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Adipose-derived adult stem cells for cartilage tissue engineering

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Abstract. Tissue engineering is a promising therapeutic approach that uses combinations of implanted cells, biomaterial scaffolds, and biologically active molecules to repair or regenerate damaged or diseased tissues. Many diverse and increasingly complex approaches are being developed to repair articular cartilage, with the underlying premise that cells introduced exogenously play a necessary role in the success of engineered tissue replacements. A major consideration that remains in this field is the identification and characterization of appropriate sources of cells for tissue-engineered repair of cartilage. In particular, there has been significant emphasis on the use of undifferentiated progenitor cells, or "stem" cells that can be expanded in culture and differentiated into a variety of different cell types. Recent studies have identified the presence of an abundant source of stem cells in subcutaneous adipose tissue. These cells, termed adipose-derived adult stem (ADAS) cells, show characteristics of multipotent adult stem cells, similar to those of bone marrow derived mesenchymal stem cells (MSCs), and under appropriate culture conditions, synthesize cartilage-specific matrix proteins that are assembled in a cartilaginous extracellular matrix. The growth and chondrogenic differentiation of ADAS cells is strongly influenced by factors in the biochemical as well as biophysical environment of the cells. Furthermore, there is strong evidence that the interaction between the cells, the extracellular biomaterial substrate, and growth factors regulate ADAS cell differentiation and tissue growth. Overall, ADAS cells show significant promise for the development of functional tissue replacements for various tissues of the musculoskeletal system.

Keywords: Articular cartilage, chondrocyte, pre-adipocyte, stromal cell, osteoarthritis, collagen, proteoglycan

1. Introduction

Under normal physiologic circumstances, articular cartilage may function for decades as a nearly frictionless articulating surface in diarthrodial joints, while exposed to loads of several times body weight. This remarkable function is attributed to the unique structure and composition that determine the mechanical properties of the cartilage extracellular matrix [51]. The cartilage extracellular matrix is maintained by the metabolic activity of a sparse population of cells (chondrocytes) embedded within the tissue. Due to its lack of vascularity and the low metabolic activity of chondrocytes, articular cartilage exhibits a limited capacity for intrinsic repair. Isolated chondral or osteochondral lesions may be a significant source of pain and loss of function, and will rarely, if ever, heal spontaneously. Even minor lesions or injuries may lead to progressive damage and joint degeneration [34,35].

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One of the potential explanations for the poor repair response of articular cartilage is the lack of a blood supply or a source of undifferentiated cells that can promote repair. To overcome these biological limitations, many surgeons have used drilling, abrasion, and microfracture of the subchondral bone to induce bleeding in a repair site in the cartilage [2,8,22,24,35]. This approach generally promotes the formation of a fibrocartilaginous repair tissue, suggesting that the cells responsible for cartilage repair do not differentiate into a true chondrocytic lineage. While the repair tissue is in some cases satisfactory and can decrease pain and morbidity in the short term, fibrocartilaginous repair tissue differs in its mechanical properties in comparison with native articular cartilage [28] and therefore may not function effectively as a long term replacement for normal tissue [55]. Other techniques for cartilage repair have included the transplantation of allograft or autograft cartilage in an effort to restore tissue function. One of the main challenges with these techniques is promoting the functional integration between the implanted graft and the host cartilage. Furthermore, the long-term efficacy of such methods remains unproven, and there is now evidence that significant morbidity may be associated with the donor site of autograft cartilage in the joint [43].

2. Tissue engineering of articular cartilage

To address the clinical need for the repair or regeneration of articular cartilage, significant attention has turned to tissue engineering approaches [3,5,13,15,20,21,23,26,33,47–49,59,65,68]. Despite rapid and exciting advances in the field, few clinical applications have been developed for cartilage tissue engineering, and tissue engineers continue to face significant challenges in repairing or replacing tissues that serve a predominantly biomechanical function. At this writing, one cell-based therapy is available clinically for cartilage repair. The Carticel^{TM} procedure (Genzyme Biosurgery, Cambridge, MA, USA) involves the isolation and amplification of autologous chondrocytes from articular cartilage of an unaffected region of the joint, followed by surgical implantation of cells into the cartilage defect [11]. A flap of autologous periosteal tissue is then used to cover the defect. Clinical outcomes of this procedure are good to excellent [10,50], although animal studies have not shown long-term success [9]. Furthermore, it is now apparent that the harvest procedure required to provide autologous cells may initiate joint degeneration [43]. Therefore there still exists a continuing and unmet need for a readily available and abundant source of chondrocyte progenitor cells for cartilage tissue engineering.

3. Adult stem cells in tissue engineering

The adult stem cell is defined as an "undifferentiated (unspecialized) cell that is found in a differentiated (specialized) tissue; it can renew itself and become specialized to yield all of the specialized cell types of the tissue from which it originated" [39]. In other words, the adult stem cell retains the capacity for self-renewal as well as the potential for differentiation into one or more specialized cell types. It is now apparent that many adult tissues harbor cells with the potential to differentiate into multiple cell types. These cells have been described most commonly as mesenchymal stem cells (or MSCs), but also as multipotential adult stem cells, human marrow stromal cells, or mesenchymal progenitors [14,16,17,37,52,58,61–63,67,72].

Due to their accessibility, adult stem cells have been used extensively in a variety of tissue engineering applications. Numerous sources of cells with multipotent or pluripotent differentiation capabilities have been found in various musculoskeletal tissues, including bone marrow [14,61,63], adipose tissue [20,29,

74,75], trabecular bone [58], periosteum [53,59], synovial tissue [18,57], muscle [36,44,73], and several other tissues. These cells have formed the basis for tissue-engineered repair of different musculoskeletal tissues [19,23,65]. However, despite several common characteristics among these cells, significant differences exist in their proliferation and differentiation capabilities, their expression of various cell surface markers, their abundance and ease of harvest, and therefore, their potential utility in tissue engineering applications.

Specific to articular cartilage, a number of tissue engineering approaches have explored embryonic or adult stem cells as a source of progenitor cells. For example, murine embryonic stem cells stimulated with bone morphogenetic protein 4 (BMP-4) have been shown to differentiate along the mesodermal lineages [40,54]. Subpopulations of the totipotent embryonic stem cells expressing the VEGF receptor (flk-1) and/or PDGFR-alpha differentiated into the chondrogenic lineage when cultured in 3D pellets and exposed to TGF-β3. The chondrogenic lineage was determined by mRNA expression of cartilage-specific genes (proteoglycans and type II collagen) and synthesis of type II collagen protein. The chondrogenic differentiation was synergistically enhanced in the presence of platelet-derived growth factor (PDGF) and the subsequent culture with BMP-4 [54]. The primary source of adult stem cells for cartilage tissue engineering has been from the bone marrow. Bone marrow-derived MSCs undergo chondrogenesis *in vitro* when maintained in pellet culture in the presence of TGF-β, based on the expression and synthesis of proteoglycans and collagen type II [7,38,46,61,72]. Other tissues have also been shown as sources of chondrocyte progenitors, including muscle [1], periosteum [60], synovium [18,57], skin/foreskin [56], and trabecular bone [58,66].

4. Adipose tissue as a source of adult stem cells

Adipose tissue harbors a population of multipotent progenitor cells that can be induced to differentiate along multiple mesodermal and ectodermal lineages under controlled *in vitro* culture conditions (Fig. 1)

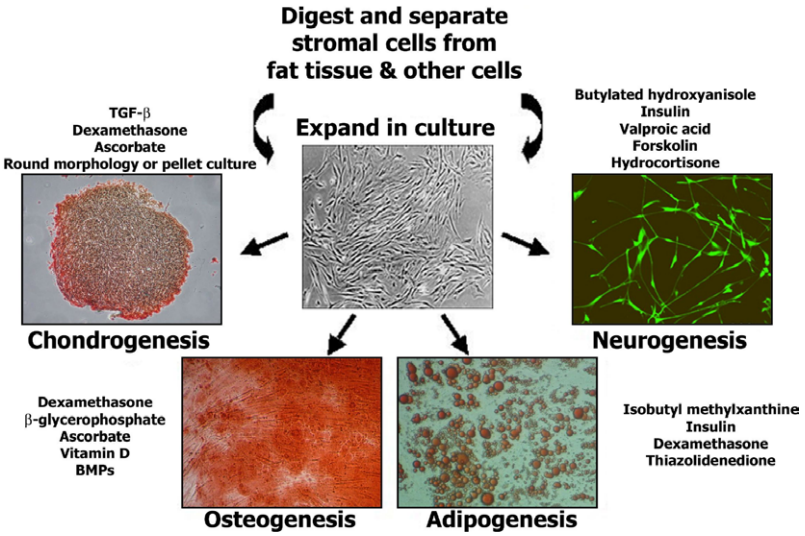

Fig. 1. Multipotent capabilities of adipose-derived adult stem (ADAS) cells. Under specific and controlled culture conditions, ADAS cells can be induced to express the phenotypic characteristics of chondrocytes, osteoblasts, adipocytes, or neurons.

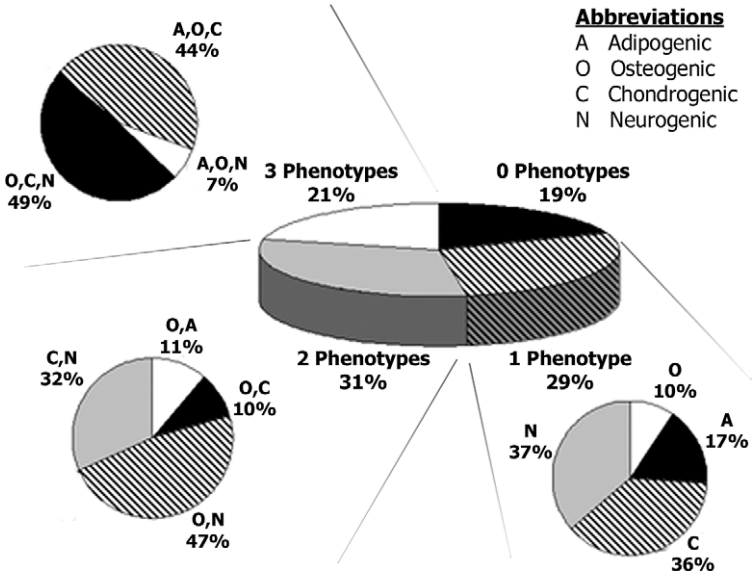

Fig. 2. Clonal analysis of the multipotent differentiation capabilities of ADAS cells [45]. The cell clones were assayed for adipogenic, osteogenic, chondrogenic, and neurogenic differentiation. 52% of the cell clones demonstrated stem cell characteristics by displaying bi- and tri-potent differentiation potential.

[4,5,20,30,31,64,70]. Furthermore, the cells seem to maintain the differentiated phenotype for long periods of time when implanted *in vivo* in a subcutaneous pouch in the mouse [20,32]. Human ADAS cells display a heterogeneous immunophenotype, expressing a panel of cell surface proteins associated with adhesion and signal transduction pathways, and displaying a remarkably similar immunophenotype to marrow derived MSCs [25,70]. Further, ADAS cells have been induced to differentiate along a number of lineage pathways relevant to musculoskeletal repair, including chondrogenic, osteogenic, myogenic, neurogenic, and adipogenic cell types (Fig. 1). Importantly, recent studies indicate that significant percentages of individual clones (Fig. 2), selected at random from primary ADAS cell populations using ring-cloning techniques, can differentiate into multiple cell types. This finding suggests that ADAS cell populations contain "true" stem cells rather than simply a mixed, heterogeneous population of unipotent progenitors [45]. The differentiation process can be controlled by biochemical factors, including the presence and exposure time of exogenous growth factors, hormones, vitamins, and other soluble mediators [4,20,31,70], as well as factors related to the physicochemical environment such as oxygen tension [69]. Furthermore, the interaction between the cells and the biomaterial substrate affects cell shape, which in turn appears to have a profound effect on the ADAS cells' phenotype [6].

5. Chondrogenic potential of *h*ADAS cells

When cultured with transforming growth factor (TGF-β1), ascorbate, and dexamethasone, human ADAS cells express biochemical markers characteristic of articular chondrocytes [4,20,70,75]. An important requirement for chondrogenic differentiation appears to be the maintenance of a rounded cell shape with a 3-dimensional culture system such as a micromass pellet [7,45] or as individual cells suspended in gel matrix such as alginate or agarose [5,20]. When maintained in pellet culture or encapsulated in alginate beads and cultured with 10 ng/ml of TGF-β1, ADAS cells express a chondrocyte-like

phenotype and synthesize collagen type II, aggrecan, link protein, and chondroitin sulfate in a time-dependent manner based on mRNA analysis, immunohistochemistry, and radiolabel incorporation [20]. The chondrogenic gene profile expressed by the ADAS cells appears similar to the chondrogenic phenotype of bone marrow derived stromal cells characterized using microarray analysis [71]. Following this *in vitro* differentiation process, human ADAS cells can retain the chondrocyte phenotype for up to 12 weeks when implanted subcutaneously *in vivo* in immunodeficient mice [20].

The influence of various growth factors and mediators on ADAS cell chondrogenesis may be additive or synergistic depending on concentration and time of exposure [4]. For example, the combination of the serum substitute ITS+ and TGF-β1 can increase ADAS cell proliferation in an additive manner in alginate beads relative to control cultures. Similarly, protein synthesis rates are increased by TGF-β1 and dexamethasone in the presence of ITS+ or fetal bovine serum (FBS). TGF-β1 significantly increased proteoglycan synthesis and accumulation by 1.5 to 2 fold, although this effect appears to be suppressed by dexamethasone (Fig. 3).

Furthermore, the choice of the biomaterial scaffold also influences the differentiation of ADAS cells and the functional properties of the tissue engineered construct when grown under *in vitro* chondrogenic culture conditions consisting of basal medium with TGF-β1, ascorbate, and dexamethasone [5, 41]. Tissue engineered constructs constructed from alginate and agarose hydrogels, and porous gelatin scaffolds (Surgifoam®) seeded with ADAS cells respond to these chondrogenic culture conditions with increased rates of protein and proteoglycan synthesis, cell proliferation, and sulfated glycosaminoglycans and hydroxyproline accumulation in the extracellular matrix compared to control culture conditions of basal medium [5]. Furthermore, chondrogenic culture conditions resulted in 86%, and 160% increases ($p < 0.05$) in the equilibrium compressive and shear moduli of the gelatin scaffolds, although they did not affect the mechanical properties of the agarose or alginate constructs over 28 days in culture [5]. Under these conditions, the equilibrium compressive and shear moduli of the gelatin scaffolds were comparable to agarose by day 28 and both were significantly greater than alginate.

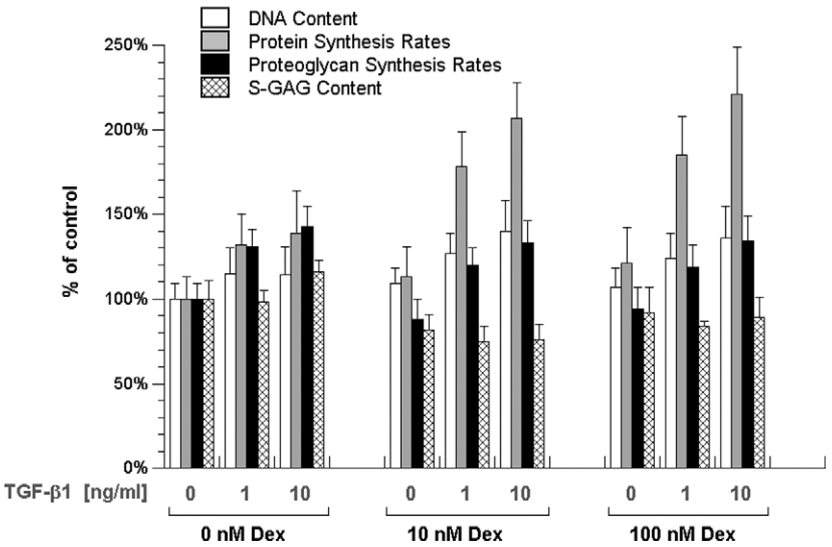

Fig. 3. Effects of Transforming Growth Factor (TGF-β1) and the glucocorticoid Dexamethasone on the growth and chondrogenic differentiation of ADAS cells seeded in alginate beads after 9 days in culture [4]. Bars represent means ($\pm$ standard error of the mean) for each culture combination with FBS and ITS+.

Interestingly, cells encapsulated in alginate or agarose exhibited a spherical cellular morphology, while cells in the gelatin scaffolds showed a more elongated shape as they exerted significant compaction on the scaffold. This elongated fibroblastic morphology did not appear to hinder the chondrogenic differentiation of the cells in the gelatin scaffold. However, immunohistochemical analysis of the constructs demonstrated that while collagen type II and chondroitin sulfate were detected in all the biomaterial scaffolds grown in chondrogenic conditions, collagen type I was also detected in the newly synthesized matrix in the gelatin constructs only (Fig. 4), suggesting that gelatin might promote the differentiation of ADAS cells into a fibrocartilaginous phenotype [41].

Although the choice of the biomaterial scaffold is important, changes in the biochemical composition of the tissue engineered cartilage constructs over time can also influence their biomechanical properties. For example, increases in the shear moduli were significantly associated with increases in S-GAG content ($R^2 = 0.36, p < 0.05$) and with the interaction between S-GAG and hydroxyproline ($R^2 = 0.34, p < 0.05$). These observations suggest that the choice of the biomaterial scaffold influences the chondrogenic differentiation of ADAS cells, and that manipulating the composition of these tissue engineered constructs may have significant effects on their mechanical and functional properties.

The biomaterial scaffold also affects the diffusion of various molecules in cartilage engineered from ADAS cells. Diffusion is likely to be the primary mechanism for macromolecular transport in tissue-engineered cartilage, and providing an adequate nutrient supply via diffusion may be necessary for cell proliferation and extracellular matrix production. The diffusion coefficients of four different sized (ranging from 3 to 500 kDa), fluorescent, uncharged dextrans were measured using fluorescence recovery after photobleaching (FRAP) in tissue engineered cartilage constructs [41]. The constructs comprised

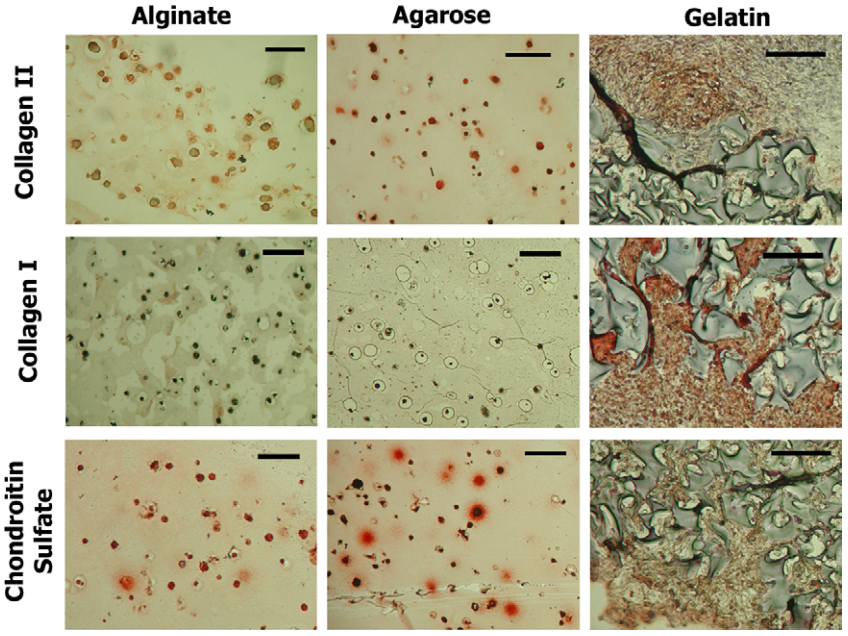

Fig. 4. Immunohistochemistry of cartilage constructs engineered with human adipose-derived adult stem cells [6]. Constructs were grown in chondrogenic media for a period of 28 days, and subsequently fixed, sectioned, and labeled against collagens type I and II and the 2B6 epitope of chondroitin sulfate. All biomaterial constructs showed synthesis of the cartilage matrix proteins collagens type II and chondroitin sulfate. However, cells within gelatin synthesized collagen type I as well, suggesting perhaps that gelatin supports more of a fibrocartilagenous differentiation of ADAS cells. Scale bar represents 100 μm.

alginate, agarose, gelatin, or fibrin scaffolds that were seeded with human adipose derived stem cells and cultured for 1 or 28 days in either chondrogenic or control conditions. Irrespective of the size of the molecule, the diffusion of uncharged dextran molecules in engineered tissue constructs depended on the biomaterial composition of the construct, the presence of cells, the culture conditions, and the culture time. By 28 days in culture, the diffusivity of the constructs decreased 42% and 27% in chondrogenic and control cultures, respectively. This decrease in diffusivity likely reflects a combination of changes that include a net increase in the synthesis, assembly, and retention of neomatrix macromolecules, which may be enhanced with chondrogenic media, over degradative changes in the scaffold. Most importantly, diffusivity in all the constructs tested up to 28 days in culture were significantly greater than those of native cartilage [42], suggesting that the transport of nutrients and metabolites to cells within the constructs might not be hindered in the early stages of tissue generation (Fig. 5).

Moreover, it is widely accepted that laboratory cell culture conditions, under which most tissue engineered constructs are grown, do not mimic the environment that would be present in the normal synovial joint. Oxygen tension is one of the most important biophysical culture variables that seem to regulate engineered tissues' growth and differentiation [69]. ADAS cells were suspended in alginate beads and cultured in control or chondrogenic media in either low oxygen (5%) or ambient oxygen tension (20%) for up to 14 days. Low oxygen tension, which would more closely resemble the *in vivo* joint environment, significantly inhibited the proliferation of ADAS cells, while inducing a two-fold increase in the rate of protein synthesis and a three-fold increase in total collagen synthesis. Low oxygen tension also increased glycosaminoglycan synthesis, as well as lactate production. Immunohistochemical analysis showed significant production of cartilage-associated matrix molecules, including collagen type II, VI, and chondroitin-4-sulfate. These findings suggest that oxygen tension may play an important role in regulating the switch between proliferation and differentiation of ADAS cells during chondrogenesis, and may provide a means of controlling cell growth and biosynthetic activity *in vitro*.

In summary, a number of previous studies show that the biochemical environment such as growth factors and hormones and biophysical environmental variables such as oxygen tension may have significant effects on the growth and chondrogenic differentiation of ADAS cells. Furthermore, the data provide strong evidence that the interplay between the cells, the extracellular biomaterial substrate, and growth factors regulate ADAS cell differentiation and tissue growth. The associated intracellular signaling mechanisms leading to this regulation remain to be elucidated and offer exciting opportunities for future research and discovery.

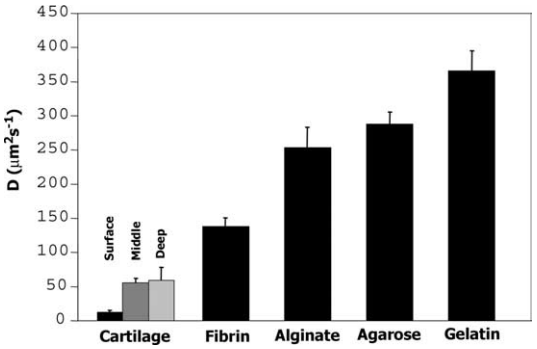

Fig. 5. Diffusion coefficients of 70 kDa dextran in the three zones of native cartilage are less than those in tissue-engineered cartilage constructs [41]. Bars represent means (± standard error of the mean) for each scaffold type averaged across all times and conditions.

6. Challenges and future goals in cartilage tissue engineering using stem cells

Adipose tissue is abundant and readily accessible as compared to most sources of viable tissue, making it a rich potential source of adult stem cells for regenerative medical applications and for functional tissue engineering approaches to cartilage repair. A number of basic and applied issues still remain regarding the functionality, safety, and cost-effectiveness of these cells, as well as any other cell source for tissue engineering.

In addition, because of the predominately mechanical role that cartilage plays in diarthrodial joints, issues of biomechanical function have been addressed by a series of guidelines and recommendations for "functional tissue engineering" proposed to promote a more rational design approach for engineered tissue replacement [12,26]. These guidelines, summarized in a recent text [27], include: (1) Improved definitions of function and success in tissue engineering; (2) A more thorough understanding of the *in vivo* biomechanical environment and mechanical properties of native tissues, and the associated design criteria that meet the biomechanical and metabolic demands of the tissue to be replaced; (3) Design and functional assessment of the biomechanical and biophysical properties of scaffolds for tissue engineering; and (4) A more thorough understanding of the role of biophysical factors on cells and tissue-engineered constructs.

The application of adult stem cells in tissue engineering shows tremendous promise for the future. Despite rapid advances and many early successes, there are few clinical applications of tissue engineering based on adult stem cells. Early work has often focused on trial-and-error methods, and there is a need to balance such an approach with more conservative "engineering" based approaches for product design and manufacture. Furthermore, other rapidly evolving new technologies, especially in biomaterials chemistry and in bioreactor design, may have a significant impact on stem-cell based tissue engineering. It is important to consider the principles of functional tissue engineering when implementing such new technologies towards the development of novel stem cell-based therapeutic products.

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