11

CELL AND TISSUE REGENERATIVE ENGINEERING

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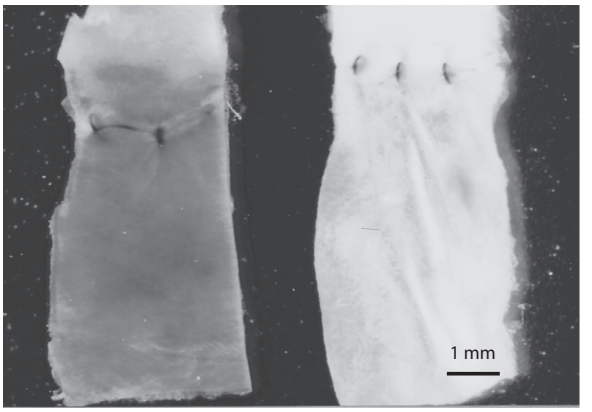
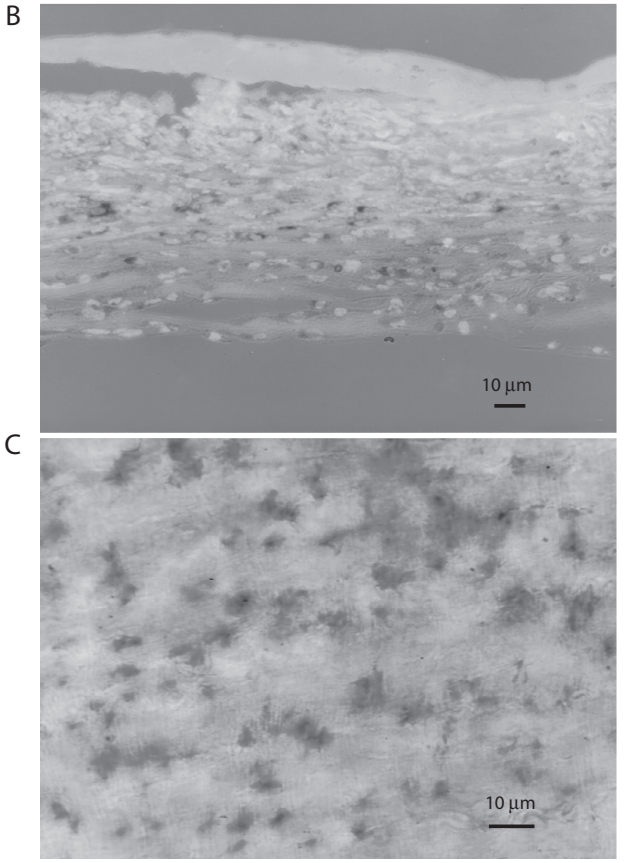


Vein graft cells transfected with a β-galactosidase (β-gal) gene vector by the mediation of electroporation. The left jugular vein of a rat was harvested, incubated in culture medium supplemented with 5 μg β-gal gene vector/100 μL, and subjected to electroporation. The transfected vein graft was incubated in culture medium for 30 min and grafted into the abdominal aorta of the rat. At day 10 after the grafting surgery, vein graft specimens were collected, fixed in 4% formaldehyde, incubated in the presence of X-gal, and observed by using an optical microscope. The blue color is indicative of the expression of the β-gal gene. (A) Vein graft specimens with (left) and without (right) β-gal gene transfection, showing the luminal surface. (B) Transverse section of a vein graft transfected with the β-gal gene. Dark blue: β-gal. Light blue: cell nuclei labeled with Hoechst 33258. Yellow: smooth muscle α-actin labeled with an anti-smooth muscle α-actin antibody. (C) En face observation of β-gal-positive cells (blue) on the luminal surface of a vein graft specimen transfected with the β-gal gene. See color insert.

Bioregenerative Engineering: Principles and Applications, by Shu Q. Liu



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An important aspect of bioregenerative engineering is to modulate, regenerate, repair, or replace disordered cells and tissues. The work at the cellular and tissue level may be considered cell and tissue regenerative engineering, respectively. However, it is often difficult to distinguish the level of the engineering work. In fact, for any given disorder, it is necessary to carry out engineering manipulations at all levels, including the cellular and tissue levels as well as the molecular level as discussed in Chapter 10. The classification of bioregenerative engineering into different levels is mostly for the convenience of description and text organization. This chapter focuses on the cellular and tissue aspects of bioregenerative engineering.

CELL REGENERATIVE ENGINEERING

Cell regenerative engineering is to regenerate, repair, or replace injured, disordered, and lost cells with functional somatic cells of the same type or stem cells that can differentiate into a desired cell type. For the majority of tissues and organs, such as the skin, intestine,

stomach, blood vessels, liver, and lung, certain types of cells including stem and progenitor cells are capable of proliferating and differentiating to replace injured and lost cells to a certain extent. However, when a pathogen is too strong and an injury is too severe, a tissue or organ may not be able to completely self-repair and regenerate. Furthermore, certain types of tissues and organs, such as the brain, spinal cord, and heart, have little capability of self-renewing and regeneration. For these systems, it is necessary to enhance the repair and regeneration processes by using engineering approaches. Cell regenerative engineering is established to achieve such a goal. To successfully conduct cell regenerative engineering, it is necessary to understand the function, organization, and development of cells and tissues, to identify and cultivate therapeutic cell lines, and to establish technologies for cell manipulation, transplantation, and functional tests.

Candidate Cell Types for Cell Regenerative Engineering [11.1]

Candidate cell types for cell regenerative engineering include somatic, stem, and progenitor cells. *Somatic cells* are defined as cells other than germ cells, including the egg and sperm, in a mature animal. Most somatic cells are specified cells that constitute tissues and organs. These cells have limited capability of proliferation, differentiation, and regeneration. Certain types of somatic cell, such as hepatocytes, smooth muscle cells, and epithelial cells, are capable of differentiating and/or proliferating within specified tissues. These cells may be used for cell regenerative engineering. For instance, hepatocytes can be used for liver repair and regeneration. Smooth muscle cells may be used for the construction of artificial blood vessels. Other types of somatic cell, such as the neurons and cardiomyocytes, exhibit minimal capacity for differentiation and proliferation. These cells are not suitable for cellular engineering. Overall, somatic cells are not considered preferred candidates for cell regenerative engineering.

Stem cells are undifferentiated cells that are capable of self-renewing and differentiating into specialized cells types. Thus, stem cells are ideal cells for the repair and regeneration of disordered or lost cells. Stem cells can be classified into three types, based on the stage of development: embryonic, fetal, and adult stem cells. *Embryonic stem cells* are cells derived primarily from an early embryonic structure, known as the inner cell mass of the blastocyst, and from embryonic germ cells. These cells are pluripotent cells that can develop into all specified cell types for peripheral tissues and organs.

Fetal stem cells are cells found in various tissues of the fetus and are committed to differentiation into specified cell types within a given tissue. Note that in humans the fetal stage is defined from the formation of the tissue and organ systems (at about the end of the second month) to the birth (at about the end of the ninth month) and the period before the fetal stage is known as the *embryonic stage*. Because of ethical concerns, fetal stem cells have not been extensively used for regenerative medicine and engineering.

Adult stem cells are committed stem cells found in a mature tissue or organ. These cells are capable of renewing and differentiating into specialized cells. Adult stem cells have been found in a number of tissues, including the bone marrow, blood, brain, liver, skin, intestine, stomach, and pancreas. While certain adult stem cells from a given tissue may be multipotent, that is, capable of differentiating into cells for a different type of tissue, most adult stem cells are committed to give rise to specialized cells in the same type of tissue. Compared to embryonic stem cells, adult stem cells are scarce and difficult

to identify. Nevertheless, because of ethical concerns about using embryonic and fetal stem cells, adult stem cells are still valuable candidates for cell regenerative engineering. Detailed descriptions for stem cells are presented on page 381.

Cell Expansion

Cell expansion via cell culture is a critical step for cell regenerative engineering. This is specially important for the preparation of stem cells. It is often difficult to collect a sufficient number of stem cells for cell repair and regeneration. For instance, each blastocyst can only provide about 30 stem cells. Stem cells are also scarce in adult tissues and organs. Thus, it is necessary to culture and expand stem cells before they are used for cellular engineering. For the three types of stem cell, including embryonic, fetal, and adult stem cells, embryonic stem cells from the human and mouse have been successfully cultured. Under an appropriate culture environment, cultured embryonic stem cells are able to grow for more than 2 years with a stable complement of chromosomes. Culture conditions may significantly influence the renewal and differentiation of the embryonic stem cells. For instance, to maintain the undifferentiated state of the embryonic stem cells, it is necessary to provide a layer of embryonic fibroblasts as feeder cells for the stem cells. When cultured in suspension without a feeder layer, the embryonic stem cells form aggregates with various cell types resembling those derived from the ectoderm, mesoderm, and endoderm.

In contrast to embryonic and fetal stem cells, adult stem cells are difficult to identify, because of their scarcity and the lack of identification markers. Adult stem cells are often dispersed in tissues. The purification of adult stem cells is a major challenge in cell regenerative engineering. Furthermore, it is difficult to maintain the undifferentiated state and to expand the number of adult stem cells in culture. These technical difficulties should be resolved before adult stem cells can be used for therapeutic purposes.

Genetic Modulation of Cells

It is often desired to have cells expressing an augmented phenotype that enhances the repair and regeneration of disordered and lost cells. Genetic modulation is an effective approach for achieving such a goal. There are two potential approaches that can be used to modulate the phenotypes of candidate cells for cell regenerative engineering: (1) enhancing or reducing the expression of desired proteins and (2) generating stem cell-like cells through somatic nuclear transfer.

As discussed in Chapter 10, the expression of a desired protein can be achieved by transferring the gene that encodes the protein. This approach can be potentially used to treat pathogenic disorders due to protein deficiency. In fact, protein deficiency is a cause for a large number of diseases. For instance, the deficiency of brain-derived growth factor in the brain is known as a cause for Alzheimer's disease. The lack of insulin causes diabetes. A reduction in elastin contributes to the development of intimal hyperplasia. By applying the principles of molecular regenerative engineering, it is possible to construct a recombinant gene encoding a desired protein and transfer it into candidate stem or progenitor cells to enhance the expression of the selected gene. Such an approach may be used to treat disorders due to protein deficiency. Furthermore, with the understanding of the regulatory mechanisms of stem cell differentiation, necessary genes can be transferred to control the differentiation of stem cells into desired cell types.

Somatic nuclear transfer is an approach established to generate cells with stem cell features in vitro by transferring a somatic cell nucleus from a patient into an embryonic stem cell derived from a blastocyst. Because the somatic nucleus carries the genome of the patient, the derived stem cells are compatible to the patient in terms of histocompatibility factors, thus reducing immune rejections. The transferred stem cells may retain their pluripotent nature. This is a potential approach for the generation of therapeutic stem cells. It is important to note that, although some techniques used for such an approach are similar to those used in reproductive animal cloning, the goal is different. Animal cloning is to reproduce an animal that is identical to the nucleus donor in genotype. An enucleated egg is used for somatic nuclear injection. The egg is implanted into the uterus and allowed to develop into progeny. In contrast, somatic nuclear transfer is for the generation of stem cells used to repair or regenerate disordered or lost cells.

Cell Transplantation

After selected stem or progenitor cells are expanded to a sufficient number, the cells can be delivered to a target tissue to replace disordered somatic cells. There are several approaches for cell delivery, including direct injection, implantation of polymer capsules with enclosed cells, and implantation of polymer scaffolds with seeded cells. The choice of delivery methods is dependent on the anatomy and function of the target tissue and organ. For cell delivery into a tissue that is difficult to access and is enclosed within a tight space, such as the brain, direct cell injection may be the method of choice. For cell delivery into the abdominal organs, such as the liver and pancreas, which reside within a relatively large space and are easy to access, scaffold implantation may be an effective approach. If immune rejection is a problem, cells may be enclosed within capsules made of porous membranes that prevent the infiltration of immune cells, but allow the transport of oxygen and nutrients into the capsules and produced proteins by the therapeutic cells out of the capsules. General criteria for cell transplantation are that the approach should not induce injury of the transplanted cells and the target tissue, and can be used to effectively deliver cells into the target tissue.

Identification of Transplanted Cells

Following cell transplantation, an important step is to identify the transplanted cells, ensuring the presence of the cells in the target tissue. The transplanted cells can be identified based on markers specific to the cells of interest. Certain cell types express protein markers unique to the cells themselves. For instance, CD34, c-Kit, Sca-1, and Thy1.1 are expressed in bone marrow-derived hematopoietic stem cells. Antibodies to these proteins can be established and used to identify hematopoietic stem cells. It is important to note that the expression of stem cell markers gradually diminishes when the stem cells are specified and differentiated. The stem cell markers may only be used within a short period after cell transplantation. Protein markers for long-term expression can be established by transfecting cells with reporter genes, such as the β-galactosidase gene, luciferase gene, CAT gene, and green or red fluorescent protein genes. These genes are not expressed in mammalian cells, and the proteins encoded by these genes can be detected for assessing cell transplantation. Methods for gene transfer are discussed on page 436.

In addition, cell morphological parameters, such as the shape and the intracellular structure of the implanted cells, the overall organization of implanted cells, and the rela-

tionship of implanted cells with neighbor cells and with extracellular matrix, can be measured and used to assess the transformation of stem cells to functional cells. For instance, the formation of cardiomyocytes can be judged by the appearance of sarcomeres and contractile filaments. Morphological information can be achieved by optical, fluorescence, and electron microscopy. Immunohistochemical methods can be used to identify specific structure and components the transplanted cells.

Functional Tests

In addition to morphological tests, another important aspect is the assessment of the function of transplanted cells. Various forms of test may be designed and performed, depending on the type and function of implanted cells. In general, functional tests may include biochemical, molecular, and physiological tests. For cells that produce necessary hormones and regulatory factors, such as hepatocytes and pancreatic β cells, biochemical assays may be designed and conducted to assess the level of a specified factor. For cells that generate forces, such as cardiac and skeletal muscle cells, mechanical tests should be carried out to assess the cell contractibility. Testing methods for specific tissues and organs are discussed in chapters addressing the organ systems through the book.

TISSUE REGENERATIVE ENGINEERING [11.2]

Tissue regenerative engineering is applied to regenerate, repair, or replace disordered and malfunctioned tissues or organs by establishing and using artificial tissue constructs based on biological or synthetic materials. Certain types of tissue construct may serve as tissue replacements that possess partial or complete function of the original tissues. These tissues are often supporting and mechanical structures. Examples of such constructs include artificial joints, bones, skin, blood vessels, and heart valves. Other types of tissue constructs may serve as scaffolds or frameworks that guide the regeneration of injured or lost tissues, which are composed of cells and glands responsible for producing and secreting hormones, enzymes, and necessary biochemical components. Examples of such constructs include liver and pancreatic scaffolds.

For most pathological disorders, tissue destruction occurs as a result of cell injury and death, which is associated with partial or complete loss of the function of the involved organ. Examples of such disorders include tissue infarction due to ischemia, severe viral and bacterial infections, and cirrhosis. When a tissue or organ system is unable to rebuild its supporting structure and framework, it is necessary to replace the malfunctioned tissue with an artificial tissue construct. For certain types of tissue, such as the hepatic, cardiac, pancreatic, and nervous tissues, it is necessary to incorporate functional cells into the tissue construct. Thus, tissue regenerative engineering is dependent on cell regenerative engineering. On the other hand, for some types of tissue, such as the bone, joint, blood vessels, and heart valves, it is not necessary to incorporate living cells into the tissue construct, although cell-based tissue constructs may improve the efficacy of tissue replacement.

On of the basis of the engineering approaches, tissue regenerative engineering can be classified into two categories: cell-based and cell-free tissue regenerative engineering. *Cell-based tissue regenerative engineering* involves the integration of living cells into

artificial tissue constructs. *Cell-free tissue regenerative engineering* does not require the use of living cells for tissue construction. When implanted into a target tissue in vivo, host cells can migrate into the tissue constructs and gradually transform the implanted construct into a functional tissue. For either cell-based or cell-free type, several procedures are necessary for the successful replacement of a malfunctioned tissue. These include tissue construction, functional test of tissue constructs in vitro, construct implantation, cell viability test in vivo for cell-based tissue constructs, as well as morphological and functional tests of tissue constructs in vivo. These aspects are briefly discussed in this section.

Tissue Construction

The construction of a specified tissue is dependent on the structure and function of the target tissue. A general standard for tissue construction is that an artificial tissue should possess the structure and function of a natural tissue. The necessity of incorporating living cells into tissue constructs is dependent on the type of target tissue. For tissues that provide the organ system with mechanical support, protection, strength, and elasticity, such as the bone, joint, and blood vessel, cell-free tissue replacements can be constructed and used. The geometry and mechanical properties of these replacements are essential factors that should be taken into consideration during construction. In contrast, for tissues that produce hormones and biochemical factors (liver and pancreas), generate forces (heart and skeletal muscle), and process electrical and chemical signals (neurons), it is necessary to construct cell-based tissue replacements. For these tissue replacements, the survival of cells and the maintenance of cell phenotypes are important issues. Various approaches can be used to achieve these goals, depending on the type of cells. Theses approaches are described in chapters corresponding to different systems through the book (e.g., for nerve regenerative engineering, see Chapter 13).

Functional Tests of Tissue Replacement

A tissue replacement should be tested for functionality before it is used for repairing or replacing a target tissue. The form of functional tests is dependent on the type of tissues and required functionality. In general, several forms of test can be performed for functional assessment, including mechanical, contractility, and biochemical tests.

Mechanical tests are designed for tissue constructs that are used to replace structures for fluid conduction and mechanical support and performance, including blood vessels, cardiac valves, bones, and joints. Common mechanical tests include the assessment of mechanical properties and maximal strength. The mechanical properties of a material can be represented by the relationship between stress and strain. *Stress* is the force per unit area applied to the tissue construct or produced by cells implanted into the construct. *Strain* is deformation induced by the applied or produced force. At a given stress level, distinct strains indicate different levels of stiffness or compliance of the tested material. The stress–strain relationship can be described by mathematical expressions known as *constitutive equations*. These equations can be used to model the mechanical properties of tissue constructs. The coefficients of the constitutive equations can be used to represent the stiffness or compliance of the material for the tissue construct. The maximal strength

of a tissue construct can be assessed by testing the yielding stress of the material used for the tissue construct. A yielding stress is the stress level at which the material breaks when subject to a continuous increasing force. The higher is the yielding stress, the stronger is the material.

Contractility tests are designed for assessing the function of contractile muscular tissues. Muscular tissue replacements can be constructed by integrating muscular stem or progenitor cells into engineered tissue scaffolds. The contractility of the constructed muscular tissue replacements can be assessed by measuring the forces generated by the integrated muscle cells and/or the deformation of the muscular replacements. The generated forces can be tested by using a force transducer and the deformation can be assessed by measuring changes in the dimensions of the muscular replacements. In practice, the contraction of the muscular cells can be induced by electrical or chemical stimulation. At a given level of stimulation, the higher is the generated force or deformation, the higher is the contractility.

Biochemical assays can be designed and conducted for testing the functions of constructed tissue replacements, such as the production of hormones, enzymes, and regulatory factors. Various assays may be established for such a purpose, depending on the functions of a specified cell type. For instance, for an artificially constructed pancreatic tissue replacement, it is necessary to test the level of insulin. For an engineered liver tissue, the level of albumin is a critical parameter indicative of the function of the tissue replacement. Various tests are described in chapters corresponding to the organ systems through the book.

Tissue Implantation

Following functional tests in vitro, a constructed tissue can be implanted into a target tissue, organ, or body cavity. Various strategies may be designed for tissue implantation, depending on the type and function of the target tissue. For tissues that provide mechanical support, strength, and function, such as the bone, joint, blood vessel, and cardiac valve, it is necessary to conduct site-specific tissue replacement, or replacing a target tissue at its original natural site. For tissues that produce hormones, enzymes, and regulatory molecules, such as the hepatic, pancreatic, and endocrine gland tissue, a tissue construct should be connected to the vascular system, so that produced hormones and biochemical factors can be directly released into the bloodstream. It is not necessary to implant these tissue constructs to the original site of the target tissue. Specific issues will be discussed in detail in chapters corresponding to various tissue and organ systems.

Morphological Tests of Implanted Tissue Constructs

After the implantation of a tissue construct, it is necessary to test its morphology and structural relationship to neighbor tissues, which provides essential information for assessing the performance and the integration of the implanted tissue construct to the host tissue. For the first step, the implanted tissue construct should be identified based on the structural properties of the material used for the tissue construct. For cell-based tissue constructs, the implanted cells can be identified by establishing specific markers, such as green fluorescent protein (GFP) and β-galactosidase as discussed on page 445. In the second step, the anatomy of the implanted tissue construct, the microstructure of implanted cells, and

associated extracellular matrix should be examined. A histological approach can be used for assessing the global morphology of the tissue construct, while an electron microscopic approach can be used for assessing the microstructure of cells and extracellular matrix. In addition, the structure and organization of inflammatory tissues and newly generated blood vessels, in response to tissue implantation, should be assessed by using similar approaches.

Test of Cell Viability and Growth

For cell-based tissue constructs, it is necessary to test the viability and growth of the seeded cells. Tissue samples can be collected at specified times for such tests. Identification of the seeded cells can be based on specific markers generated by transfected genes, such GFP and β-galactosidase genes. Several approaches can be used for testing cell viability and growth, including the test of cell density, proliferation, and apoptosis. Cell density can be assessed by labeling and counting the nuclei of mononucleated cells per unit area. Cell nuclei can be labeled with DNA-specific fluorescent dyes, such as Hoechst 33258, and histological dyes, such as hematoxylin. Cell density is easy to measure and is a reliable index for assessing the cell viability. A cell proliferation test, such as the BrdU assay, can be conducted to assess whether the cells in the implanted tissue construct undergo cell division and growth. A cell apoptosis test, such as the TUNEL assay, can be used to estimate the rate of cell death. These methods are described on page 307.

Functional Tests for Implanted Tissue Constructs

The most important task of all is to test the function of the implanted tissue constructs. Various testing strategies can be designed and conducted, depending on the type and function of the target tissue. For tissues that provide mechanical performance and support, such as the bone and joint, the mechanical performance of the implanted tissue construct should be examined under a given condition at appropriate observation times following tissue implantation. For tissue constructs that conduct fluids, such as vascular substitutes, it is necessary to test the patency of the implanted vascular constructs. For tissue constructs that assist the contraction of the heart, it is important to examine the cardiac ejection function. For tissue constructs that generate hormone and regulatory factors, biochemical and molecular approaches should be used to detect the level of a specified hormone or factor.

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