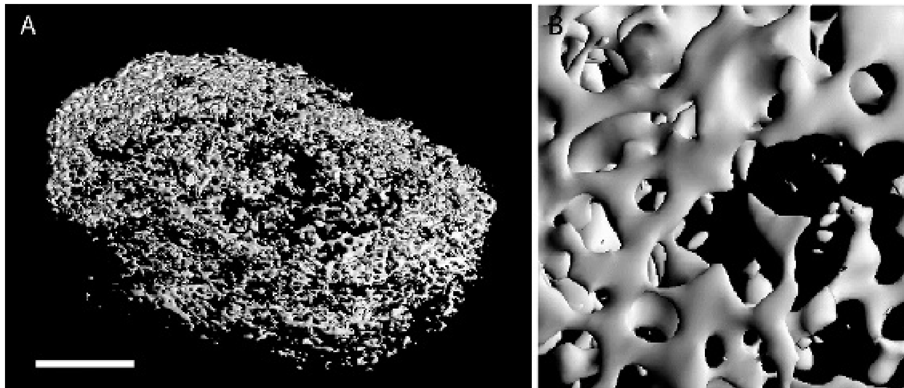

Figure 13.3. Tissue engineering of bone-like tissue. Bar = 5 mm (A). Magnification shows trabecula-like structure (B).

groups that allow an easy manipulation of the biomaterial. Silks, as do other proteins, provide all kinds of functional groups, allowing covalent decoration through easy chemical reaction. Therefore, silks offer an advantage because of their easy manipulation to serve as functionalized biomaterials for the engineering of complex composite tissues.

Diffusional limitations during culture, although in part overcome by the use of bioreactors, still remain the major challenge in tissue engineering and generally result in bone tissues with a thickness of less than 0.5 mm [Ishaug et al., 1997]. Although these protocols serve as a basis to engineer trabecular networks of more than 3 mm, the connectivity, trabecular surface, and volume are higher at the outer areas of the scaffold as compared to the center section. We have tried to overcome these limitations in earlier perfusion studies by forcing the medium flow through collagen [Meinel et al. 2004b] and silk scaffolds (unpublished data). However, mineralization was significantly less compared to the use of spinner flasks, and bone formation was restricted to mineralized rods with the absence of a trabecular network. A possible avenue to address the problem of restricted diffusion mass transport may be mechanical stimulation. Silks may again be particularly well suited for mechanical stimulation, which would allow a series of mechanical simulations without altering the mechanical integrity. In addition to the restricted mass transport, inhomogeneous seeding of the scaffold with cells can contribute to the variances in mineralization [Vunjak-Novakovic et al., 1996, 1998, 1999; Vunjak-Novakovic, 2003].

Despite all the advantages, silks as natural products have an intrinsic batch-to-batch variability, depending on the sericulture of the silkworms, nutrition, and temperature of silkworm culture. Aside from existing natural sources of silk, future options provided by the availability of reasonable quantities of genetically engineered silk variants would expand the set of structures available for use in vivo, including spider silk. These techniques result in homogenous and reproducible fabrication of silks, overcoming natural variability.

In summary, silks provide interesting and unique properties for bone tissue engineering promoting orderly tissue regeneration. Bone formation results in complex trabecular networks, predetermined by the lattice structure of the silk scaffolds. This allows the custom-made fabrication of cancellous bone implants, highly resembling the geometry at the implantation site. Furthermore, the unique properties of silks offer the advantage of prolonged mechanical competence together with the possibility of exposing the biomaterial to repetitive cycles of mechanical stimulation without compromising mechanical integrity.

SOURCES OF MATERIALS

Materials	*Suppliers*
Acetic acid	Sigma
Accuspin™ tubes	Sigma Diagnostics
ALP Kit: Alkaline phosphatase (ALP)	Sigma Diagnostics
AMP (2-Amino-2-methylpropanol), alkaline buffer solution A9226 or as provided in the ALP Kit	Sigma
Antibodies for FACS analysis:	
CD31-PE (555446)	Pharmingen (BD Biosciences)
CD34-APC (555824)	Pharmingen (BD Biosciences)
CD14-FITC (555397)	Pharmingen (BD Biosciences)
CD71-APC (551374)	Pharmingen (BD Biosciences)
Mouse anti-human CD105 (555690)	Pharmingen (BD Biosciences)
CD45-APC (555485)	Pharmingen (BD Biosciences)
CD44-FITC (555478)	Pharmingen (BD Biosciences)
Ascorbic acid phosphate	Sigma
bFGF (human, recombinant) (basic fibroblast growth factor)	Invitrogen
BMP-2, bone morphogenetic protein	Wyeth
Bone marrow	Cambrex
BupH™ MES buffered saline	Pierce
Calcium binding reagent	Sigma Diagnostics
Calcium buffer reagent	Sigma Diagnostics
Calcium-phosphorus combined standard, 10 mg/dl calcium	Sigma Diagnostics
Chloroform	Sigma
Chondroitin sulfate A, sodium salt from bovine trachea, 70%	Sigma
Cocoons from *Bombyx mori* (Linne, 1758)	Individual sources
Collagen: Avitene Ultrafoam collagen sponge	Bard
Cysteine	Sigma
Cytoseal™ 60	Microm
DEPC, diethylpyrocarbonate	Sigma
Dermal puncher	Miltey
Dexamethasone	Sigma
1,9-Dimethylmethylene blue dye (“Eschenmoser’s salt”)	Sigma-Aldrich
Disodium hydrogen phosphate	Sigma

Materials	*Suppliers*
DMSO, dimethyl sulfoxide	Sigma-Aldrich
Dulbecco's modified Eagle's medium (DMEM)	Invitrogen
EDC, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide	Pierce
EDTA disodium salt	Sigma
Ethanol	Sigma
Fast Green FCF	Sigma
Fetal bovine serum (FBS)	Invitrogen
Formalin, 10% neutral buffered	Sigma
Fungizone	Invitrogen
Glutamic acid	Sigma
β-Glycerophosphate	Invitrogen
Glycine	Sigma
Hematoxylin solution, Harris modified	Sigma
HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol	Sigma-Aldrich
HistoGel™	Lab Storage Systems Inc.
Histopaque®-1077	Sigma
Hydroxylamine hydrochloride	Pierce
Lithium bromide	Sigma
Matrigel, basement membrane matrix, from BD Biosciences	
Magnesium chloride	Sigma
MEM nonessential amino acids solution	Invitrogen
2-Mercaptoethanol	Sigma
Methanol	Sigma
Millex-SV syringe-driven filter unit, pore size 5 μm	Millipore
MiniBeadBeater™	Biospec
Nalgene™ Cryo 1 °C Freezing Container	Nalge Nunc
Nuclear Fast Red	Sigma
Papain suspension, activates to ≥20 units per mg protein	Worthington
PBSA: phosphate-buffered saline, w/o calcium or magnesium	Invitrogen
Penicillin-streptomycin, liquid	Invitrogen
PicoGreen® dsDNA Quantitation Kit	Molecular Probes
RGD: GRGDS: H-Gly-Arg-Gly-Asp-Ser-OH	Calbiochem
RNAseZap®	Ambion
Rneasy Mini Kit	Qiagen
RPMI 1640	Invitrogen
Safranin O	Sigma
Silver nitrate	Sigma
Slide-a-Lyzer® Dialysis Cassette, cut-off 2000 g/mol	Pierce
Sodium carbonate	Sigma
Sodium chloride USP granular	Fisher
Sodium chloride	Sigma
Sodium hydroxide	Sigma
Sodium thiosulfate	Sigma
Spinner flask	Bellco
Sucrose	Fluka
Sulfo-NHS, *N*-Hydroxysulfosuccinimide	Pierce

Materials	*Suppliers*
SuperScript™ First-Strand Synthesis System for RT-PCR	Invitrogen
TaqMan 2× PCR Master Mix	Applied Biosystems
TCA, trichloroacetic acid	Sigma
Teflon PFA container, 5-ml round vials with snap cap	Cole Parmer
TGF-β_1, transforming growth factor-β_1, human recombinant	R&D systems
Triton X-100	Sigma
TRIzol	Invitrogen
Trypan Blue	Invitrogen
Trypsin-EDTA, liquid, 0.25% trypsin, 1 mM EDTA	Invitrogen
Tween 80	Sigma
Xylene (mixed xylene isomers, X2377)	Sigma

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