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Considerations for the creation of cellular bone substitutes for large defects in load bearing bones through tissue engineering

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CONSIDERATIONS FOR THE CREATION OF CELLULAR BONE
SUBSTITUTES FOR LARGE DEFECTS IN
LOAD BEARING BONES THROUGH
TISSUE ENGINEERING

Thesis

Submitted to

The Graduate School of the
UNIVERSITY OF DAYTON

in Partial Fulfillment of the Requirements for

The Degree

Master of Science in Biology

By


Maria Lynch

UNIVERSITY OF DAYTON

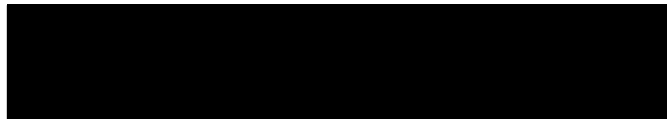
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May 2008

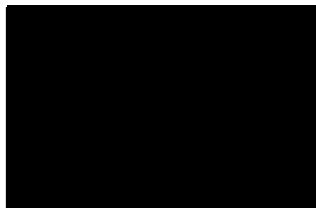
APPROVAL



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ABSTRACT

There are over one million cases of bone injury that require a bone graft or implant each year. Current technology relies on living grafts taken from the patient or a donor or synthetic implants. These options can be limited by one or more of the following: supply, disease transmission, immune response, a lack of plasticity, poorly matched mechanical strength. Engineered cellular bone constructs have merits in that they do not transmit disease, are available in sufficient supply, elicit a biological response, have sufficient mechanical strength and display plasticity. The constructs are created via tissue engineering, which requires a scaffold, cells and a place to allow the construct to mature, known as a bioreactor. The scaffold should be a synthetic, degradable polymer such as polyglycolic acid. It must be created in such a way as to produce the desired microstructure and maintain the polymer's mechanical properties, for example via solvent casting / particulate leaching. The cells used should be mesenchymal stem cells. The cells must come from the patient or be modified to match to the patient's HLA type. The bioreactor should be a dynamic, closed system. Being a dynamic system, it will not have concentration gradients and will avoid feed/famine cycles. Being a closed system, it will remain sterile. The most promising option is a perfusion reactor, as it can be optimized to provide mechanical stimulation. Additionally the bioreactor should be monitored so the state of the construct is known and to know when the construct is mature enough for implantation. This technology is not at the point of clinical application, but other tissues such as skin and the bladder have been successfully grown extracorporeally and implanted. The technology to create engineered bone is still in its infancy, but success with other tissues, indicate results will ultimately be successful.

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TABLE OF CONTENTS

APPROVAL.....	II
ABSTRACT.....	III
ACKNOWLEDGEMENTS.....	IV
TABLE OF CONTENTS.....	V
LIST OF ILLUSTRATIONS.....	VI
LIST OF TABLES	VII
CHAPTER I	1
BACKGROUND INFORMATION.....	1
INTRODUCTION.....	1
BONE GROWTH, HEALING.....	3
CURRENT TECHNOLOGIES FOR BONE REPAIR.....	6
CHAPTER II	10
SCAFFOLDS.....	10
INTRODUCTION.....	10
DESIRED PROPERTIES.....	11
TYPES OF SCAFFOLDS.....	12
MANUFACTURING SCAFFOLDS.....	17
CHAPTER III	22
CELLULAR COMPONENTS	22
INTRODUCTION.....	22
CELL SOURCES.....	22
IMMUNE SYSTEM.....	24
MAINTENANCE ISSUES.....	24
MITIGATING IMMUNOGENICITY	25
CHAPTER IV	28
BIOREACTORS	28
INTRODUCTION.....	28
SEEDING CELLS ONTO A SCAFFOLD.....	28
BIOREACTORS CHARACTERISTICS.....	29
TYPES OF BIOREACTORS	31
EVALUATION	33
FINAL PRODUCT	34
CHAPTER V.....	36
CONCLUSIONS.....	36
CURRENT STATE OF AFFAIRS.....	36
FUTURE RESEARCH OPPORTUNITIES.....	37
BIBLIOGRAPHY.....	39

LIST OF ILLUSTRATIONS

Figure 1 Proposed Steps of Osteoblast Differentiation from an MSC	4
Figure 2 Continuum of Injury Treatment.....	6
Figure 3 Polymer Scaffold	11
Figure 4 Seeding Cell Scaffolds.....	29
Figure 5 Perfusion Culture Setup.....	32

LIST OF TABLES

Table 1 Immunologically Relevant Properties of Natural Polymers.....	15
Table 2 Mechanical Properties of Material Classes for Bone Substitution	16
Table 3 Summary of Properties of Different Bioreactors	33

CHAPTER I

BACKGROUND INFORMATION

Introduction

There are approximately one million cases of skeletal defects a year that require bone-graft procedures (Salgado, Coutinho and Reis 2004, 743-765). Large amounts of bone may need to be replaced in the event of fractures with defects, fractures that are delayed in healing, resection of bone tumors, changing of an endoprosthesis, pseudoarthroses, an extension osteotomy, and treatment of osteomyelitis among others (Schlickewei and Schlickewei 2007, 10-23). These defects are being treated by tissue grafts. However, there is a limited amount of bone that can be harvested from the patient and there is a limited supply of donor material. In response to these limitations synthetic grafts are also being used to treat defects. However, these synthetic grafts are not able to display the mechanical *and* biological characteristics desired. These problems are leading scientists to the creation of synthetic cellular bone constructs using bone tissue engineering. These constructs will overcome the limited supply of natural grafts and be able to combine both the necessary mechanical and biological properties, unlike synthetic implants. It is important to note that these constructs are not entire bones or mature pieces of bone to be implanted, but components that will assimilate to be indistinguishable from the patient's natural bone.

The desired characteristics of a replacement material cover a number of areas as bone is a complex, living tissue. The implant should provide temporary mechanical strength to the region and allow or induce the region to reconstitute itself with new bone. The scaffolding should degrade into non-toxic molecules that the body can metabolize or excrete so bone can go through its usual process of remodeling. (Yaszemski et al. 1996, 175) This will leave the patient's own bone where the implant once was.

To create engineered bone, cells are harvested and placed on a scaffold, a temporary place for the cells to proliferate and differentiate. The construct created is placed in a bioreactor, which is simply a controlled environment, where the construct can grow and mature. The scaffold, cell source, and bioreactor are simple in concept, but there are numerous complications within the details.

In this section, bone physiology, as well as, the current and historic range of bone substitutes will be reviewed. This knowledge is an important foundation for the next generation of repair of large bony defects. In the next section, scaffold properties and manufacture will be discussed. This will be followed by a discussion of cell sources and immunological issues. Finally, the bioreactor will be defined and optimization and monitoring will be discussed and a brief conclusion section will follow

Bone Growth, Healing

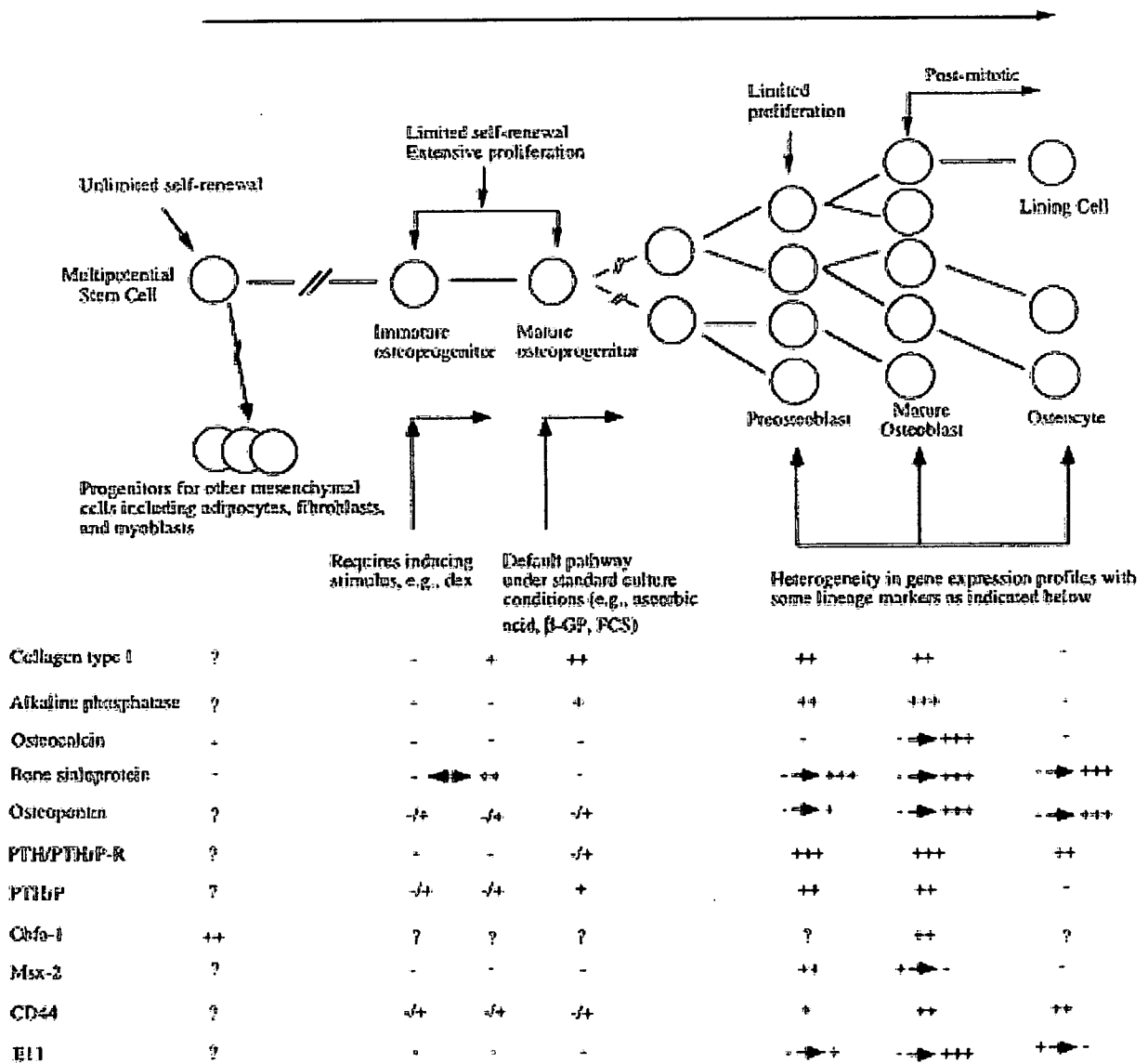
To create a viable substitute, it is important to appreciate and understand how bone is made and its role in the body.

In the body, bone is formed from osteoprogenitor cells which are found primarily in the bone marrow. Osteoprogenitors ultimately find their source in mesenchymal stem cells (MSC). MSCs have the ability to differentiate into a number of cell types including adipose, muscle, cartilage and bone. Moreover, MSCs can self renew. MSCs, driven by gene activation turn, into bone.

In vivo genes drive differentiation by creating different stimuli. Some of the stimuli produced to aid in the differentiation of MSCs are detectable at a specific stage or stages of differentiation. In the Figure 1, common markers are imposed over the various stages of differentiation including: Collagen Type X, Alkaline phosphatase, osteocalcin, bone sialoprotein, osteopontin, PTH/PTHrP-R, PTHrP, Cbfa-1, Msx-2, CD44 and E11.

As MSCs turn to bone there are distinct stages as shown in Figure 1. First MSCs turn into immature osteoprogenitors which are limited to self renewal or differentiation to a mature osteoprogenitor. Stepwise, MSCs differentiate to immature osteoprogenitors, mature osteoprogenitors, and finally bone cells.

There are three types of bone cells: osteoblasts are bone forming cells; osteocytes are mature osteoblasts; and osteoclasts function in the resorption of bone.



Postulated steps in the osteoblast lineage implying recognizable stages of differentiation as detectable from *in vitro* and *in vivo* experiments. Superimposed on this scheme are several well-established markers of the osteoblast and current thinking as to how their expression changes through differentiation stages. -, no detectable expression; +/+ - + + +, expression ranging from detectable to very high, → + + +, heterogeneous expression in individual cells.

Figure 1 Proposed Steps of Osteoblast Differentiation from an MSC

This figure shows proposed steps of osteoblast differentiation from mesenchymal stem cells. Additionally, it shows markers that are expressed at each step. This figure is used with the kind permission of Dr Aubin, University of Toronto. (Aubin 1998, 73-82)

Bone has a number of physiological roles. It is the primary reservoir of calcium within the body and its hematopoietic marrow produces red and white blood cells.

Bone, however, is most well known for providing mechanical support and providing sites of muscle attachment that allow for movement. When a graft or implant is needed, support and attachment are the only functions it assumes, because relatively small amounts of bone are replaced.

Normally, when injured, bone heals without a fibrous scar as most other tissues do. Instead, new bone is regenerated and that new bone is remodeled. Fracture repair follows the stages of inflammation, repair, and remodeling. Inflammation is the shortest stage. During the inflammation stage, escaped blood forms a haematoma that is organized to fill the gap and necrotic tissue is removed. Next, new bone organic matrix is synthesized and the matrix is ossified to form new woven bone, this is the repair stage. It results in a combination of osteoblasts, cartilaginous material and woven bone, called the fracture callus. The fracture callus is the product of repair and is the raw material for remodeling. Remodeling is the longest stage of bone healing. In remodeling, unorganized, woven bone is transformed to concentric sheets of bone, known as lamellar bone, through sequential resorption and deposition. (Yaszemski et al. 1996, 175).

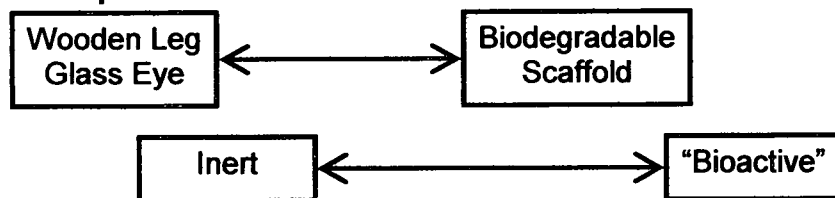
When the body's normal healing process cannot repair the injury to the bone surgeons use grafts or implants. The graft or implant is incorporated by the same stages of inflammation, repair and remodeling. However, one major difference for incorporating graft tissue is the need for the graft to become

vascularized. It is helpful to appreciate that the surrounding area is re-injured by the surgery. As such, the blood clotting cascade is activated and a fibrin clot forms. This is critical for the graft to revascularize, because it attracts epithelial cells used to create new vascular tissues. Depending on the type of graft, the process of revascularization could take hours to months. The graft is neovascularized and then bone forming cells appear. Only after the graft has undergone inflammation and repair will it be remodeled from organized bone to lamellar bone as in normal healing. (Yaszemski et al. 1996, 175)

Current Technologies for Bone Repair

There is a continuum of ways to treat injury to the human body from replacement, to repair, to regeneration (See Figure 2). As medicine steadily marches onward, the line between repair and regeneration is beginning to blur.

- **Full spectrum of materials and influences**



- Assist in regeneration
- Initiate a biological response
 - adhesion
 - proliferation
 - differentiation

- **Progression of technology**



Figure 2 Continuum of Injury Treatment

Injury treatment is increasingly relying on bioactive materials to help the body regenerate rather than simply using inert materials to replace broken parts. This diagram was created by Dr Ryan

Miller based on Enderle, Blanchard & Bronzino (2005) Introduction to Biomedical Engineering, 2nd. It is used with Dr Miller's kind permission.

The first option for large bony defects, still considered to be the gold standard, is an autograft. An autograft is a tissue graft in which tissue is harvested from one site in the patient to be used elsewhere. Since the tissue is from the patient there is no risk of immune rejection. Additionally, bone formation is immediately possible since the cells are living, and there is no risk of disease transmission from the graft. However, autografts are limited in their size, shape, quantity and quality as bone is usually taken from the iliac crest. Furthermore, there are additional incisions, increased operative time, and an increased potential for infection and/or deformity due to the harvest site (Hsu, Zucherman and White 1995, 870).

Another option to obtain a graft is to take them from another member of the same species, an allograft (as opposed to xenografts which are grafts taken from a different species). Allograft tissues are usually harvested from cadavers. They are preserved in a variety of ways including being fresh, frozen, freeze dried or demineralized. They are readily available and come in a wide variety of shapes and sizes and provide a scaffolding that is gradually replaced with the patient's own bone. However, as a consequence of the preservation and sterilization it is unable to contribute to osteogenesis, and incorporation of the graft and vascular penetration are slower. Additionally, there may be immunologic rejection of the graft. Furthermore, there is a risk of transmitting diseases via an allograft (Hsu, Zucherman and White 1995, 870). Xenografts carry a higher risk for rejection

and disease transmission. Therefore they are used far less frequently than allografts.

Synthetic implants can surmount the problems of limited supply, size and shapes, activation of immune response and pathogen transmission presented by living grafts, but have limitations of their own. Synthetic implant materials include metal, ceramic, polymers, and composites. Initially, implants were made of materials off the shelf, because these were well characterized and readily available. For example, hip replacements were initially made out of the same materials as airplane parts. The goal of these materials was to meet the mechanical requirements while being as inert as possible. The second generation of materials moved beyond inertness to being "bioactive," meaning they would elicit a physiological response. For example, bioactive glasses, (a class of ceramics), are osteoconductive, meaning they provide a framework for bone growth. Prostheses with bioactive glasses have a stronger material interface (Hench and Polak 2002, 1014). Second and third generation materials are ultimately limited because they cannot respond to changing loads and stimuli in the body. They cannot repair themselves and as such, their life spans are limited.

A third generation of materials is needed that meet the mechanical needs, elicit a physiological reaction, *and* shows plasticity once implanted. This is a new strategy where the implant will help the body heal itself. In tissue engineering

cells are implanted on a scaffold and the construct is grown in a bioreactor until the construct is ready for implantation. While simple in concept, there are many details to be worked out. The ultimate goal is to imitate nature (Hench and Polak 2002, 1014). The details will be explored in sections on scaffolds, cells, and bioreactors (in that order). Relevant issues will also be touched upon.

CHAPTER II

SCAFFOLDS

Introduction

Scaffolding is used in construction as a temporary platform where workers sit or stand while working (Merriam-Webster 2003). In the same way, scaffolds used in tissue engineering are places where cells attach, so they may proliferate and differentiate. Imperfect examples of scaffolds are a brillo pad or a honey comb. The Figure 3 shows a magnified view of a polymer scaffold. In this section the properties of an ideal scaffold will be explored and the manufacturing processes available to create scaffolds will be reviewed.



Figure 3 Polymer Scaffold

This is a picture of a polymer, PLGA, used as a scaffold. It is used with the kind permission of Prof. John E Davies from the University of Toronto. (Salgado, Coutinho and Reis 2004, 743-765)

Desired Properties

Without a scaffold cells would not attain the necessary three-dimensional architecture. There are a number of properties important to the ideal scaffold for bone tissue engineering.

- ◇ Foremost, a scaffold must be biocompatible. Biocompatibility can be equated with having a low level of immunogenicity.

- ◇ It is important that the scaffold has mechanical characteristics that are fairly well matched to the surrounding tissue. Mechanical properties/strength is a general term referring to compressive strength, tensile strength, modulus or other descriptors of the material. If a material is too hard it can injure surrounding tissue; if it is too soft it will not perform its desired roles.
- ◇ When a construct is implanted, it should be able to support the operative load of the treated area. However, it should degrade so that the implant can take over. In the genesis of bone, in particular, the cells must be mechanically loaded to function properly.
- ◇ The scaffold must encourage cells to attach and grow (Wiesmann, Joos and Meyer 2004, 523-530).
- ◇ Since the ultimate goal is to surgically implant the construct, it should be easily adapted to existing surgical procedures and tools. (Ahsan and Nerem 2005, 134-140, Yaszemski et al. 1996, 175)
- ◇ Additionally, the scaffold should have an acceptable shelf life.

Types of Scaffolds

There will be no singularly ideal material. There are a number of materials that possess some combination of the desired properties including metals, ceramics, polymers and composites.

Metals and ceramics have limited applicability as scaffolds (Liu and Ma 2004, 477-486). Metals, while providing excellent mechanical properties, tend to

cause stress shielding, a condition where bone mass is decreased (Berube 2001). Ceramics, while very similar in microstructure to bone, tend to lack the mechanical properties to act in a load bearing capacity.

Polymers are the preferred material because the range and combinations of properties they can express. Polymers chains are both jumbled like a bowl of spaghetti and possess regions that are quite well ordered. The percentage of the polymer that is well ordered is said to be crystalline. Crystalline regions add to the mechanical strength of material, but increase the difficulty of degradation.

Degradable polymeric scaffolds may also be classified as synthetic, natural, or composite. Natural polymers are created by a living source and are harvested in their complete form. Synthetic polymers are man-made from monomers.

Natural polymers that are candidates for bone tissue engineering scaffolds include polysaccharides (such as chitosan, starch, and hyaluronic acid) and proteins (such as fibrin, silks, and collagen)(Rezwan et al. 2006, 3413-3431). Natural materials have the benefit of being bioactive and presenting receptor-binding ligands to cells. Materials such as starch and collagen are in nearly unlimited supply. Additionally, natural materials tend to degrade into innocuous molecules that can be readily excreted after implantation. On the other hand, natural materials do not tend to have the mechanical strength necessary to act as bone scaffold. Additionally, they do not have predictable and reproducible

mechanical and physical properties from batch to batch. To avoid denaturing or damaging the molecule of interest, the purification process is relatively mild. As the purification process is mild, the molecule of interest may not be fully isolated or purified. So they may elicit an immune response and/or be able to carry pathogens especially between species (Rezwan et al. 2006, 3413-3431). See Table 1. As such, natural materials are an option for building scaffolds but their drawbacks have led researchers into the area of synthetic materials.

Table 1 Immunologically Relevant Properties of Natural Polymers

	Immunogenic	Source	Potential Impurities	Can it Carry Pathogens?
Proteins				
Fibrin	No	Blood Plasma	Viruses	Yes
Collagen	Sometimes	Tendons and Ligaments (Human, Porcine, Bovine etc)		
Polysaccharides				
Chitosan	No, but impurities may be	Crustacean Shells (byproducts of food production)	Protein, Bacteria, Yeast, Mold, Endotoxin, Heavy Metals	Yes
Hyaluronic Acid		Chemically modified natural HA from rooster combs and bovine vitreous humor, recently fermentation		

This table expresses the immunological potential of natural polymers. The immune response may be activated by impurities and pathogens.

Synthetic polymers may also be used for the creation of a scaffold. Synthetic polymers can be produced by binding together monomers. Because the production process is known, synthetic materials are reproducible on a large

scale with controlled properties including strength, degradation rate and microstructure. Synthetic materials can be manufactured to have the compressive strength necessary to serve as a bone substitute. Unfortunately, the production of a synthetic polymer can leave behind harmful molecules such as organic solvent, monomers, and catalysts. The safety of the degradation products and any intermediates must also be considered.

Table 2 Mechanical Properties of Material Classes for Bone Substitution

	Degradable	Variation between batches	Mechanical Strength	Microstructure similar to bone
Metal	—	-	+++	-
Ceramic	-/+	+	--	+++
Polymer (Natural)	++	---	++	- ↔ ++
Polymer (Synthetic)	- ↔ ++	- / +	++	- ↔ ++

This table reviews the mechanical properties of broad material classes with respect to being a bone substitute.

Based on all the information, synthetic degradable polymers are the strongest candidates for scaffolding. Synthetic polymers that are candidates for scaffolds for bone tissue engineering include poly- α -hydroxy acids, polyanhydrides, polycarbonates, polyfumarates, polyphosphazenes and polyphosphoesterases. Poly- α -hydroxyacids are strong candidates because of their mechanical strengths and some practical considerations. Practically, poly- α -hydroxyacids have been studied the most among the candidates for bone tissue engineering scaffolds. Polyglycolic acid (PGA) for example has FDA approval for use in the human body. Also, it is widely used, making it commercially available for study in pure and various forms. PGA is broken down into glycine which can be

excreted in the urine or converted to carbon dioxide and water via the citric acid cycle. However, debris formed mainly from crystalline regions of the polymer and acidic degradation products have been implicated in adverse tissue reactions. Large amounts of biodegradable polymer may overwhelm the body's ability to clear degradation products. (Agrawal and Ray 2001, 141-150). Another disadvantage is that poly- α -hydroxy acids in undergoing bulk degradation can fail prematurely. In bulk degradation polymer bonds throughout the scaffold are attacked at the same rate. (Rezwan et al. 2006, 3413-3431)

To circumvent the limitations of PGA – or other polymers for that matter – composites and copolymers are being manufactured. Composites are composed of two classes of materials, such as polymers and ceramics. Copolymers are composed of two polymer types. Composites and copolymers display mechanical properties different than those of either of the individual materials. Materials lacking strength but having bioactivity such as ceramics can be combined with high strength materials such as metals and polymers to create a strong bioactive composite.

Manufacturing Scaffolds

The processes used to make the polymer greatly influence the material's final properties. This includes both the creation of the material and the molding or shaping of the material to its final form. The ideal manufacturing process would lead to:

- ◇ Accurate and consistent porosity, pore size, pore distribution and interconnectivity. Though there is some debate, most scientists agree that the pores should be between 200 μ m and 400 μ m (Burg, Porter and Kellam 2000, 2347-2359) to accommodate osteoblasts and to aid in the delivery of a nutrient supply. The size and interconnectivity are important to nutrient supply to cells on the inside of the scaffold.
- ◇ Minimal variation between batches.
- ◇ No adverse effect to the material properties such as, mechanical stability, degradation rate and surface chemistry for cell adhesion and function.

There are a number of ways to manufacture scaffolds for bone tissue engineering. They include solvent casting/particulate leaching, and solid freeform fabrication (three dimensional printing, fused deposition modeling, three dimensional plotting). (Liu and Ma 2004, 477-486, Rezwan et al. 2006, 3413-3431, Salgado, Coutinho and Reis 2004, 743-765) Additionally, rapid prototyping techniques are being developed to make custom shaped and sized scaffolds and implants.

Solvent casting/particulate leaching is the most widely used method for preparing bone tissue engineering scaffolds. In this method, mineral or organic particles are dispersed in the polymer solution. The dispersion is cast into a predefined three dimensional mold to produce the porous scaffold. The solvent is allowed to evaporate and the particles are leached out of the solid polymer. This leaves

pores where the mineral or organic particles were. This method is quite easy and requires no specialized equipment. Also, porosity can be controlled by variation of the ratio of porogen to polymer used. However, this method uses toxic solvents which may be retained in the polymer and be harmful to the cells seeded onto the scaffold. Additionally, the shape of the scaffold is limited. This method is limited to the production of thin wafers or membranes.

There are variations on this procedure that address some of its shortcomings, for example phase inversion/particulate leaching and thermally induced phase separation. Phase inversion/particulate leaching is similar to solvent casting / particulate leaching. However, instead of allowing the solvent to evaporate, the solution is placed in water. A phase inversion causes the polymer to precipitate. It has many of the same benefits and limitations as solvent casting/particulate leaching. However, crystal deposition is avoided and thicker samples can be made (Rezwan et al. 2006, 3413-3431, Salgado, Coutinho and Reis 2004, 743-765). In thermally induced phase separation the polymer is dissolved in an organic solvent. The solution is quenched in liquid nitrogen. The solvent is sublimated under vacuum and then dried. Again, many of the same benefits and drawbacks of solvent casting/particulate leaching are seen. The main improvement is that high porosities and high interconnectivity are achieved. (Liu and Ma 2004, 477-486)

Solid free form techniques allow for custom implants to be made. They are highly computer driven, using CAD (computer aided drafting) and CAM (computer aided modeling) along with MRI (magnetic resonance imaging) or CT (computed tomography) imagery. These techniques include three dimensional printing, three dimensional plotting and fused deposition modeling. Taking three dimensional printing as an example, a printer head is used to print liquid binder onto thin layers of powder following the profile generated by a CAD file. The subsequent stacking and printing of material layers recreates the full structure. Solid free form techniques provide control over shape, porosity and pore architecture including size, geometry, orientation, branching, and interconnectivity. Thus the porous structure can be tailored to the host tissue. Due to the low processing temperatures, proteins and cells may be encapsulated within the scaffold. However, three dimensional printing takes a great deal of time. Furthermore, this technology is in its infancy. (Rezwan et al. 2006, 3413-3431, Salgado, Coutinho and Reis 2004, 743-765)

After manufacture, scaffolds are sometimes further modified. Common modifications include the addition of molecules to increase bioactivity or change surface properties. (Liu and Ma 2004, 477-486) However, this can lead to clogged pores. Also, the added molecules may not adhere to the scaffold strongly enough.

The scaffold is a place for cells to adhere as they proliferate and differentiate. The final properties of a scaffold are determined by the material chosen *and* the manufacturing process used. In the next section the cellular component of tissue engineering will be explored.

CHAPTER III

CELLULAR COMPONENTS

Introduction

Cells are the second component in engineered bone. The source and type of cells best suited to this application will be explored. Whatever type of cells are used there may be immunological issues. Ways to interrupt the immune response will be explored.

Cell Sources

Whether cells are autologous, allogenic or xenogenic, scientist use cells in three main categories to generate osseous tissue: unpurified primary tissue cultures, embryonic stem cells (ESCs) and mesenchymal stem cells (MSC). The cell type used should allow for extensive proliferation and differentiation.

It initially makes sense to create bone from cells harvested from existing bone tissue. Osteoblasts, chondroblasts, marrow and stem cells are harvested from periosteum, cortical bone and cancellous bone. (Meyer, Joos and Wiesmann 2004, 325-332, Ng et al. 2005, 192-199). These cells can be harvested easily and autologously. However, expansion rates *in vitro* are low (Salgado, Coutinho

and Reis 2004, 743-765). Also, cells from different locations in the vasculature can have phenotypic and functional characteristics that are very different. There can also be differences with age of the patient, including cellular functionality and availability.

To avoid functional and phenotypic variations, embryonic stem cells could be used. The public and scientific communities are currently fascinated with ESCs as they can differentiate to any cell type. Even more, ESCs can create cells like themselves to maintain their existence. However, ESCs are the subject of much ethical and legal debate, as their creation destroys a young human embryo. Currently in the US, research on ESCs is not federally funded. In addition to the ethical debate, there are some fears that ESCs will give rise to tumors because of their known growth potential. ESCs may not maintain may not retain their function because of their plasticity.

Mesenchymal stem cells balance the benefits and drawbacks of both an unpurified primary tissue culture and ESCs. Like ESCs, MSCs can self renew or differentiate. Unlike ESCs, harvesting MSCs does not harm the source. MSCs are easily harvested and can be harvested autologously. There are a number of sources of MSCs with the traditional sources of MSCs is of course bone marrow (Meyer, Joos and Wiesmann 2004, 635-641). While MSCs are relatively rare within bone marrow, at a 1:100,000 ratio (Salgado, Coutinho and Reis 2004, 743-765), MSCs have been researched extensively and purification protocols are

well developed. It is important to isolate these cells from the surrounding tissue from the standpoints of purity, yield, and simplicity. Because of the extensive research, MSCs do quite well *in vitro*, though they do require induction. The specific signals given are an imitation of nature. As scientists come to better understand what is happening as cells differentiate, there will be ever greater control *in vitro*.

Immune System

One of the main jobs of the immune system is to distinguish self from non-self. When tissue engineered cells are implanted in the body, the immune system reacts naturally reacts first with the inflammatory process, as part of the innate immune system and possibly with the adaptive arm of the immune system. In tissue engineering it is desirable for the length and severity of the immune response to be limited. The best scenario in tissue engineering is to avoid the response all together. If the response cannot be avoided it can be limited by changing the patient or the implant

Maintenance Issues

Cells that are extensively cultured *in vitro* may cause an immunogenic reaction upon implantation. A virus or toxin could invade the cell line. Alternatively, after implantation, cellular component released at cell lysis or cell death may activate an immune response. Additionally, as cells are grown in fetal bovine serum, the resulting tissue from this culture may contain bovine proteins. One solution would be to use human serum. However, blood banks are already frequently in

crisis so obtaining serum from human donors is impractical. What is feasible is the creation of a synthetic serum where the components that are necessary in serum are added individually. Determining and creating the components needed could prove to be a daunting task

Mitigating Immunogenicity

It has been mentioned that autologous cells are the gold-standard for implants because of their compatibility with the host. Much thought has gone into ways to alleviate and/or block the immune response to avoid implant rejection. Below are several ideas:

- ◇ Classic Immunosuppression The host may simply be immunosuppressed. While the medications are well known and commercially available, the side effects they produce are generally unacceptable. Associated complications include drug toxicities, opportunistic infections and an increase in the incidence of tumors. Considering these complications and decreased quality of life, this is usually an unacceptable option (Batten, Rosenthal and Yacoub 2007, 1343-1356, Kindt, Goldsby and Osborne 2007, 574)
- ◇ Creating a Cell Library. In this option, cells with known HLA types would be isolated and stored. HLA, Human Leukocyte Antigen, is the name of the human major histocompatibility complex, a group of genes that codes for cell surface proteins. The HLA type is a reflection of the markers that an individual expresses in "self" cells. The closer the HLA match, the more likely it is for an implant to succeed. An undifferentiated population

of ESCs that could be matched to any HLA background is a enticing proposition. This option could virtually assure success of an implant, but creating a cell bank would require a great deal of time and effort, as pure populations must be isolated, characterized and maintained (Batten, Rosenthal and Yacoub 2007, 1343-1356). Since there are so many HLA types this is not a feasible.

- ◇ 'Therapeutic Cloning' In this option, the nuclear genome of the patient would be placed in an oocyte where the nucleus was removed, that is nuclear transfer. This would yield embryonic stem cells with the same genetic material as the patient minus the mitochondrial DNA (Batten, Rosenthal and Yacoub 2007, 1343-1356). It is thought that the cells harvested from this method would be indistinguishable from "self" cells, leading to near-100% acceptance of the implanted cells. Creating an embryo and destroying it for therapeutic uses raises a maelstrom of ethical issues, making this option untenable
- ◇ Induction of tolerance of the host It was found that cotransplanting stem-cell-derived graft and bone marrow from the same donor into the patient, at the same time, will avoid rejection. The coexistence of donor and patient marrow cells is known as chimerism (Batten, Rosenthal and Yacoub 2007, 1343-1356, Ahsan and Nerem 2005, 134-140). This is a tantalizing option, and is currently under investigation.
- ◇ Immunomodulation of the graft Stem cells can be readily transduced by a variety of vectors and maintain expression after differentiation. Using

genetic modification, a universal donor cell could be engineered. One route is to eliminate MHC class I molecules. Such a drastic modification could impact the cells in unknown ways and may lead to indirect allo-recognition-mediated rejection and / or destruction. Another route is to target specific molecules or genes. These molecules or proteins would offer protection against rejection (Batten, Rosenthal and Yacoub 2007, 1343-1356). Genetic modification may have unexpected side effects but, the more scientist understand the immune system and the effects of genetic modification, the safer and more practical this method will become. Immunomodulation shows the greatest potential to mitigate the immune response at this time. I.

Cellular components are key to tissue engineering. MSCs are the suited to bone tissue engineering because their drawbacks have already largely been addressed. Attenuating immune response will broaden the possibilities for bone tissue engineering. At this point, the cells and the scaffold must be brought together. A friendly environment for cells to proliferate and differentiate should be used for the construct.

CHAPTER IV

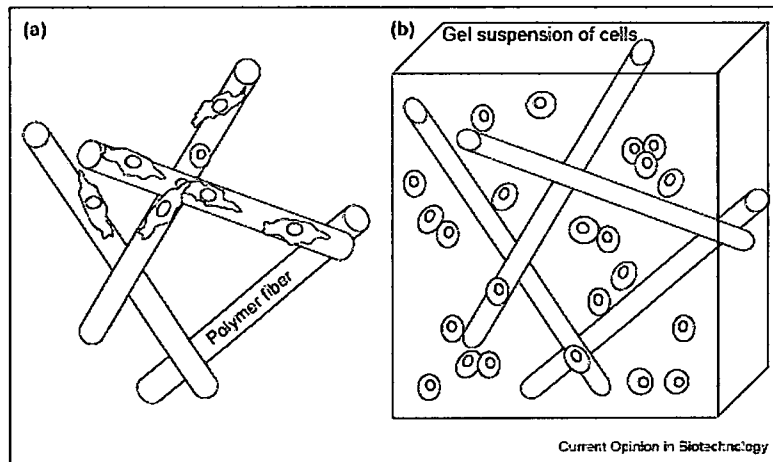
BIOREACTORS

Introduction

To generate a functional synthetic cellular bone construct, the components of the implant, scaffold and cells, are brought together in a process referred to as seeding. The seeded scaffold must be placed in an environment that encourages the cells to proliferate and differentiate, a bioreactor. Optimization of this basic bioreactor will be briefly explored and the practical issues of quality control through monitoring will be discussed.

Seeding Cells onto a Scaffold

A wide variety of methods have been developed to uniformly distribute cells and ensure cells penetrate the depths of the scaffold. Cells may be directly seeded onto the matrix or they can be suspended in a gel that the scaffold is covered by. (See Figure 4). (Sittinger, Hutmacher and Risbud 2004, 411-418). Static seeding (in which the cell solution are brought together and not mixed or stirred) is most widely used. Dynamic methods, such as simple mixing, seeding in spinner flask and convective seeding in perfusion reactors, are gaining wider use. Dynamic methods produce higher attachment efficiencies and more uniform distribution of cells.



Options for cell-seeding in scaffolds. (a) Cells are seeded onto the inner surface of the scaffold material. Cells attach and spread on the surface structures such as fibers. (b) Cells are distributed in the interconnecting cavities of a porous scaffold structure using a viscous embedding matrix component. Cells are not directly attached to the inner surface of the scaffold structure.

Figure 4 Seeding Cell Scaffolds

This figure depicts direct seeding of cells onto a scaffold and the gel suspension of cells. This picture is used with the kind permission of Dr Michael Sittinger of Charité University Medicine Berlin and German Rheumatism Research Center(Sittinger, Hutmacher and Risbud 2004, 411-418)

Bioreactors Characteristics

The seeded scaffold needs to mature through cell differentiation and proliferation. This occurs in a bioreactor. A bioreactor is simply a device in which biological and /or biochemical processes are performed under controlled conditions. (Meyer and Wiesman 2006, 264). Bioreactors, in the form of large stirred vats, are used industrially to grow eukaryotic or prokaryotic cells to produce some metabolic product, enzyme, or recombinant gene product. Industrial bioreactors have shown that the challenge of growing one specific cell or factor is achievable. The challenge in tissue engineering is the growth of a multitude of cell types in an organized, interactive, three dimensional structure (Ellis, Jarman-Smith and Chaudhuri 2005, 1). In tissue engineering, it is important for bioreactors to mimic

in vivo conditions: temperature, pH, nutrient supply (oxygen, glucose amino acids and proteins) and waste removal (carbon dioxide, lactate and urea) and physical and chemical stimulation (Ellis, Jarman-Smith and Chaudhuri 2005, 1) As tissue culture is a non-steady state process, with some parameters continually changing, mathematical modeling will be an invaluable tool in quantifying these variables.

Biochemical stimulation uses the signaling sequences that turn MSCs to bone. Many of the signals are known and using these prompts would give scientists a great deal of control over the maturation of the construct. Currently, biochemical prompts are being administered to cells chronically and in high doses. Cells are showing positive responses, but the practicality of using these prompts *in vitro* is questionable. Cells in the population of interest may be at different stages of differentiation. Furthermore, the creation or isolation of these molecules could be quite costly (Meyer and Wiesman 2006, 264)

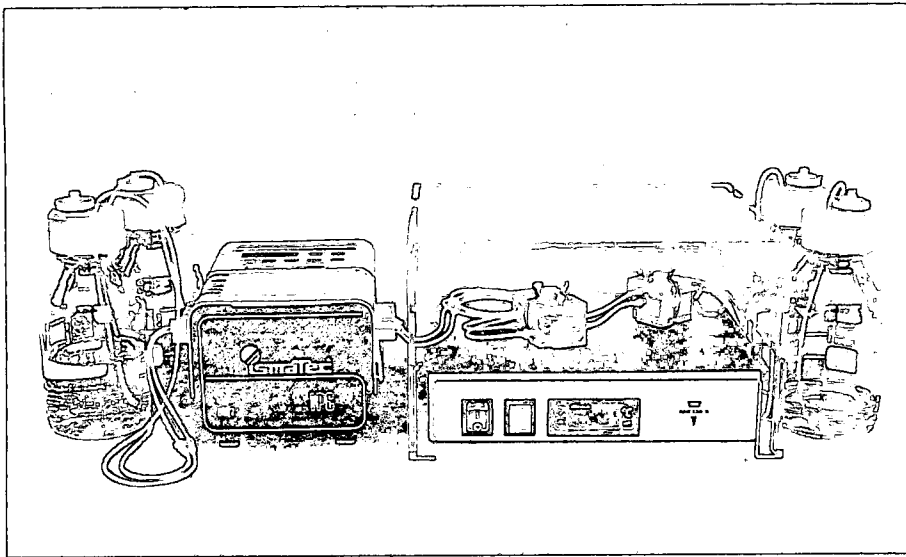
In addition to the biochemical ways to impact cell growth and development, there are biophysical cues being explored. For example, electric fields improve biomineral formation and enhance extracellular matrix synthesis. More commonly mechanical stimulation may come from hydrostatic pressure, or fluid flow induced shear stress. Shear stresses are generated by the flow of fluid through the reactor. They are dictated by the bioreactor set-up and can be controlled by the flow rate. Mechanical force is necessary for normal physiology

and morphology of bone cells *in vivo*. It is thought that these forces effect proliferation, cell orientation, gene activity and other cellular activities. (Meyer and Wiesman 2006, 264) It is logical and effective to apply force *in vitro* to increase cell differentiation and proliferation. If mechanical forces can be sufficiently well understood, they may negate the need for biochemical stimuli, which are expensive (Cartemell and El Haj 2005, 193). Again tissues at different stages of development require different conditioning. Little is know about the specific parameter or system of application (magnitude, frequency, duration and mode of load application) that provides optimal stimulation for bone tissue.

Types of Bioreactors

There is not a singal model of bioreactor being used throughout the community. Rather, most systems are created bespoke for the needs of an individual laboratory. The simplest and most widely used bioreactor is the culture plate, (Recall that bioreactors' primary purpose is to provide a controlled environment). It is easy to handle and economical to manufacture. However, they are of limited value when large numbers of cells are needed. They required individual manual handling for medium exchange, cell seeding etc. Additionally, the culture plate is an open, static system. In an open system, sterility cannot be assured. A static system is not stirred or mixed creating nutrient depletion and by-product build up cycles. Human cells can be sensitive to these cycles (Ellis, Jarman-Smith and Chaudhuri 2005, 1)

Dynamic systems relieve feed/famine cycles. They can be created by simply stirring the media. The simplest way to mix a system is to use a stir bar. Cells embedded in dense extracellular matrices experience enhanced transport of large molecules, like proteins and growth factors, with increased mixing. However, there is a limit to the intensity of mixing that can be used because of the shear fields generated.



Perfusion culture setups are self-contained and can be used on a laboratory table. A peristaltic pump transports the media (1 ml/h) to a gas expander module and then to a gradient container. The waste medium is collected in the bottles on the right side. A heating plate and a Plexiglas lid maintain the desired temperature.

Figure 5 Perfusion Culture Setup

This picture is of a small scale, closed bioreactor. It is used with the kind permission of Dr Minuth, University of Regensburg. (Minuth and Strehl, R., Schumacher, K 2005, 19)

Perfusion bioreactors, in which media is allowed to flow steadily over or through a bed of cells, show great promise for bone tissue engineering. Perfusion removes the feed / famine cycles of static cultures and they increase the mass transport of nutrients and oxygen especially into central parts of the cell-scaffold construct. The reactor can be optimized to balance nutrient supply, transport of

metabolites away, and shear stress induced. (Meyer and Wiesman 2006, 264)..

Furthermore perfusion reactors can accommodate a three dimensional construct.

Finally perfusion reactors are closed systems assuring sterility of the implant.

Table 3 Summary of Properties of Different Bioreactors

	Dynamic	Closed	Nutrient Gradient	Economical	Shear Fields	Available Off-the Shelf	Allows for Cell Stimulation	Three Dimensional Growth
Petri Dish			X	X		X		
Perfusion Bioreactor	X	X			X		X	X

This table summarizes the properties of different types of bioreactors

Evaluation

To determine the state of the construct in the bioreactor, in-line, nondestructive evaluation must be used. Parameters, such as cell number and cell differentiation should be monitored. Direct observation of the cells should be used as is possible, however, they are increasingly difficult to make as cells are inside the scaffold and are proliferating into a three-dimensional tissue. Indirect markers can be used to assess cell health and differentiation while maintaining a closed system. Having these data aids in adjusting culture conditions to actual tissue needs. Conditions within the bioreactor should be monitored including temperature, pH, O₂ and CO₂ concentration, nutrient composition, media flowrate, metabolite concentration and specific tissue markers. These markers include proteins secreted by the cells as they go through distinct phases in their growth. Additional technologies must be developed for observations to be made

in-line(Ellis, Jarman-Smith and Chaudhuri 2005, 1, Meyer and Wiesman 2006, 264).

Though observations are important to make to know the progression the construct, they are also important to verification of a quality product. Currently, the FDA has not adequately dealt with approval issues surrounding medical devices that combine materials and cells. Other industry standards such as quality assurance (QA) and good manufacturing practices (GMP) are being used as a stopgap measure. These guidelines ensure consistent quality. They apply to cell sourcing and expansion process, scaffold production, and growth of the construct (Ellis, Jarman-Smith and Chaudhuri 2005, 1)

Final Product

Following incubation in the bioreactor for approximately two weeks, a mature construct emerges. The engineered bone will be sterilely packed and stored until a surgeon uses it to repair a large section of bone. The construct does not look all that different from the scaffold at this point. After implantation the engineered bone will continue to mature until it is indistinguishable from the patient's natural bone.

Like an autograft, engineered bone carries a low risk of pathogen transmission or immune reaction. It is immediately able to contribute to osteogenesis. The engineered bone is ultimately indistinguishable from healthy bone. However, engineered bone is not limited in size, shape, quality or quantity. Engineered

bone provides the advantages of the industry's gold standard *and* mitigates some of the autografts shortfalls.

CHAPTER V

CONCLUSIONS

Current State of Affairs

Many strides have been made in the arena of bone tissue engineering for weight bearing bone. Materials engineers have developed scaffolds that are mechanically sound. Biologists are mastering culture of stem cells to bone cells. There are innumerable reports, books and articles on the subject. What is left is for scientist and engineers from mechanical engineering to chemistry from biology to materials engineering to look at the puzzle as a whole.

There have been clinical successes using tissue engineering.

- ◇ The FDA has approved a living skin product
- ◇ University of Massachusetts medical School in Worcester has grown cartilage in the shapes of ears and noses
- ◇ A bladder has been successfully grown *in vitro* and implanted.
- ◇ Joseph Vacanti has grown intestines within the abdominal cavity and then spliced it into existing intestinal tissue (Mooney 1999, 60)
- ◇ Additionally functional arteries have been made (Shastri 2006, 828-834)

There are successes in and towards replacing large amounts of bone.

- ◇ Foam impregnated with proteins is being clinically used for the reconstruction of non load bearing bone. (Hsu, Zucherman and White 1995, 870).
- ◇ Vascularization of a scaffold through localized delivery of growth factors(Shastri 2006, 828-834)

Future Research Opportunities

Tissue engineering is a field in its infancy. It seeks to tie together a number of diverse fields. This leaves the researcher a number questions and the employee job security. Questions must be considered across a number of discipline and have no singular or straightforward answers.

- ◇ With regards to bone physiology: What are the mechanisms of MSC to osteoblast differentiation and proliferation? What are the ideal mechanisms of stimulation *in vitro*? What proteins mark each stage?
- ◇ With regard to scaffolding: What are ideal scaffolding materials? How can scaffolds be produced with a minimum of toxic molecules? How can scaffolds be manufactured to a maximum of desired characteristics? What composite materials would be appropriate for scaffolding?
- ◇ With regard to the cellular portion: How can issues of immunogenicity acceptably be mitigated? How can cells be passaged multiple times without immortalizing the cell line? How can MSC identification processes be improved upon?
- ◇ With regard to bioreactors: How can time in the bioreactor be minimized? What is the appropriate system of stimulation for cells in the bioreactor (both

mechanical and chemical)? Through modeling what are optimum conditions within the bioreactor(e.g. temperature, pH)? What monitoring equipment will be developed to monitor constructs in-line? What should the regulatory standards be for tissue engineered bone?

These questions are already beginning to be asked in the community. Individual scientists and engineers are working the answers to these questions. There is no doubt that this will be standard treatment in the future.

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