Part II

Tissue Engineering

6

Tissue Engineering: Basic Considerations

Gordana Vunjak-Novakovic

Department of Biomedical Engineering, Columbia University, 363G Engineering Terrace, Mail Code 8904, 1210 Amsterdam Avenue, New York, NY 10027, gv2131@columbia.edu

1. Introduction 132
2. In vitro Cultivation of Engineered Tissues 132
   2.1. Overall Approach 132
   2.2. Functional Tissue Engineering 133
   2.3. Model System 133
3. Cells 135
   3.1. Basic Requirements 135
   3.2. Overview of Cell Sources 136
4. Media 137
   4.1. Chondrocytes 137
   4.2. MSCs 137
   4.3. Cardiac Myocytes 138
5. Biomaterial Scaffolds 138
   5.1. Basic Requirements 138
   5.2. Overview of Scaffolds 138
6. Bioreactors 139
   6.1. Basic Requirements 139
   6.2. Overview of Bioreactor Types 140
7. Scaffold Seeding 142

Culture of Cells for Tissue Engineering, edited by Gordana Vunjak-Novakovic and R. Ian Freshney

7.1. Basic Requirements 142
7.2. Dynamic Seeding in Spinner Flasks 142
Protocol 6.1. Dynamic Seeding in Spinner Flasks for Cartilage Tissue Engineering 142
7.3. Gel-Cell Seeding of Porous Scaffolds 144
Protocol 6.2. Gel-Cell Seeding of Porous Scaffolds for Cardiac Tissue Engineering 144
Protocol 6.3. Perfusion of Porous Scaffolds for Tissue Engineering 145
8. Bioreactor Cultivation 146
8.1. Hydrodynamic Environment 146
8.2. Growth Factors 146
8.3. Physical Signals 147
8.4. Structural and Functional Assessment of Engineered Constructs 148
9. Summary 149
Acknowledgments 150
Sources of Materials 150
References 150

1. INTRODUCTION

Tissue engineering combines the principles of biology, engineering, and medicine to create biological substitutes for lost or defective native tissues. One approach to tissue engineering involves the generation of immature but functional tissue grafts in vitro and their maturation after implantation in vivo. Constructs engineered in vitro can also serve as high-fidelity models for quantitative studies of cell and tissue responses to genetic alterations, drugs, hypoxia, and mechanical stimuli. Extensive reviews of the methods and principles of tissue engineering can be found elsewhere [Lanza et al., 2000; Atala and Lanza, 2002]. In this book, we focus on selected examples of functional tissues engineered in vitro. Chapters 7–15 review the approaches used for successful tissue engineering of cartilage, bone, ligaments, blood vessels, cardiac muscle, skeletal muscle, and liver. In this introductory chapter to Part II, we review the principles and representative methods of tissue engineering, using cartilage and cardiac muscle as examples of two distinctly different tissues of substantial clinical interest that impose different requirements for the design and operation of tissue engineering systems.

2. IN VITRO CULTIVATION OF ENGINEERED TISSUES

2.1. Overall Approach

There is a serious lack of suitable donor tissues for transplantation, along with an increasing number of patients in need of transplantable tissues and organs. The disparity between the need and availability of donor tissues has motivated the tissue engineering approach, aimed at creating cell-based substitutes of native tissues. The overall concept of tissue engineering is depicted in Fig. 6.1. Living cells are

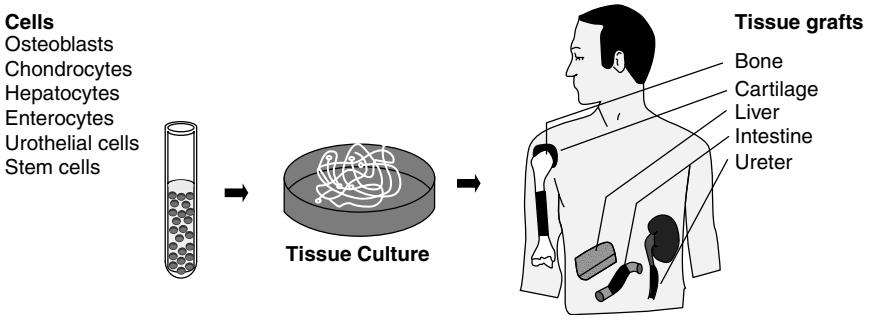


Figure 6.1. Overall approach to tissue engineering. Cells are cultured on a three-dimensional scaffold to engineer a graft that can replace a lost or damaged tissue. (Adapted from Langer and Vacanti, 1994).

obtained from a tissue harvest (either from the patient to engineer an *autograft*, or from a different person to engineer an *allograft*) and cultured in vitro on a three-dimensional scaffold to obtain a tissue construct suitable for transplantation. This approach, pioneered by Langer and Vacanti [1993] has been explored in numerous variations that differ from each other with respect to the cell source, the biomaterial scaffold, the conditions and duration of tissue culture, and the type and properties of the tissue being engineered.

2.2. Functional Tissue Engineering

In this book, we focus on *functional tissue engineering*, defined as the in vitro cultivation of tissue constructs with the structural and functional properties of the tissue being replaced. As compared to the transplantation of cells alone, engineered tissue constructs have the potential advantage of *immediate functionality*. As compared to transplantation of native tissues, engineered tissues can alleviate donor-recipient compatibility and disease transmission (for allografts), and donor site morbidity (for autografts). Engineered tissues can also serve as physiologically relevant models for *controlled studies of cells and tissues* under normal and pathological conditions [Vunjak-Novakovic and Goldstein, 2005].

Ideally, a lost or damaged tissue could be replaced by an engineered graft that can reestablish appropriate structure, composition, cell signaling, and function of the native tissue. In light of this paradigm, the clinical utility of tissue engineering will likely depend on our ability to replicate the site-specific properties of the particular tissue across different size scales. An engineered graft should provide regeneration, rather than repair, and undergo orderly remodeling in response to environmental factors in order to provide normal function in the long term (Table 6.1).

2.3. Model System

Tissue engineering generally attempts to recapitulate certain aspects of the environment present during normal development in order to stimulate functional assembly of the cells into specialized tissues. This involves the presence of

Table 6.1. Repair, regeneration, and remodeling.

1. *Repair* is rapid replacement of the damaged, defective, or lost tissue with functional new tissue that resembles, but does not replicate the structure, composition, and function of the native tissue.
2. *Regeneration* is slow restoration of all components of the repair tissue to their original condition such that the new tissue is indistinguishable from normal tissue with respect to structure, composition, and functional properties.
3. *Remodeling* is the change in tissue structure and composition in response to the local and systemic environmental factors that alter the functional tissue properties.

Buckwalter and Mankin, 1998; Einhorn, 1998; O'Driscoll, 2001.

reparative cells, the use of scaffolds (designed to provide a structural and logistic template for tissue development and to biodegrade at a controlled rate), and bioreactors (designed to control cellular microenvironment, facilitate mass transport to and from the cells, and provide the necessary biochemical and physical regulatory signals). Many different tissues have been engineered by utilizing variations of the "biomimetic" approach depicted in Fig. 6.2. In each case, the material properties of the native tissue provide the basis for establishing tissue engineering requirements and standards of success.

We will describe the components of this model system by referring to cartilage and myocardium, which perform structural and mechanical functions vital for health and survival and are of high clinical interest because of their inability for self-repair and distinctly different in many respects. Articular cartilage is an avascular tissue containing only one cell type, the chondrocyte, at a very low concentration. Chondrocytes maintain an extracellular matrix (ECM) consisting of a fibrous network of collagen type II and glycosaminoglycan (GAG)-rich proteoglycan [Buckwalter and Mankin, 1997]. Cartilage covers the surfaces of our joints, and its main function is to transfer compressive and shear forces during joint loading. The myocardium (cardiac muscle) is a highly vascularized muscular organ composed of cardiac myocytes, fibroblasts, and macrophages present at very high

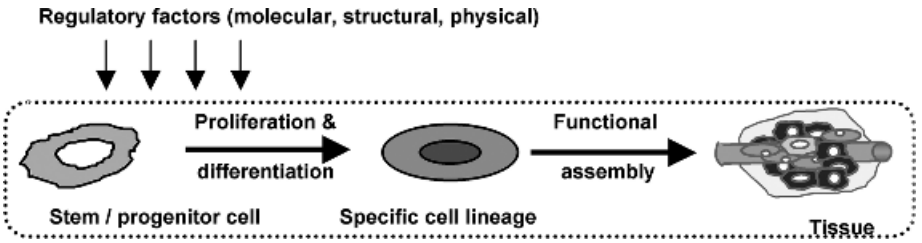


Figure 6.2. Environmental factors for functional tissue assembly. Cell function in vivo depends on a number of factors: a three-dimensional template; normal physicochemical milieu (temperature, pH, osmolality); exchange of nutrients, oxygen, and metabolites; and the presence of biochemical and physical regulatory factors. The design of tissue engineering systems is governed by the need to mimic these environmental factors in vitro and thereby direct the cells to assemble functional tissue structures.

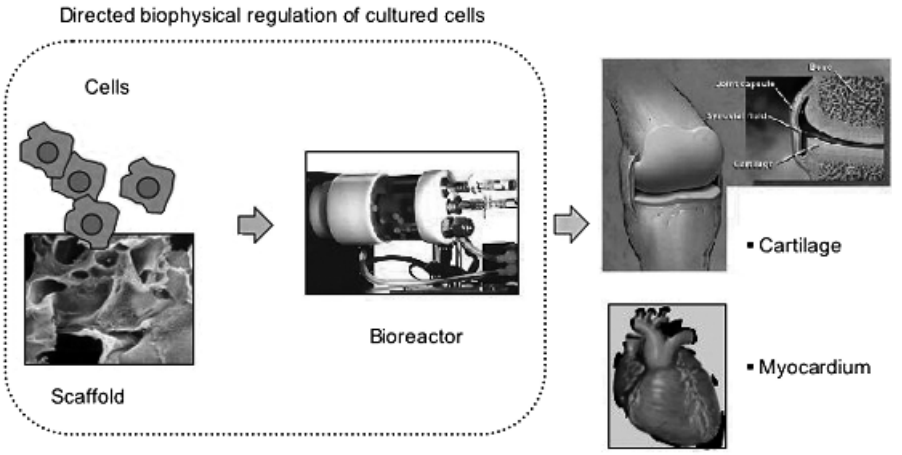


Figure 6.3. Model system. The cell–scaffold–bioreactor system for tissue engineering has been designed to modulate cell differentiation and functional assembly by providing environmental factors described in Fig. 6.2.

concentrations [MacKenna et al., 1994; Brilla et al., 1995]. The myocytes form a three-dimensional syncytium that enables propagation of electrical signals across specialized intracellular junctions to produce coordinated mechanical contractions that pump blood.

The cell–scaffold–bioreactor system (Fig. 6.3) was designed to utilize the factors thought to play regulatory roles during tissue development (See Fig. 6.2). This system has been extensively studied in vitro [See, e.g., Carrier et al., 1999, 2002a,b; Freed and Vunjak-Novakovic, 2000a,b] and in vivo [Schaefer et al., 2002]. It involves bioreactor cultivation of dissociated cells seeded at a high initial density on a three-dimensional scaffold.

Once implanted, an engineered tissue should (i) develop and integrate with adjacent host tissues and (ii) provide some minimal level of function immediately postimplantation that should improve progressively until normal function has been restored. All engineered tissues should possess a certain minimal size, thickness, and mechanical integrity to allow for handling and should permit construct survival under physiological conditions (e.g., in an articular joint for engineered cartilage or the myocardial wall for engineered cardiac tissue), as well as specific functional requirements (i.e., engineered cartilage should withstand and transmit loads, and engineered cardiac tissue should contract in a coordinated manner). We discuss here the general requirements for tissue engineering as well as the additional requirements for engineering cartilage and cardiac muscle.

3. CELLS

3.1. Basic Requirements

An ideal cell source for tissue engineering should have the capacity to proliferate and then differentiate in vitro, in a manner that can be reproducibly controlled. For

cartilage tissue engineering, these criteria can be met by either articular chondrocytes or bone marrow-derived mesenchymal stem cells (MSC) [Freed et al., 1999; Meinel et al., 2004a], and in vivo studies have shown that both chondrocyte- and MSC-based grafts can be used to repair large, full-thickness cartilage defects in rabbit knee joints [Caplan et al., 1997; Kawamura et al., 1998; Schreiber et al., 1999]. For cardiac tissue engineering, the above criteria have not yet been met, because precursor cell sources have not been established and in vivo studies in immunocompetent animals have not yet been published.

3.2. Overview of Cell Sources

The cells used thus far to engineer cartilage have varied with respect to donor age (embryonic, neonatal, immature, or adult), differentiation state (precursor or phenotypically mature), and method of preparation (selection, expansion, gene transfer). The sources of chondrogenic cells have included bovine chondrocytes [See, e.g., Buschmann et al., 1992; Freed et al., 1998; Pei et al., 2002b], rabbit chondrocytes [See, e.g., Fedewa et al., 1998], equine chondrocytes [See, e.g., Heath, 2000; Litzke et al., 2004], embryonic chick limb bud cells [See, e.g., Elder et al., 2000], human chondrocytes [See, e.g., Sittinger et al., 1994] and mesenchymal stem cells derived from bone marrow [Meinel et al., 2004a]. The choice of cell type can affect in vitro culture requirements (e.g., medium supplements, structure, and degradation rate of scaffold) and in vivo function (e.g., potential for integration) of engineered cartilage constructs.

Articular chondrocytes are phenotypically stable if cultured under appropriate conditions (e.g., up to 7–8 months in vitro [Hauselmann et al., 1994; Freed et al., 1997]) and can be used to engineer mechanically functional cartilaginous constructs [Buschmann et al., 1995; Freed et al., 1997; Vunjak-Novakovic et al., 1999; Obradovic et al., 2001; Schaefer et al., 2002]. However, adult chondrocytes are not easily harvested; cells from younger donors tend to be more responsive to environmental stimuli [(See, e.g., Heath, 2000]), and cell expansion responds to growth factors [Martin et al., 1999, 2001b; Pei et al., 2002a]. Articular chondrocytes are obtained by enzymatic digestion of full-thickness articular cartilage harvested from 2- to 4-week-old bovine calves [Freed et al., 1993] or 2- to 8-month old New Zealand White rabbits [Freed et al., 1994a]. For both sources, the cell yield is $3–5 \times 10^7$ cells per gram of wet tissue. Primary chondrocytes can be directly seeded onto scaffolds, or first amplified by subculture in the chondrocyte medium (See below) supplemented with 5 ng/ml of fibroblast growth factor FGF-2 [Martin et al., 1999]. Expanding chondrocytes undergo approximately 10 doublings, in two passages. During monolayer culture, chondrocytes can be transfected, for example, by human insulin-like growth factor I [Madry and Trippel, 2000].

Precursor cells from the bone marrow are relatively easier to harvest and expand in culture, remain metabolically active in older donors [Haynesworth et al., 1998],

and can recapitulate some aspects of skeletal tissue development [Caplan et al., 1997]. Bone marrow-derived MSC are obtained from 16-day embryonic chicks [Martin et al., 1998], 2- to 4-week old bovine calves [Martin et al., 2001a], or human bone marrow aspirates [Meinel et al., 2004b]. BMSC are selected from the mixed-cell population based on their ability to adhere to the dish, fully characterized (for the presence of surface receptors and ability for selective differentiation [Meinel et al., 2004b]), and passaged in monolayers (twice, to undergo 10–20 doublings).

In the case of engineered cardiac tissue, heart cells were obtained from embryonic chicks and fetal or neonatal rats [See, e.g. Carrier et al., 1999; Li et al., 1999; Zimmermann et al., 2000]. In vivo studies avoided immunorejection by using donor heart cells from inbred rats [Li et al., 1999]; in general, the younger the cell donor, the higher the proliferative capacity and metabolic activity of the harvested cells. Whereas all cell types used to engineer cartilage were able to proliferate in vitro, only embryonic and fetal heart cells proliferated in vitro [Li et al., 1999; Fink et al., 2000].

Primary cardiac myocytes are obtained by enzymatic digestion of heart ventricles harvested from 1- to 2-day neonatal rats [Bursac et al., 1999; Carrier et al., 1999] or 14- to 15-day embryonic chicks [Carrier et al., 1999]. Cell yields range from 1.5 to 7×10^6 cells/heart [Springhorn and Claycomb, 1989; Toraason et al., 1989; Barnett et al., 1993]. The fraction of cardiac myocytes in the cell preparation can be increased by preplating, which allows preferential attachment of fibroblasts [Maki et al., 1996]. After two 1-h periods, the cells that remain unattached are used to seed polymer scaffolds.

4. MEDIA

4.1. Chondrocytes

Chondrocytes are cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose and 4 mM glutamine supplemented with 10% fetal bovine serum (FBS), 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 0.1 mM nonessential amino acids (NEAA), 0.4 mM proline, 0.2 mM (50 μg/ml) ascorbic acid 2-phosphate, 100 U/mL penicillin, 100 μg/ml streptomycin, and 0.5 μg/ml Fungizone (optional) [Sah et al., 1989].

4.2. MSCs

Bone marrow-derived precursor cells are cultured in DMEM containing 4.5 mg/ml glucose and 4 mM glutamine supplemented with 10% FBS, 0.1 mM NEAA, 0.2 mM (50 μg/ml) ascorbic acid 2-phosphate, 10 nM dexamethasone, 5 μg/ml insulin, 5 ng/ml TGF-β1, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.5 μg/ ml Fungizone (optional) [Meinel et al., 2004b].

4.3. Cardiac Myocytes

Neonatal rat cardiac myocytes are cultured in DMEM containing 5.5 mM (1 mg/ml) glucose, 1 mM pyruvate, 4 mM glutamine, and 25 mM HEPES and supplemented with 100 U/ml penicillin (optional) and 0.5 μg/ml Fungizone (optional) [Bursac et al., 1999; Carrier et al., 1999]. Medium can be further supplemented with 10% FBS or 2% adult horse serum (HyClone) [Papadaki et al., 2001]. Embryonic chick cardiac myocytes are cultured in medium supplemented with 6% FBS [Barnett et al., 1993].

5. BIOMATERIAL SCAFFOLDS

5.1. Basic Requirements

Most studies suggest that the scaffold is essential for promoting orderly regeneration of cartilage, in vivo and in vitro. A biomaterial scaffold provides a template for cell attachment and tissue development, and it biodegrades in parallel with the accumulation of tissue components. Scaffold structure determines the transport of nutrients, metabolites, and regulatory molecules to and from the cells, and the scaffold chemistry may have an important signaling role. Ideally, scaffolds are made of materials that are biocompatible and biodegradable, preferentially those already used in products approved by the Food and Drug Administration. To achieve isomorphous tissue replacement, the scaffold should biodegrade at a rate matching the rate of extracellular matrix deposition and without any toxic or inhibitory byproducts. The maintenance or the rate of decline of the mechanical properties of the scaffold may be critical for its efficacy, as well as for the modulation of the stress-strain environment at the cellular and tissue levels.

5.2. Overview of Scaffolds

Scaffolds used in tissue engineering vary with respect to material chemistry (e.g., collagen, hydrogels, or synthetic polymers), geometry (e.g., gels, fibrous meshes, porous sponges), structure (e.g., porosity, pore size, pore distribution, orientation, and connectivity), mechanical properties (e.g., tension, compression, resistance to shear, and permeability), and the sensitivity to and rate of degradation [See, e.g., Freed and Vunjak-Novakovic, 2000b for review].

A variety of scaffolds have been used to engineer cartilage, including gels of agarose [Buschmann et al., 1995; Lee and Bader, 1997; Mauck et al., 2000] collagen [Wakitani et al., 1994, 1998], and chitosan [Di Martino et al., 2005]; meshes of collagen [Grande et al., 1997] and polyglycolic acid (PGA) [Freed et al., 1997, 1998; Grande et al., 1997; Carver and Heath, 1999; Obradovic et al., 1999; Vunjak-Novakovic et al., 1999; Schaefer et al., 2000a]; and sponges of PLA [Chu et al., 1997]. Scaffolds used to engineer cardiac tissues include gels of collagen with or without Matrigel® [Eschenhagen et al., 1997; Fink et al., 2000; Zimmermann et al., 2000], meshes of PGA with or without laminin coating [Freed et al., 1997; Bursac et al., 1999; Carrier et al., 1999; Papadaki et al., 2001], sponges

of native collagen (Ultrafoam®) [Radisic et al., 2003, 2004a,b], sponges of denatured collagen (Gelfoam®) [Li et al., 1999], and fibers of collagen and polystyrene beads [Akins et al., 1999] (See Fig. 6.4 for representative structures of a fibrous mesh and a sponge).

One representative fibrous scaffold is a nonwoven mesh made of PGA fibers (13 μm fiber diameter, >95% void volume) [Freed et al., 1994a]. In most cases, the mesh is punched into 1- to 5-mm-thick disks, 5–10 mm in diameter. Other shapes are used for other applications (e.g., tubular scaffolds made of 1-mm-thick mesh to engineer small-caliber arteries [Niklason et al., 1999]). In culture, fibrous PGA loses its mechanical integrity over 12 days [Niklason et al., 1999] and degrades to approximately 50% of the initial mass over 4 weeks [Freed et al., 1994c]. PGA mesh can be surface-hydrolyzed to increase hydrophilicity [Gao et al., 1998] and then coated with laminin to enhance cell attachment [Papadaki et al., 2001] (Fig. 6.4).

One representative porous scaffold is the Davol Ultrafoam™, a clinically used hemostatic sponge made of a water-insoluble hydrochloric acid salt of purified bovine corium collagen, in the form of 3-mm-thick sheets. For tissue engineering studies, this sheet is punched into 13-mm-diameter disks; after hydration in culture medium, disks reduce their diameters to approximately 10 mm. For rapid cell inoculation, Ultrafoam™ is used in conjunction with Matrigel®, a basement membrane preparation that is liquid at low temperatures (2–8 °C) and gels at 22–35 °C (Fig. 6.4).

6. BIOREACTORS

6.1. Basic Requirements

Ideally, a bioreactor should provide an in vitro environment for rapid and orderly development of functional tissue structures by isolated cells on three-dimensional

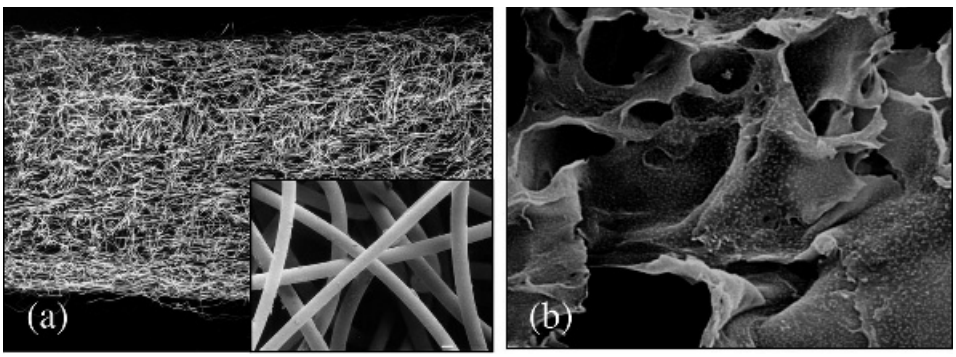


Figure 6.4. Biomaterial scaffolds. a) Fibrous mesh made of polyglycolic acid. b) Collagen sponge.

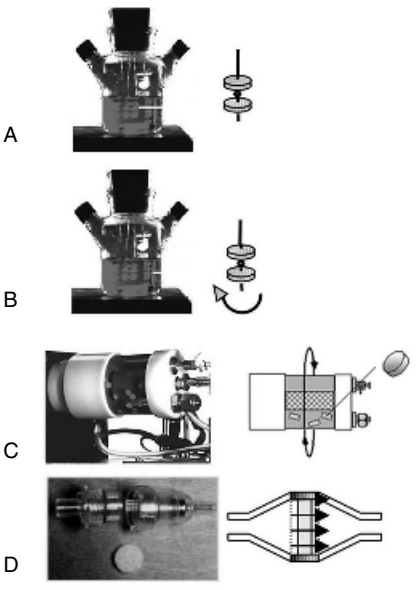
scaffolds. In a general case, bioreactors are designed to perform one or more of the following functions:

1. Establish spatially uniform concentrations of cells within biomaterial scaffolds.
2. Control conditions in culture medium (e.g., temperature, pH, osmolality, levels of oxygen, nutrients, metabolites, regulatory molecules).
3. Facilitate mass transfer between the cells and the culture environment.
4. Provide physiologically relevant physical signals (e.g., interstitial fluid flow; shear, and compression for cartilage; pulsatile pressure and stretch for cardiac muscle).

6.2. Overview of Bioreactor Types

Representative culture vessels that are frequently used for tissue engineering (static flasks, spinner flasks, rotating vessels) are compared in Fig. 6.5. All culture vessels are operated in incubators (to maintain the temperature and CO_2, and, thereby, control pH), with continuous gas exchange and periodic medium replacement.

Starter dishes are 6-well or 96-well plates containing one scaffold per well seeded with 0.06 ml (96-well plate) to 6 ml of cell suspension (6-well plate). Dishes are used static [Freed et al., 1993], on an orbital shaker mixed at 50–75 rpm [Freed et al., 1994b], or on an XYZ gyrator mixed at 60 rpm [Papadaki et al., 2001].



Flow conditions	Mixing mechanism	Mass transfer	Fluid shear
Static flask	Diffusion	Slow	None
Mixed flask	Convective flow	Fast	Turbulent, steady
Rotating vessel	Construct settling	Fast	Laminar, dynamic
Perfused cartridge	Convective flow	Fast	Laminar, steady

Figure 6.5. Bioreactors. a) Static flask. b) Mixed flask. c) Rotating vessel. d) Perfused cartridge.

Spinner flasks (100-ml nominal capacity) are 6.5 cm in diameter × 12 cm high, filled with 120 ml of medium, and fitted with a stopper. Flasks can be operated statically or mixed at 50–75 rpm, using a nonsuspended 0.8-cm-diameter × 4-cm-length stir bar. Gas exchange is via surface aeration through the sidearms [Vunjak-Novakovic et al., 1996, 1998].

Rotating vessels. The Slow Turning Lateral Vessel (STLV) and the High Aspect Ratio Vessel (HARV) were developed at NASA. The STLV is configured as two concentric cylinders that have diameters of 5.75 cm and 2 cm. The annular space, approximately 110 ml in volume, is used for tissue culture, and the inner cylinder serves as a membrane gas exchanger. The HARV is configured as a cylinder 1.3 cm high and 10 cm in diameter, approximately 110 ml in capacity, with one base serving as a membrane gas exchanger. Both vessels are primed with medium to displace all air and mounted on a base that simultaneously rotates the vessel around its central axis at the desired rate (10–45 rpm) and pumps filter-sterilized incubator air over the gas exchange membrane at a rate of about 1 l/min [Freed and Vunjak-Novakovic, 1995].

Perfusion cartridges are small, 10-mm-diameter, 42-mm-length, 1.5-ml-volume polycarbonate vessels made by Advanced Tissue Sciences to culture one scaffold apiece [Dunkelman et al., 1995]. For cell seeding, each cartridge is fitted with two stainless steel screens (with 85% void area) supporting the scaffold during perfusion, each with a silicone gasket (1 mm thick, 10-mm OD, 5-mm ID) preventing the bypass of medium around the scaffold (See Fig. 6.2). Each cartridge is filled with medium to displace all air and connected to a recirculation loop containing two gas exchangers (each configured as a coil of platinum-cured silicone tubing, 80 cm long, 1.6-mm ID, 3.2-mm OD), one on each side of the cartridge. Medium is recirculated by a push/pull syringe pump that can operate two loops at a time (Fig. 6.6).

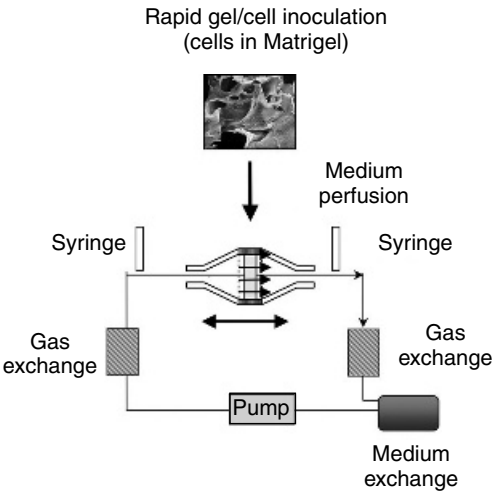


Figure 6.6. Perfused cartridges with interstitial flow of culture medium.

7. SCAFFOLD SEEDING

7.1. Basic Requirements

Cell seeding of 3D scaffolds is the first step of bioreactor cultivation of engineered tissues. Seeding requirements include (a) high seeding efficiency, to maximize cell utilization; (b) high kinetic rate of cell attachment, to minimize the time in suspension for anchorage-dependent cells; and (c) high and spatially uniform distribution of attached cells, for rapid and uniform tissue assembly [Vunjak-Novakovic and Radisic, 2004a].

Before cell seeding, scaffolds must be thoroughly prewetted with culture medium such that all air is displaced. Methods in use include the application of pressure (by a small forceps) or vacuum (by a pipette) on scaffolds immersed in culture medium. Synthetic scaffolds can be first exposed to 70% ethanol to increase wettability [Freed et al., 1993]. Scaffolds are incubated in culture medium for 2–24 h before cell seeding. Incubation in medium containing serum can further enhance cell attachment.

After cell inoculation in a laminar hood, scaffolds are incubated in a humidified, 37 °C incubator containing 5–10% CO_2 in air. The duration of attachment ranges from 1 h to 3 days, depending on the cells, scaffold, seeding vessel, and specific method used. We and others have studied a number of seeding methods for different cell types, initial cell concentrations ($5–50 \times 10^6$ cells per cm^3 of scaffold volume), and scaffolds (fibrous and porous; 5–10 mm in diameter $\times$ 1–5 mm thick). Thin (<2 mm) scaffolds can be seeded statically; spatially uniform cell seeding of thicker ($\geq$2 mm) scaffolds requires mixing. A variety of mechanisms was utilized to provide mixing and flow of culture medium, in order to suspend the isolated cells and to generate convective motion of the cells into the scaffold interior. We present two well-characterized cell seeding protocols that give consistently good results, one for cartilage tissue engineering and one for cardiac tissue engineering. [See Vunjak-Novakovic et al., 1998; Radisic et al., 2003; Vunjak-Novakovic and Radisic, 2004a for more details.]

7.2. Dynamic Seeding in Spinner Flasks

Seeding in spinner flasks is the preferred system for seeding fibrous scaffolds. This is a well-characterized method that results in a relatively uniform spatial distribution of attached cells within a period of 24 hours at a yield of essentially 100% [Vunjak-Novakovic et al., 1998]. The main steps of scaffold seeding in spinner flasks are as follows.

Protocol 6.1. Dynamic Seeding in Spinner Flasks for Cartilage Tissue Engineering

Reagents and Materials

Sterile

- ❑ Growth medium (See Section 4)

- ❑ Spinner flask assembly (See Fig. 6.5):
  - (i) Spinner flask, 100-ml nominal capacity, containing a 4-cm-long magnetic stir bar.
  - (ii) Silicone stopper, 5-cm diameter, into which four 4-in.-long, 22-gauge needles have been symmetrically placed (See Fig. 6.5).
  - (iii) Sterilize the flask by autoclaving with the stopper in place such that the needles extend into the flask.
- ❑ Polymer scaffold disks: 10-mm diameter × 5-mm thick, 10 mm × 2 mm thick, or 5 mm × 2 mm thick, sterilized with ethylene oxide (ETO, for 12 h), aerated (for at least 24 h), packaged in trilaminate aluminum foil pouches in a dry box to minimize hydrolytic degradation, and stored at room temperature
- ❑ Silicone tubing, #13, 3-mm lengths, sterilized by autoclaving
- ❑ Gloves

Nonsterile

- ❑ Magnetic stirrer

Protocol

(a) Wearing sterile gloves, thread prewetted polymer scaffolds onto the needles such that each needle contains 2 disks that are 10 mm in diameter × 2–5 mm thick, or 3 disks that are 5 mm in diameter × 2 mm thick.
(b) Position disks, using 3-mm-long segments of #13 silicone tubing.
(c) Place the stopper in the mouth of the flask.
(d) Add 120 ml medium via one of the sidearms. Remove any droplets and flame to ensure sterility.
(e) Leave the sidearm caps slack to allow gas exchange.
(f) Transfer the assembled stirrer flask onto a magnetic stirrer in a humidified 37 °C, 5% CO_2 incubator. Set stirring rate to 50–75 rpm and leave overnight.
(g) When a suspension of freshly isolated cells is prepared, remove 16 ml medium from the flask, through the sidearm, using a pipette. Flame the sidearm after removing the cap.
(h) Add cells (e.g., 16 ml of 4×10^6 cells/ml to seed 12 scaffolds that are 5 mm in diameter × 2 mm thick).
(i) Flame the sidearm and replace the cap (leave loosely capped, to allow gas exchange).
(j) Transfer flask onto the magnetic stirrer in a 37 °C, 5% humidified CO_2 incubator.
(k) Completely replace medium after 1–3 days.
(l) Continue to culture cell-polymer constructs in flasks, or transfer into a different culture vessel.

Dynamic seeding in flasks has been extensively used to seed fibrous PGA scaffolds with primary and passaged chondrocytes [Vunjak-Novakovic et al., 1999; Schaefer et al., 2000b; Gooch et al., 2001b; Obradovic et al., 2001], embryonic chick and bovine MSCs [Martin et al., 1998, 1999], and cardiac myocytes [Carrier

et al., 1999; Papadaki et al., 2001]. Spinner flasks are also used to seed porous scaffolds made of poly(lactic-co-glycolic acid) (PLGA) and polyethylene glycol (PEG) with bovine MSC [Martin et al., 2001a] and periosteal cells [Schaefer et al., 2000b]. Seeding efficiency (percentage of total cells that attached to scaffolds) is essentially 100% for chondrocytes on PGA scaffolds [Vunjak-Novakovic et al., 1998], approximately 60% for cardiac myocytes on PGA scaffolds [Carrier et al., 1999], and 30–60% for periosteal cells on PLGA-PEG scaffolds [Schaefer et al., 2000b].

In spinner flasks, mixing during cell seeding maintains the cells in suspension and provides relative velocity between the cells and the scaffolds. The probable mechanism by which cells populate the scaffold interior is convective motion of suspended cells into the scaffold followed by inertial impacts between the cells and the fibers and cell attachment [Vunjak-Novakovic et al., 1998].

7.3. Gel-Cell Seeding of Porous Scaffolds

Rapid inoculation of metabolically active cells at high initial densities can be achieved by using gels as cell delivery vehicles. Collagen gel can be used to seed mesenchymal stem cells onto biodegradable sutures [Awad et al., 2000]. Fibrin gel can be used for rapid inoculation of PGA scaffolds with bovine articulate chondrocytes [Ameer et al., 2002]. We used Matrigel® to inoculate Ultrafoam™ scaffolds (10-mm diameter × 3-mm thickness) with cardiac myocytes at the approximate density of 70×10^6 cells/cm^3 according to the procedure described in Protocol 6.2 [Radisic et al., 2003, 2004a,b].

Protocol 6.2. Gel-Cell Seeding of Porous Scaffolds for Cardiac Tissue Engineering

Reagents and Materials

Sterile or aseptically prepared

- ❑ Counted cell suspension
- ❑ Growth medium (See Section 4)
- ❑ Matrigel®
- ❑ Scaffold disks (as in Protocol 6.1)
- ❑ Petri dishes, 9 cm
- ❑ Kimwipes

Protocol

(a) Thaw Matrigel overnight in a 4 °C refrigerator.

(b) Prewet the scaffolds with culture medium and place into a 37 °C, 5% CO_2 incubator for $\geq$2 h before inoculation.

(c) Blot-dry the scaffolds with sterile Kimwipes, and transfer them to empty Petri dishes (4–5 scaffolds/dish.)

(d) Centrifuge the cell suspension at 1000 rpm for 10 min in a 15-ml conical tube. Place the tube on ice in the laminar hood and aspirate off the supernatant.
(e) Using an automatic micropipette, resuspend a cell pellet in Matrigel. It is recommended that between 5 and 10 μl Matrigel per 10^6 cells be used.
(f) Load the homogeneous cell suspension onto a scaffold as uniformly as possible.
(g) Place the Petri dish with inoculated scaffolds for 10 min in the 37 °C, 5% CO_2 humidified incubator to allow gelation to occur.

To avoid diffusional limitations of mass transfer during scaffold seeding, the scaffolds are directly perfused with culture medium such that the transport of oxygen from the medium to the cells occurs via both diffusion and convection. For seeding in perfusion, the system shown in Fig. 6.6 is used with one scaffold per cartridge in an alternating flow regime according to Protocol 6.3 [See Radisic et al., 2003 for more details].

Protocol 6.3. Perfusion of Porous Scaffolds for Tissue Engineering

Reagents and Materials

Sterile

- ❑ Growth medium (See Section 4)
- ❑ Silicone tubing, screens, gaskets, and cartridges (See Protocol 6.2), sterilized by autoclaving (20 min autoclaving/20 min drying on a dry cycle, 121 °C)
- ❑ Syringes, 10 ml, 2

Protocol

(a) Assemble the seeding loops according to Fig. 6.6 in a laminar flow hood, using sterile technique.
(b) Using one syringe, prime one loop with total of 8 ml culture medium (5.5 ml in perfusion cartridge and tubing; 2.5 ml in one reservoir syringe; the other syringe is empty).
(c) Place the primed loop in the 37 °C, 5% CO_2 incubator for minimum 2 h before seeding.
(d) In the laminar flow hood, carefully open a perfusion cartridge and, using forceps, place the gel-cell inoculated scaffold between two silicone gaskets and stainless steel screens.
(e) Close the perfusion cartridge, and remove any air bubbles by injecting culture medium from syringe 2 into syringe 4 (See Fig. 6.6) while holding the cartridge in a vertical position so that bubbles can escape upward.
(f) Place the whole assembly in a 37 °C, 5% CO_2 incubator and insert the seeding loop into the Push/Pull pump. Program the pump to perfuse the medium at a desired flow rate (0.5–1.5 ml/min) with flow direction reversal after every 2.5 ml for a period of 1.5–4.5 h.

Perfusion of gel-cell inoculated scaffolds helps maintain viability, metabolic activity, and uniform cell distribution [Radisic et al., 2003]. It is also possible to seed cells directly from suspension (omitting the gel inoculation step) to PGA scaffolds in a perfusion loop [Kim et al., 2000]. The procedure yields constructs with relatively uniform distribution of metabolically active cells, but the cell density and seeding efficiency are significantly lower than in gel-cell inoculated scaffolds.

8. BIOREACTOR CULTIVATION

8.1. Hydrodynamic Environment

Convective mixing improves the kinetic rate, efficiency, and spatial uniformity of cell seeding on three-dimensional polymer scaffolds and improves tissue structure and composition by enhancing mass transport within the culture medium and at the tissue surfaces [Vunjak-Novakovic et al., 1998; Radisic et al., 2003]. Hydrodynamic factors present during culture can modulate cell function and tissue development in at least two ways: via associated effects on mass transport between the developing tissue and culture medium (e.g., oxygen, nutrients, growth factors) and by physical stimulation of the cells (e.g., shear, pressure). The composition, morphology, and mechanical properties of engineered tissues grown in hydrodynamically active environments were generally better than in static environments. For cultivations in vessels described in Fig. 6.5, construct compositions and mechanical properties were better in stirred flasks and rotating vessels than in static flasks.

Direct perfusion through cultured tissue constructs stimulated chondrogenesis, presumably because of the combined effects of enhanced mass transport, pH regulation, and fluid shear in the cell microenvironment [Sittinger et al., 1994; Dunkelman et al., 1995; Pazzano et al., 2000], in particular at physiological interstitial flow velocities [Maroudas, 1979; Mow et al., 1980, 1991; Vunjak-Novakovic et al., 1996]. However, physiologically thick, functional cartilage could be engineered with diffusional transport within the cultured tissue constructs (e.g., in rotating bioreactors, [Vunjak-Novakovic et al., 1999; Pei et al., 2002a]). In contrast, the same culture conditions yielded engineered cardiac tissue in which the functional layer was only approximately 0.1 mm thick [Carrier et al., 1999]. This finding could be attributed to diffusional limitations of oxygen transport to the cells, as supported by calculations that showed that the pO_2 would decrease to zero at a depth of approximately 0.1 mm [Carrier et al., 2002b]. The engineering of a thick layer of functional cardiac tissue requires the perfusion of oxygen-rich culture medium directly through the growing construct [Radisic et al., 2004a]. The combination of rapid cell inoculation and immediate establishment of medium perfusion enabled physiological densities of viable cells in engineered cardiac constructs, because of the maintenance of efficient oxygen supply to the cells at all times of cultivation.

8.2. Growth Factors

Growth factors (e.g., FGF-2, TGF-β1) are generally required to engineer cartilaginous tissues starting from bone marrow-derived mesenchymal stem cells

[Johnstone et al., 1998; Martin et al., 1998; Meinel et al., 2004a] and expanded chondrocytes [Martin et al., 1999, 2001b]. Specific combinations of bioactive factors were shown to promote chondrocytes cells to first dedifferentiate during expansion in monolayers and then redifferentiate and regenerate cartilaginous tissues during subsequent cultivation on biomaterial scaffolds [Pei et al., 2002a]. Growth factors supplemented sequentially to culture medium (TGF-β/FGF-2 early, IGF-I later) markedly and significantly improved the compositions and mechanical properties of engineered cartilage [Pei et al., 2002a]. Gene transfer of human IGF-I augmented the structural and functional properties of cartilaginous constructs grown in bioreactors, suggesting that spatially defined overexpression of growth factors may be advantageous for cartilage tissue engineering [Madry et al., 2001].

Importantly, the mechanical environment and supplemental growth factors independently modulate the growth and mechanical properties of engineered cartilage, interact to produce results not suggested by the independent responses, and in certain combinations can produce tissues superior to those obtained by utilizing these factors individually [Gooch et al., 2001a]. Beneficial effects of growth factors can be amplified by dynamic mechanical loading. TGF-β and IGF-I interacted with dynamic loading applied during culture in a synergetic manner and improved the compositions and mechanical properties of cultured constructs to an extent greater than the sum of effects of either stimulus applied alone [Mauck et al., 2000, 2002, 2003]. Likewise, the hydrodynamically active environment present in rotating bioreactors amplified the beneficial effects of polymer scaffolds on construct compositions and mechanical properties and yielded engineered cartilage that had equilibrium moduli of 400–540 kPa after only 4 weeks of bioreactor cultivation [Pei et al., 2002b].

8.3. Physical Signals

Chondrogenesis in vitro and remodeling in vivo of native and engineered cartilage has been studied with a variety of physical signals, including fluid flow [Wu et al., 1999; Pazzano et al., 2000], dynamic fluctuations in hydrodynamic shear and pressure [Freed et al., 1997; Vunjak-Novakovic et al., 1999], cyclic hydrostatic pressure [Carver and Heath, 1999], cyclic mechanical compression [Buschmann et al., 1995; Mauck et al., 2000; Seidel et al., 2004], and cyclic stretch [Wu and Chen, 2000]. In vitro, dynamic compression enhanced synthesis of proteoglycans in cartilage explants [Sah et al., 1989], improved the mechanical function of engineered cartilage [Buschmann et al., 1992, 1995; Lee and Bader, 1997; Carver and Heath, 1999; Mauck et al., 2000], and enhanced chondrogenesis of chick limb bud cells [Elder et al., 2000, 2001]. In vivo, cyclic loading caused the mesenchymal cells to differentiate into cartilage overlaying bone [Tagil and Aspenberg, 1999]. The application of mechanical strain during cultivation improved the properties of engineered cardiac constructs, in vitro and in vivo [Zimmermann et al., 2002a,b].

Mechanical stimulation can cause multiple changes to the extracellular environment (a) by direct effects on cell shape and interfibrillar spacing, (b) by increase

in hydrostatic pressure, (c) by fluid flow that can enhance mass transport to and from the cells, or (d) by change in fluid volume that can cause changes in concentrations of chemical and ionic species [Mow et al., 1999]. All these effects can modulate the synthesis, breakdown, and structural adaptations of the ECM, which in turn serves as a transducer of mechanical and electrochemical signals, and thereby mediate the catabolic and anabolic changes in cell metabolism (See Vunjak-Novakovic and Goldstein, 2005 for detailed review).

Most recently, cardiac constructs prepared by seeding collagen sponges with neonatal rat ventricular cells were stimulated during cultivation with suprathreshold square biphasic pulses (2-ms duration, 1 Hz, 5 V) [Radisic et al., 2004b]. Over only 8 days of culture, stimulation resulted in significantly better contractile responses to pacing as compared to unstimulated controls, as evidenced by the sevenfold higher amplitude of contractions in response to pacing. Excitation-contraction coupling of cardiac myocytes in stimulated constructs was evidenced by transmembrane potentials that were similar to the action potentials reported previously for cells from mechanically stimulated constructs [Zimmermann et al., 2002b]. Stimulated constructs exhibited higher levels of cardiac markets and a remarkable level of ultrastructural organization. These studies suggest that electrical stimulation of construct contractions during cultivation enhanced the properties of engineered myocardium at the cellular, ultrastructural, and tissue levels [Radisic et al., 2004b].

8.4. Structural and Functional Assessment of Engineered Constructs

For histologic assessment, engineered constructs are fixed in neutral buffered formalin, embedded in paraffin, and sectioned (5–8 μm thick). Engineered cartilage sections are stained with hematoxylin and eosin (H&E) for cells, safranin-O for GAGs, and monoclonal antibodies for collagen types I, II, IX, and X [Freed et al., 1998; Martin et al., 1998; Riesle et al., 1998]. Engineered cardiac tissue sections are stained with H&E for cells and monoclonal antibodies for sarcomeric α-actin, cardiac troponin I, sarcomeric tropomyosin, and connexin 43 [Carrier et al., 1999; Papadaki et al., 2001; Radisic et al., 2004a,b]. Construct size and distributions of cells and tissue components are assessed by image analysis [Freed et al., 1998; Vunjak-Novakovic et al., 1998]. Ultrastructural analyses include scanning and transmission electron microscopy (SEM, TEM) [Riesle et al., 1998; Carrier et al., 2002a,b; Radisic et al., 2004b].

For biochemical evaluation, cartilage constructs are digested with papain or protease-K, and lyophilized [Freed et al., 1993, 1994c, 1998]. The amount of DNA is determined fluorometrically with Hoechst 33258 dye [Kim et al., 1988]. Sulfated GAG content is determined spectrophotometrically by Dimethylmethylene Blue dye binding [Farndale et al., 1986]. Total collagen content is determined spectrophotometrically from hydroxyproline content after acid hydrolysis and reaction with *p*-dimethylaminobenzaldehyde and chloramine-T [Woessner, 1961]. Type II collagen content is determined by inhibition ELISA [Hollander et al., 1994; Freed et al., 1998; Riesle et al., 1998]. The presence of other collagens (e.g., I, IX, X) is semiquantitatively measured by SDS-PAGE and Western blot [Riesle et al., 1998].

GAG distribution is determined by magnetic resonance imaging (MRI) [Bashir et al., 1996; Williams et al., 1998]. Synthesis rates of GAG, total protein, and collagen are measured by incorporation and release of radiolabeled tracers [Freed et al., 1998].

The molar ratio of lactate production to glucose consumption ($\sim$1 for aerobic, $\sim$2 for anaerobic cell metabolism) and the rate of ammonia production are determined from concentrations measured in culture medium at timed intervals with blood gas and lactate analyzers [Obradovic et al., 1997; Radisic et al., 2004a]. Cell number and cell viability are assessed with ethidium monoazide bromide (EMA) in conjunction with fluorescence-activated cell sorting (FACS [Radisic et al., 2003]. Cell cycle analysis was done by FACS after incubation with propidium iodide [Radisic et al., 2004a].

For engineered cartilage, mechanical construct properties (e.g., compressive modulus, dynamic stiffness, hydraulic permeability, streaming potential) are measured in static and dynamic radially confined compression [Frank and Grodzinsky, 1987; Vunjak-Novakovic et al., 1999, Vunjak-Novakovic and Goldstein, 2005]. The capacity for integration with native cartilage is evaluated in vitro in bioreactors [e.g., Obradovic et al., 2001]) and in vivo in an animal model [e.g., Schaefer et al., 2002].

For engineered cardiac tissue, electrophysiological studies are determined by measuring signal propagation over macroscopic distances with a linear array of extracellular microelectrodes [Bursac et al., 1999]. At the cellular level, transmembrane potentials (action potentials) are measured under physiologic conditions and in response to a potassium current blocker [Bursac et al., 2003]. The contractile function of engineered cardiac constructs is evaluated by measuring contraction amplitude and frequency in response to electrical stimulation, using video microscopy and image processing[Radisic et al., 2003, 2004a,b].

9. SUMMARY

The primary functions of cartilage and myocardium, biomechanical for cartilage and contractile for myocardium, drive tissue engineering toward the restoration of the functions inherent in the tissue being replaced. The current paradigm is that the restoration of normal tissue function can be best achieved by using in vitro or in vivo engineered constructs that can regenerate the exact site-specific properties (molecular, structural, functional) of native tissues across different size scales [Vunjak-Novakovic and Goldstein, 2005].

Cells, biomaterial scaffolds, and regulatory factors (biochemical and physical) have been utilized in a variety of ways, in vitro with bioreactors and in vivo by implantation, to engineer functional tissues. In a general sense, tissue engineering tends to recapitulate some aspects of the environment present in vivo during tissue development and thereby stimulate the cells to regenerate functional tissue structures. Cultivation of biosynthetically active cells on an appropriate scaffold, facilitated mass transport, and the provision of physical regulatory signals are among common requirements for rapid and orderly tissue regeneration.

ACKNOWLEDGMENTS

Research described in this chapter has been conducted by research teams supported by grants from NASA and NIH. The expert help of Sue Kangiser with manuscript preparation is greatly appreciated.

SOURCES OF MATERIALS

Item	*Supplier*
Ascorbic acid 2-phosphate	Sigma
Dexamethasone	Sigma
DMEM	Invitrogen (Gibco)
FBS	Invitrogen (Gibco)
Fungizone	Invitrogen (Gibco)
Gyratory mixer	Boeker Scientific
High Aspect Ratio Vessel (HARV)	Synthecon
Horse serum	HyClone
Insulin	Sigma
Matrigel®	B-D Biosciences
Multiwell plates or dishes, 6 well or 96 well	Corning Costar
Nonessential amino acids	Invitrogen (Gibco)
Orbital shaker	Belco
Penicillin	Invitrogen (Gibco)
Perfusion cartridges, polycarbonate	Advanced Tissue Sciences
PGA fibrous scaffold	Albany International
Platinum-cured silicone	Cole Parmer
Slow Turning Lateral Vessel (STLV)	Synthecon
Spinner flasks	Belco
Streptomycin	Invitrogen (Gibco)
Syringe pump, PHD 2000	Harvard Apparatus
Syringes	Becton Dickinson
TGF-β1	Invitrogen (Gibco)
Three-way stopcocks	Baxter Healthcare
Ultrafoam™	Davol

REFERENCES

Akins, R.E., Boyce, R.A., Madonna, M.L., Schroedl, N.A., Gonda, S.R., McLaughlin, T.A., Hartzell, C.R. (1999) Cardiac organogenesis in vitro: Reestablishment of three-dimensional tissue architecture by dissociated neonatal rat ventricular cells. *Tissue Eng*. 5: 103–118.

Ameer, G.A., Mahmood, T.A., Langer, R. (2002) A biodegradable composite scaffold for cell transplantation. *J. Orthop. Res.* 20: 16–19.

Atala, A., and Lanza, R.P., Eds. (2002) *Methods of Tissue Engineering*. San Diego, Academic Press.

Awad, H.A., Butler, D.L., Harris, M.T., Ibrahim, R.E., Wu, Y., Young, R.G., Kadiyala, S., Boivin, G.P. (2000) In vitro characterization of mesenchymal stem cell-seeded collagen scaffolds for tendon repair: Effects of initial seeding density on contraction kinetics. *J. Biomed. Mater. Res.* 51: 233–240.

Barnett, J.V., Taniuchi, M., Yang, M.B., Galper, J.B. (1993) Co-culture of embryonic chick heart cells and ciliary ganglia induces parasympathetic responsiveness in embryonic chick heart cells. *Biochem. J.* 292: 395–399.
Bashir, A., Gray, M.L., Burstein, D. (1996) Gd-DTPA2 as a measure of cartilage degradation. *Magn. Reson. Med.* 36: 665–673.
Brilla, C.G., Maisch, B., Rupp, H., Sunck, R., Zhou, G., Weber, K.T. (1995) Pharmacological modulation of cardiac fibroblast function. *Herz* 20: 127–135.
Buckwalter, J.A., and Mankin, H.J. (1997) Articular cartilage, part I: Tissue design and chondrocyte-matrix interactions. *J. Bone Joint Surg. Am.* 79A: 600–611.
Buckwalter, J.A., and Mankin, H.J. (1998) Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and transplantation. *Instr. Course Lect.* 47: 487–504.
Bursac, N., Papadaki, M., Cohen, R.J., Schoen, F.J., Eisenberg, S.R., Carrier, R., Vunjak-Novakovic, G., Freed, L.E. (1999) Cardiac muscle tissue engineering: toward an in vitro model for electrophysiological studies. *Am. J. Physiol. Heart Circ. Physiol.* 277: H433–H444.
Bursac, N., Papadaki, M., White, J.A., Eisenberg, S.R., Vunjak-Novakovic, G., Freed, L.E. (2003) Cultivation in rotating bioreactors promotes maintenance of cardiac myocyte electrophysiology and molecular properties. *Tissue Eng.* 9: 1243–1253.
Buschmann, M.D., Gluzband, Y.A., Grodzinsky, A.J., Kimura, J.H., Hunziker, E.B. (1992) Chondrocytes in agarose culture synthesize a mechanically functional extracellular matrix. *J. Orthop. Res.* 10: 745–758.
Buschmann, M.D., Gluzband, Y.A., Grodzinsky, A.J., Hunziker, E.B. (1995) Mechanical compression modulates matrix biosynthesis in chondrocyte/agarose culture. *J. Cell Sci.* 108: 1497–1508.
Caplan, A.I., Elyaderani, M., Mochizuki, Y., Wakitani, S., Goldberg, V.M. (1997) Principles of cartilage repair and regeneration. *Clin. Orthop. Rel. Res.* 342: 254–269.
Carrier, R.L., Papadaki, M., Rupnick, M., Schoen, F.J., Bursac, N., Langer, R., Freed, L.E., Vunjak-Novakovic, G. (1999) Cardiac tissue engineering: cell seeding, cultivation parameters and tissue construct characterization. *Biotechnol. Bioeng.* 64: 580–589.
Carrier, R.L., Rupnick, M., Langer, R., Schoen, F.J., Freed, L.E., Vunjak-Novakovic, G. (2002a) Effects of oxygen on engineered cardiac muscle. *Biotechnol. Bioeng.* 78: 617–625.
Carrier, R.L., Rupnick, M., Langer, R., Schoen, F.J., Freed, L.E., Vunjak-Novakovic, G. (2002b) Perfusion improves tissue architecture of engineered cardiac muscle. *Tissue Eng.* 8: 175–188.
Carver, S.E., and Heath, C.A. (1999) Semi-continuous perfusion system for delivering intermittent physiological pressure to regenerating cartilage. *Tissue Eng.* 5: 1–11.
Chu, C., Dounchis, J.S., Yoshioka, M., Sah, R.L., Coutts, R.D., Amiel, D. (1997) Osteochondral repair using perichondrial cells: a 1 year study in rabbits. *Clin. Orthop. Rel. Res.* 340: 220–229.
Di Martino, A., Sittinger, M., Risbud, M.V. (2005) Chitosan: A versatile biopolymer for orthopaedic tissue-engineering. *Biomaterials* May 12; [Epub ahead of print].
Dunkelman, N.S., Zimber, M.P., Lebaron, R.G., Pavelec, R., Kwan, M., Purchio, A.F. (1995) Cartilage production by rabbit articular chondrocytes on polyglycolic acid scaffolds in a closed bioreactor system. *Biotechnol. Bioeng.* 46: 299–305.
Einhorn, T.A. (1998) The cell and molecular biology of fracture healing. *Clin. Orthop. Rel. Res.* 355S: S7–S21.
Elder, S.H., Kimura, J.H., Soslowsky, L.J., Lavagnino, M., Goldstein, S.A. (2000) Effect of compressive loading on chondrocyte differentiation in agarose cultures of chick limb-bud cells. *J. Orthop. Res.* 18: 78–86.
Elder, S.H., Goldstein, S.A., Kimura, J.H., Soslowsky, L.J., Spengler, D.M. (2001) Chondrocyte differentiation is modulated by frequency and duration of cyclic compressive loading. *Ann. Biomed. Eng.* 29: 476–482.
Eschenhagen, T., Fink, C., Remmers, U., Scholz, H., Wattchow, J., Woil, J., Zimmermann, W., Dohmen, H.H., Schafer, H., Bishopric, N., Wakatsuki, T., Elson, E. (1997) Three-dimensional reconstitution of embryonic cardiomyocytes in a collagen matrix: a new heart model system. *FASEB J.* 11: 683–694.
Farndale, R.W., Buttler, D.J., Barrett, A.J. (1986) Improved quantitation and discrimination of sulphated glycosaminoglycans by the use of dimethylmethylene blue. *Biochim. Biophys. Acta* 883: 173–177.

Fedewa, M.M., Oegema, T.R., Jr., Schwartz, M.H., MacLeod, A., Lewis, J.L. (1998) Chondrocytes in culture produce a mechanically functional tissue. *J. Orthop. Res.* 16: 227–236.

Fink, C., Ergun, S., Kralisch, D., Remmers, U., Weil, J., Eschenhagen, T. (2000) Chronic stretch of engineered heart tissue induces hypertrophy and functional improvement. *FASEB J.* 14: 669–679.

Frank, E.H., and Grodzinsky, A.J. (1987) Cartilage electromechanics II. A continuum model of cartilage electrokinetics and correlation with experiments. *J. Biomech.* 20: 629–639.

Freed, L.E., Marquis, J.C., Nohria, A., Emmanual, J., Mikos, A.G., Langer, R. (1993) Neocartilage formation in vitro and in vivo using cells cultured on synthetic biodegradable polymers. *J. Biomed. Mater. Res.* 27: 11–23.

Freed, L.E., Grande, D.A., Lingbin, Z., Emmanual, J., Marquis, J.C., Langer, R. (1994a) Joint resurfacing using allograft chondrocytes and synthetic biodegradable polymer scaffolds. *J. Biomed. Mater. Res.* 28: 891–899.

Freed, L.E., Marquis, J.C., Vunjak-Novakovic, G., Emmanual, J., Langer, R. (1994b) Composition of cell-polymer cartilage implants. *Biotechnol. Bioeng.* 43: 605–614.

Freed, L.E., Vunjak-Novakovic, G., Biron, R., Eagles, D., Lesnoy, D., Barlow, S., Langer, R. (1994c) Biodegradable polymer scaffolds for tissue engineering. *Bio/Technology* 12: 689–693.

Freed, L.E., and Vunjak-Novakovic, G. (1995) Cultivation of cell-polymer constructs in simulated microgravity. *Biotechnol. Bioeng.* 46: 306–313.

Freed, L.E., Langer, R., Martin, I., Pellis, N., Vunjak-Novakovic, G. (1997) Tissue engineering of cartilage in space. *Proc. Natl. Acad. Sci. USA* 94: 13885–13890.

Freed, L.E., Hollander, A.P., Martin, I., Barry, J.R., Langer, R., Vunjak-Novakovic, G. (1998) Chondrogenesis in a cell-polymer-bioreactor system. *Exp. Cell Res.* 240: 58–65.

Freed, L.E., Martin, I., Vunjak-Novakovic, G. (1999) Frontiers in tissue engineering: in vitro modulation of chondrogenesis. *Clin. Orthop. Rel. Res.* 367S: S46–S58.

Freed, L.E., and Vunjak-Novakovic, G. (2000a) Tissue engineering bioreactors. In Lanza, R.P., Langer, R., Vacanti, J., eds, *Principles of Tissue Engineering*. San Diego, Academic Press, pp. 143–156.

Freed, L.E., and Vunjak-Novakovic, G. (2000b) Tissue engineering of cartilage. In Bronzino, J.D., ed, *The Biomedical Engineering Handbook*. Boca Raton, CRC Press, pp. 124–126.

Gao, J., Niklason, L., Langer, R. (1998) Surface hydrolysis of poly(glycolic acid) meshes increases the seeding density of vascular smooth muscle cells. *J. Biomed. Mater. Res.* 42: 417–424.

Gooch, K.J., Blunk, T., Courter, D.L., Sieminski, A.L., Bursac, P.M., Vunjak-Novakovic, G., Freed, L.E. (2001a) IGF-I and mechanical environment interact to modulate engineered cartilage development. *Biochem. Biophys. Res. Commun.* 286: 909–915.

Gooch, K.J., Kwon, J.H., Blunk, T., Langer, R., Freed, L.E., Vunjak-Novakovic, G. (2001b) Effects of mixing intensity on tissue-engineered cartilage. *Biotechnol. Bioeng.* 72: 402–407.

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

Hauselmann, H.J., Fernandes, R.J., Mok, S.S., Schmid, T.M., Block, J.A., Aydelotte, M.B., Kuettner, K.E., Thonar, E.J.M. (1994) Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads. *J. Cell Sci.* 107: 17–27.

Haynesworth, S.E., Reuben, D., Caplan, A.I. (1998) Cell-based tissue engineering therapies: The influence of whole body physiology. *Adv. Drug Deliv. Res.* 33: 3–14.

Heath, C.A. (2000) The effects of physical forces on cartilage tissue engineering. *Biotechnol. Genet. Eng. Rev.* 17: 533–551.

Hoemann, C.D., Sun, J., Legare, A., McKee, M.D., Ranger, P., Buschmann, M.D. (2001) A thermosensitive polysaccharide gel for cell delivery in cartilage repair. *Trans. Orthop. Res. Soc.* 26.

Hollander, A.P., Heathfield, T.F., Webber, C., Iwata, Y., Bourne, R., Rorabeck, C., Poole, R.A. (1994) Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay. *J. Clin. Invest.* 93: 1722–1732.

Johnstone, B., Hering, T.M., Caplan, A.I., Goldberg, V.M., Yoo, J.U. (1998) In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp. Cell Res.* 238: 265–272.

Kawamura, S., Wakitani, S., Kimura, T., Maeda, A., Caplan, A.I., Shino, K., Ochi, T. (1998) Articular cartilage repair-rabbit experiments with a collagen gel-biomatrix and chondrocytes cultured in it. *Acta Orthop. Scand.* 69: 56–62.

Kim, S.S., Sundback, C.A., Kaihara, S., Benvenuto, M.S., Kim, B.S., Mooney, D.J., Vacanti, J.P. (2000) Dynamic seeding and in vitro culture of hepatocytes in a flow perfusion system. *Tissue Eng.* 6: 39–44.

Kim, Y.J., Sah, R.L., Doong, J.Y.H., Grodzinsky, A.J. (1988) Fluorometric assay of DNA in cartilage explants using Hoechst 33258. *Anal. Biochem.* 174: 168–176.

Langer, R., and Vacanti, J.P. (1993) Tissue engineering. *Science* 260: 920–926.

Lanza, R.P., Langer, R., Vacanti, J., Eds. (2000) *Principles of Tissue Engineering*. San Diego, Academic Press.

Lee, D.A., and Bader, D.L. (1997) Compressive strains at physiological frequencies influence the metabolism of chondrocytes seeded in agarose. *J. Orthop. Res.* 15: 181–188.

Li, R.-K., Jia, Z.Q., Weisel, R.D., Mickle, D.A.G., Choi, A., Yau, T.M. (1999) Survival and function of bioengineered cardiac grafts. *Circulation* 100: II63–II69.

Litzke, L.E., Wagner, E., Baumgaertner, W., Hetzel, U., Josimovic-Alasevic, O., Libera, J. (2004) Repair of extensive articular cartilage defects in horses by autologous chondrocyte transplantation. *Ann. Biomed. Eng.* 32: 57–69.

MacKenna, D.A., Omens, J.H., McCulloch, A.D., Covell, J.W. (1994) Contribution of collagen matrix to passive left ventricular mechanics in isolated rat heart. *Am. J. Physiol Heart Circ. Physiol.* 266: H1007–H1018.

Madry, H., and Trippel, S.B. (2000) Efficient lipid-mediated gene transfer to articular chondrocytes. *Gene Ther.* 7: 286–291.

Madry, H., Padera, R., Seidel, J., Freed, L.E., Langer, R., Trippel, S.B., Vunjak-Novakovic, G. (2001) Tissue engineering of cartilage enhanced by the transfer of a human insulin-like growth factor-I gene. *Trans. Orthop. Res. Soc.* 26: 289.

Maki, T., Gruver, E., Davidoff, A., Izzo, N., Touplin, D., Colucci, W., Marks, A. (1996) Regulation of calcium channel expression in neonatal myocytes by catecholamines. *J. Clin. Invest.* 97: 656–663.

Maroudas, A. (1979) Physiochemical properties of articular cartilage. In Freeman, M.A.R., ed, *Adult articular cartilage*, Pitman Medical, pp. 215–290.

Martin, I., Padera, R.F., Vunjak-Novakovic, G., Freed, L.E. (1998) In vitro differentiation of chick embryo bone marrow stromal cells into cartilaginous and bone-like tissues. *J. Orthop. Res.* 16: 181–189.

Martin, I., Vunjak-Novakovic, G., Yang, J., Langer, R., Freed, L.E. (1999) Mammalian chondrocytes expanded in the presence of fibroblast growth factor-2 maintain the ability to differentiate and regenerate three-dimensional cartilaginous tissue. *Exp. Cell Res.* 253: 681–688.

Martin, I., Shastri, V.P., Padera, R.F., Yang, J., Mackay, A.J., Langer, R., Vunjak-Novakovic, G., Freed, L.E. (2001a) Selective differentiation of mammalian bone marrow stromal cells cultured on three-dimensional polymer foams. *J. Biomed. Mater. Res.* 55: 229–235.

Martin, I., Suetterlin, R., Baschong, W., Heberer, M., Vunjak-Novakovic, G., Freed, L.E. (2001b) Enhanced cartilage tissue engineering by sequential exposure of chondrocytes to FGF-2 during 2D expansion and BMP-2 during 3D cultivation. *J. Cell. Biochem.* 83: 121–128.

Mauck, R.L., Soltz, M.A., Wang, C.C.B., Wong, D.D., Chao, P.G., Valhmu, W.B., Hung, C.T., Ateshian, G.A. (2000) Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels. *J. Biomech. Eng.* 122: 252–260.

Mauck, R.L., Seyhan, S.L., Ateshian, G.A., Hung, C.T. (2002) Influence of seeding density and dynamic deformational loading on the developing structure/function relationships of chondrocyte-seeded agarose hydrogels. *Ann. Biomed. Eng.* 30: 1046–1056.

Mauck, R.L., Nicoll, S.B., Seyhan, S.L., Ateshian, G.A., Hung, C.T. (2003) Synergistic action of growth factors and dynamic loading for articular cartilage tissue engineering. *Tissue Eng.* 9: 597–611.

Meinel, L., Hofmann, S., Karageorgiou, V., Zichner, L., Langer, R., Kaplan, D., Vunjak-Novakovic, G. (2004a) Engineering cartilage-like tissue using human mesenchymal stem cells and silk protein scaffolds. *Biotechnol. Bioeng.* 88: 379–391.

Meinel, L., Karageorgiou, V., Fajardo, R., Snyder, B., Shinde-Patil, V., Zichner, L., Kaplan, D., Langer, R., Vunjak-Novakovic, G. (2004b) Bone tissue engineering using human mesenchymal stem cells: Effects of scaffold material and medium flow. *Ann. Biomed. Eng.* 32: 112–122.

Mow, V.C., Kuei, S.C., Lai, W.M., Armstrong, C.G. (1980) Biphasic creep and stress relaxation of articular cartilage in compression: Theory and experiments. *J. Biomech. Eng.* 102: 73–84.

Mow, V.C., Ratcliffe, A., Rosenwasser, M.P., Buckwalter, J.A. (1991) Experimental studies on the repair of large osteochondral defects at a high weight bearing area of the knee joint: a tissue engineering study. *Trans. Am. Soc. Mech. Engr.* 113: 198–207.

Mow, V.C., Wang, C.B., Hung, C.T. (1999) The extracellular matrix, intersitial fluid and ions as a mechanical signal transducer in articular cartilage. *Osteoarthr. Cartilage* 7: 41–58.

Niklason, L.E., Gao, J., Abbott, W.M., Hirschi, K.K., Houser, S., Marini, R., Langer, R. (1999) Functional arteries grown in vitro. *Science* 284: 489–493.

Obradovic, B., Freed, L.E., Langer, R., Vunjak-Novakovic, G. (1997) Bioreactor studies of natural and engineered cartilage metabolism. In Peppas, N.A., Mooney, D.J., Mikos, A.G., Brannon-Peppas, L., eds, *Proceedings of the Topical Conference on Biomaterials, Carriers for Drug Delivery, and Scaffolds for Tissue Engineering*. AIChE, p. 335.

Obradovic, B., Carrier, R.L., Vunjak-Novakovic, G., Freed, L.E. (1999) Gas exchange is essential for bioreactor cultivation of tissue engineered cartilage. *Biotechnol. Bioeng.* 63: 197–205.

Obradovic, B., Martin, I., Padera, R.F., Treppo, S., Freed, L.E., Vunjak-Novakovic, G. (2001) Integration of engineered cartilage. *J. Orthop. Res.* 19: 1089–1097.

O'Driscoll, S.W. (2001) Preclinical cartilage repair: current status and future perspectives. *Clinical Orthopaedics* 391 Suppl: S397–S401.

Papadaki, M., Bursac, N., Langer, R., Merok, J., Vunjak-Novakovic, G., Freed, L.E. (2001) Tissue engineering of functional cardiac muscle: molecular, structural and electrophysiological studies. *Am. J. Physiol. Heart Circ. Physiol.* 280: H168–H178.

Pazzano, D., Mercier, K.A., Moran, J.M., Fong, S.S., DiBiasio, D.D., Rulfs, J.X., Kohles, S.S., Bonassar, L.J. (2000) Comparison of chondrogensis in static and perfused bioreactor culture. *Biotechnol. Prog.* 16: 893–896.

Pei, M., Seidel, J., Vunjak-Novakovic, G., Freed, L.E. (2002a) Growth factors for sequential cellular de- and re-differentiation in tissue engineering. *Biochem. Biophys. Res. Commun.* 294: 149–154.

Pei, M., Solchaga, L.A., Seidel, J., Zeng, L., Vunjak-Novakovic, G., Caplan, A.I., Freed, L.E. (2002b) Bioreactors mediate the effectiveness of tissue engineering scaffolds. *FASEB J.* 16: 1691–1694.

Radisic, M., Euloth, M., Yang, L., Langer, R., Freed, L.E., Vunjak-Novakovic, G. (2003) High density seeding of myocyte cells for tissue engineering. *Biotechnol. Bioeng.* 82: 403–414.

Radisic, M., Yang, L., Boublik, J., Cohen, R.J., Langer, R., Freed, L.E., Vunjak-Novakovic, G. (2004a) Medium perfusion enables engineering of compact and contractile cardiac tissue. *Am. J. Physiol. Heart Circ. Physiol.* 286: H507–H516.

Radisic, M., Park, H., Shing, H., Consi, T., Schoen, F.J., Langer, R., Freed, L.E., Vunjak-Novakovic, G. Functional assembly of engineered myocardium by electrical stimulation of cardiac myocytes cultured on scaffolds. *Proc. Natl. Acad. Sci. USA.* 101(52): 18129–18134 (2004b).

Riesle, J., Hollander, A.P., Langer, R., Freed, L.E., Vunjak-Novakovic, G. (1998) Collagen in tissue-engineered cartilage: Types, structure and crosslinks. *J. Cell. Biochem.* 71: 313–327.

Sah, R.L.Y., Kim, Y.J., Doong, J.Y.H., Grodzinsky, A.J., Plaas, A.H.K., Sandy, J.D. (1989) Biosynthetic response of cartilage explants to dynamic compression. *J. Orthop. Res.* 7: 619–636.

Schaefer, D., Martin, I., Heberer, M., Jundt, G., Seidel, J., Bergin, J., Grodzinsky, A.J., Vunjak-Novakovic, G., Freed, L.E. (2000a) Tissue engineered composites for the repair of large osteochondral defects. *Trans. Orthop. Res. Soc.* 25: 619.

Schaefer, D., Martin, I., Shastri, V.P., Padera, R.F., Langer, R., Freed, L.E., Vunjak-Novakovic, G. (2000b) In vitro generation of osteochondral composites. *Biomaterials* 21: 2599–2606.

Schaefer, D., Martin, I., Jundt, G., Seidel, J., Heberer, M., Grodzinsky, A.J., Bergin, I., Vunjak-Novakovic, G., Freed, L.E. (2002) Tissue engineered composites for the repair of large osteochondral defects. *Arthritis Rheum.* 46: 2524–2534.

Schreiber, R.E., Ilten-Kirby, B.M., Dunkelman, N.S., Symons, K.T., Rekettye, L.M., Willoughby, J., Ratcliffe, A. (1999) Repair of osteochondral defects with allogeneic tissue-engineered cartilage implants. *Clin. Orthop. Rel. Res.* 367S: S382–S395.

Seidel, J.O., Pei, M., Gray, M.L., Langer, R., Freed, L.E., Vunjak-Novakovic, G. (2004) Long-term culture of tissue engineered cartilage in a perfused chamber with mechanical stimulation. *Biorheology* 41: 445–458.

Sittinger, M., Bujia, J., Minuth, W.W., Hammer, C., Burmester, G.R. (1994) Engineering of cartilage tissue using bioresorbable polymer carriers in perfusion culture. *Biomaterials* 15: 451–456.

Springhorn, J.P., and Claycomb, W.C. (1989) Prepoenkephalin mRNA expression in developing rat heart and in cultured ventricular cardiac muscle cells. *Biochem. J.* 258: 73–78.

Tagil, M., and Aspenberg, P. (1999) Cartilage induction by controlled mechanical stimulation in vivo. *J. Orthop. Res.* 17: 200–204.

Toraason, M., Luken, M., Breitenstein, M., Krueger, J., Biagini, R. (1989) Comparative toxicity of allylamine and acrolein in cultured myocytes and fibroblasts from neonatal rat heart. *Toxicology* 56: 107–113.

Vunjak-Novakovic, G., Freed, L.E., Biron, R.J., Langer, R. (1996) Effects of mixing on the composition and morphology of tissue-engineered cartilage. *AIChE J.* 42: 850–860.

Vunjak-Novakovic, G., Obradovic, B., Bursac, P., Martin, I., Langer, R., Freed, L.E. (1998) Dynamic cell seeding of polymer scaffolds for cartilage tissue engineering. *Biotechnol. Prog.* 14: 193–202.

Vunjak-Novakovic, G., Martin, I., Obradovic, B., Treppo, S., Grodzinsky, A.J., Langer, R., Freed, L.E. (1999) Bioreactor cultivation conditions modulate the composition and mechanical properties of tissue engineered cartilage. *J. Orthop. Res.* 17: 130–138.

Vunjak-Novakovic, G., and Radisic, M. (2004) Cell seeding of polymer scaffolds. *Methods Mol. Biol.* 238: 131–146.

Vunjak-Novakovic, G., and Goldstein, S. (2005) Biomechanical principles of cartilage and bone tissue engineering. In: *Basic Orthopaedic Biomechanics and Mechanobiology*, Mow, V.C., and Huiskes, R. 3rd Ed., Lippincot-Williams and Wilkens, Chapter 8, pp. 343–408.

Wakitani, S., Goto, T., Pineda, S.J., Young, R.G., Mansour, J.M., Caplan, A.I., Goldberg, V.M. (1994) Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. *J. Bone Joint Surg. Am.* 76A: 579–592.

Wakitani, S., Goto, T., Young, R.G., Mansour, J.M., Goldberg, V.M., Caplan, A.I. (1998) Repair of large full-thickness articular cartilage defects with allograft articular chondrocytes embedded in a collagen gel. *Tissue Eng.* 4: 429–444.

Williams, S.N.O., Burstein, D., Freed, L.E., Gray, M.L., Langer, R., Vunjak-Novakovic, G. (1998) MRI measurements of fixed charge density as a measure of glycosaminoglycan content and distribution in tissue engineered cartilage. *Trans. Orthop. Res. Soc.* 23: 203.

Woessner, J.F. (1961) The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch. Biochem. Biophys.* 93: 440–447.

Wu, F., Dunkelman, N., Peterson, A., Davisson, T., De La Torre, R., Jain, D. (1999) Bioreactor development for tissue-engineered cartilage. *Ann. N. Y. Acad. Sci.* 875: 405–411.

Wu, Q.-q., and Chen, Q. (2000) Mechanoregulation of chondrocyte proliferation, maturation and hypertrophy: ion-channel dependent transduction of matrix deformation signals. *Exp. Cell Res.* 256: 383–391.

Zimmermann, W.H., Fink, C., Kralish, D., Remmers, U., Weil, J., Eschenhagen, T. (2000) Three-dimensional engineered heart tissue from neonatal rat cardiac myocytes. *Biotechnol. Bioeng.* 68: 106–114.

Zimmermann, W.H., Didie, M., Wasmeier, G.H., Nixdorff, U., Hess, A., Melnychenko, I., Boy, O., Nehuber, W.L., Weyand, M., Eschenhagen, T. (2002a) Cardiac grafting of engineered heart tissue in syngenic rats. *Circulation* 106: I151–I157.

Zimmermann, W.H., Schneiderbanger, K., Schubert, P., Didie, M., Munzel, F., Heubach, J.F., Kostin, S., Nehuber, W.L., Eschenhagen, T. (2002b) Tissue engineering of a differentiated cardiac muscle construct. *Circ. Res.* 90: 223–230.