Recent Patents in Cell-Based Strategies for Soft Tissue Engineering in Plastic and Reconstructive Surgery

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Received: ???? 7, 2010; Accepted: ???? 12, 2010; Revised: ???? 7, 2010

Abstract: Reconstructive surgery is performed on abnormal or damaged soft tissues, caused by trauma, burns, congenital defects, tumours or disease. Damage to the underlying fatty tissues results in scar tissue formation and deformity, as well as the potential for reduced mobility if the injury occurs near a joint. The general aim in the clinic is to rebuild the affected tissues, and by doing so, to recover or improve function. Relevant clinical procedures include the revision of scar tissue from burns or trauma, laceration repair, the reconstruction of the breast after mastectomy or lumpectomy, and the restoration of the normal body structure following the removal of sarcomas or skin cancers. Since adipose tissue comprises the bulk of the adult tissues treated in these reconstructive approaches, the importance of vascularized fat in the field of plastic and reconstructive surgery is well established. Soft tissue engineering holds great promise for the improvement of standard reconstructive methodologies. In this context, two main approaches have arisen, which can be employed individually or in combination: (i) cell-based therapies and (ii) biologically compatible tissue scaffolds. This review provides a brief description of recent patents and findings in soft tissue regeneration for plastic and reconstructive surgery.

Keywords: Adipose tissue, cell therapy, plastic and reconstructive surgery, scaffolds, stem cells, tissue engineering, volume augmentation, wound healing.

INTRODUCTION

Reconstructive (or plastic) surgery is an option when trauma, burns, birth defects, tumours or disease influence the normal contours and structures of the body's soft tissues [1, 2]. Patients with soft tissue damage often experience anxiety, depression and poor self-image related to their physical scars, and reconstruction can have a major positive impact on their psychological and emotional health, and consequently, quality of life [3]. Plastic surgeons are skilled in techniques including volume augmentation, skin grafting, scar tissue revision and other approaches to facilitate wound healing [4]. In particular, soft tissue augmentation is indicated when there is a loss of tissue volume, potentially due to trauma, congenital abnormalities or surgical procedures, such as tumour resection (such as mastectomy or lumpectomy). Over 5.2 million reconstructive procedures were performed in the U.S. in 2009, including >86,000 breast reconstructions, >21,000 burn treatments, and >3.9 million tumour removals [5].

Currently, autologous tissue transfer using vascularized flaps of skin, fat, and muscle represents the gold standard treatment for large-volume reconstruction [6]. However, harvesting tissues creates large donor site defects, is associated with further scarring and deformity, and has high costs in terms of hospitalization and treatment time. When tissue availability is limited, synthetic implants are an alternative; however, these implants become encapsulated in fibrous tissue, and do not integrate into the host, which can result in migration or rupture [7, 8]. Soft tissue augmentation can also be performed for cosmetic purposes, such as in the case of breast augmentation, lip enhancement and wrinkle removal. More than 12.5 million cosmetic procedures were conducted in the U.S. alone in 2009, including more than 1.7 million procedures that specifically required soft tissue fillers [5]. Fat transfer has been studied for these smaller-volume applications, as most patients have expendable adipose tissue (fat) that can be harvested by liposuction [9, 10]. However, this approach is associated with unpredictable graft resorption, and repeated treatments are required to maintain the tissue volume [11]. Skin grafting is another procedure commonly used to treat various pathological conditions resulting from diseases or trauma where the normal architecture of the dermis is disrupted or damaged, such as in the case of severe burns, trauma, piebaldism, naevoid skin disease and vitiligo. To date, numerous biological skin substitutes have been investigated, but have generally failed to match the wound healing properties of grafted autologous tissues [12]. Overall, there is a significant clinical need for the development of new therapies in plastic and reconstructive surgery, to facilitate predictable, long-term soft tissue regeneration without scarring.

Recent research has demonstrated that acute and chronic wound healing can be supported, and in some cases accelerated, using new approaches in regenerative medicine [13]. Acute wounds are defined as those that physiologically heal

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1874-7647/10 $100.00+.00

within 30 days, such as minor contusions, lacerations, puncture wounds and crushing injuries. In contrast, chronic wounds are more severe, are slower to heal, and can be associated with life-threatening infections and severe pain. Some examples of potential non-healing chronic wounds include surgical wounds, diabetic ulcers, severe thermal or chemical burns, compound fractures, long-term catheter sites and major injuries due to projectiles or explosions [14]. All of these conditions impact, to a varying degree, the structure and composition of the subcutaneous adipose tissues, as well as the skin (dermis and epidermis). Therefore, advancements in soft tissue engineering would ideally target the regeneration of the complete integumentary system, including the underlying fatty tissues [1, 15]. While many approaches in the past have focused solely on dermal augmentation as a means to temporarily fill tissue voids, promising new strategies to facilitate adipose regeneration have emerged over the past 10 years [16-18]. The ultimate objective will be to facilitate complete and predictable healing without scarring, with long-term maintenance of the desired volume and shape [19]. Methods that promote tissue revascularization are likely to be most successful, as angiogenesis is key for both wound healing and stable fat formation, and impaired vascularisation is often a root problem in chronic wounds, such as in the case of diabetics and severe burn patients [12, 13, 20].

The majority of patents and findings in the field of soft tissue engineering for plastic surgery can be grouped into one of three main categories, corresponding to the primary approaches undertaken in the scientific community:

1) *Cell-based strategies*, such as those incorporating mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) or differentiated/adult populations
2) *Biocompatible tissue substitutes*
3) Combination strategies involving *cell-seeded biocompatible materials*

This review provides an overview of recent patents, findings and improvements in soft tissue engineering, with a focus on cell-based approaches, either alone or with biomaterial scaffolds, for adipose tissue regeneration.

1. CELL-BASED THERAPEUTIC STRATEGIES

A key concept in the emerging field of regenerative medicine is the selection of suitable cells for repopulating, renewing or reconstructing tissues [13]. Candidate populations for new cell-based therapeutic strategies include adult stem cells, ESCs and iPSCs, all of which have associated strengths and limitations [21-26]. The differentiation potential of ESCs and iPSCs is enormous, but ethical issues andsafety concerns limit their practical application. While adult stem cells derived from tissues circumvent these ethical problems, they maybe associated with the potential for immunogenic reaction, especially if allogenic or xenogenic sources are implemented [27]. Further, hurdles related to cell isolation, purification, culture and controlled differentiation remain to be resolved before these adult stem cells can be effectively and broadly applied in the clinic [13]. It is now well established in the literature that MSCs are found in an array of adult tissues including skin, brain, blood, fat and bone marrow, with the cells believed to be residing in a perivascular niche [28-30]. In fact, it has been demonstrated that these cellular reservoirs are key to normal wound healing and tissue turnover, with controlled MSC recruitment, mobilization and differentiation, when there is a need for a given type of cellular repopulation in an associated organ system [31-34]. Despite the aforementioned limitations, one of the main approaches investigated for promoting soft tissue regeneration and wound healing is the employment of stem cells or somatic cells in cell-based therapeutic strategies. The underlying concept is to use the intrinsic properties of the cells to improve and/or accelerate the desired physiological processes involved in wound healing. Strategies relying on cell delivery alone are more promising for smaller-volume applications in plastic and reconstructive surgery, where there are no large defects or voids that would create significant contour deformities.

1.1. Stem Cells for Wound Healing and Tissue Regeneration

Adipose-derived Stem Cells

Dr. A. J. Katz *et al.* are inventors on a patent related to the use of adipose-derived stem cells (ASCs), the multipotent stem cell population isolated from fat [35]. Fat is a relatively abundant, easily accessible and uniquely expendable source of stem cells for the development of new regenerative therapies for a broad range of applications, including in cosmetic and reconstructive surgery [36-39]. The inventors describe methods for isolating, purifying and culturing the ASC population, which can be stimulated to differentiate along multiple lineages including fat, bone, cartilage, muscle, and nerve. The inventors claim that the cells have specific cluster designation (CD) marker profiles, including varying patterns of expression of Stro-1, CD29, CD44, and CD49D, amongst other markers. In addition to using the cells themselves in the development of cell-based therapies, the inventors describe using the cultured ASCs to produce hormones and conditioned medium to support other cultured cell populations. There has been recent litigation surrounding the inventorship on this patent, and the United States Patent and Trademark Office decided in 2008 that only the two scientists at the University of Pittsburgh, Dr. A. Katz and Dr. R. Llull, were inventors [40].

A natural evolution of this approach is described in the recent patent application entitled “Methods of Preparing and Characterizing Mesenchymal Stem Cell Aggregates and Uses Thereof” [41]. The basis of this new methodology is that a self-generated extracellular matrix may provide an ideal microenvironment for the ASCs to grow and differentiate, in order to regenerate a given tissue. The approach involves culturing the ASCs under conditions that induce them to form three-dimensional (3-D) multicellular aggregates. The cells and aggregates can be implanted to enhance soft tissue repair, regeneration, or augmentation processes, potentially in combination with biocompatible materials, also described in other related patent applications from the same group [41-44]. Inventors from Korea have patented similar methods for culturing human ASCs in the form of cell pellets or spheres, to obtain homogeneous and highly-proliferative

stem cell populations for use in cell-based therapies, including for breast reconstruction [45].

Cytori Therapeutics Inc. holds a patent [46] invented by Dr. M. Hedrick and Dr. J. Fraser pertaining to the use of regenerative cells from processed adipose tissue, including the ASC population, for augmenting autologous adipose tissue transfer. The patent describes a "self-contained adipose-derived stem cell processing unit," which is a closed sterile system that can be used to isolate and concentrate autologous ASCs. The concentrated stem cells can be mixed with unprocessed adipose tissue, and applied in a range of applications in plastic surgery, including breast reconstruction, the recontouring of soft tissue defects, and as a bulking material for the treatment of urinary incontinence. Growth factors and cytokines may also be incorporated to stimulate cell growth and/or differentiation. The company also holds a similar patent on an ASC extraction and purification device, involving a group including the same inventors [47].

PrimeGen Biotech LLC filed a patent application [48] in 2006 pertaining to the use of ASCs for wound healing and the reduction of scar tissue formation in mammals, with a focus on skin regeneration. In addition to the ASCs, the inventors specify a "side population"(ASC-SP) of regenerative cells that can be isolated from mammalian adipose tissues. More precisely, using flow cytometry, these cells have the unique immunopheno type Lin^{-}, $Sca\text{-}1^{+}$, $CD90^{+}$, $CD34^{+/low}$, $CD13^{+/low}$, $CD117^{-}$ and $CD18^{+/low}$, where Lin is a mixture of lineage markers that identifies precursor cells committed to the hematopoietic lineages. The ASC-SP are indicated to exist in an undifferentiated state, and like the ASCs, are able to differentiate along the osteogenic, chondrogenic, neurogenic, and adipogenic lineages. Acute and chronic wounds may be treated with ASCs and ASC-SP, using a delivery device to perform subcutaneous, intramuscular, or intravenous injection, depending on the localization and therapeutic dosage required for each individual patient. The inventors state that the cell-based therapies may incorporate a delivery vehicle formulation or pharmaceutical cell carrier, potentially including bioactive agents, such as growth factors, to further promote regeneration.

In addition to the potential for direct integration through differentiation, the mechanisms through which ASCs support wound healing include growth factor and cytokine secretion to promote the revascularization of the affected tissues and the migration of host regenerative populations to the site of injury [49-51]. Moreover, related advantages of implementing cell-based strategies with the ASCs include the potential for accelerating the wound healing process, and consequently, lowering the risk of infection. Further, implementing ASCs can promote more complete tissue regeneration without scarring, which is particularly attractive in the field of plastic surgery.

An interesting extension of the use of ASCs has been developed by Dr. R. Tamarat and Dr. M. Benderitter from the Institute de Radioprotection et de Sûreté Nucléaire, who have patented [52] applying the cells, either intravenously or directly on a wound bed, to improve or accelerate healing following radiation damage resulting from radiotherapy or accidental exposure.

The optimization of the yield of multipotent progenitor cells from fat is the main topic of the patent application "Multipotent progenitor cell derived from adipose tissue" in which Dr. A. Matsuyama *et al.* [53] disclose methods to eliminate contaminating erythrocytes and vascular endothelial cells from the adipose tissue by hemolysis or by filtration and the addition of a chelator, such as ethylendiamine tetraacetic acid (EDTA), respectively.

Dr. W. Wilkison *et al.* [54] recently filed a patent application for using differentiated ASCs for the treatment, repair, correction, or regeneration of cosmetic soft-tissue defects. The proposed regenerative cell population is characterized by increased extracellular matrix protein production and a lower intracellular lipid content, relative to mature adipocytes. The application includes methods for *in vitro* cell expansion and adipogenic differentiation.

Mesenchymal Stem Cells

Bone marrow-derived MSCs are an alternative to ASCs that can be applied for tissue regeneration purposes in reconstructive and cosmetic plastic surgery [13]. Similar to ASCs, bone marrow-derived MSCs have multipotent differentiation capacity, including along the adipogenic, chondrogenic, osteogenic, and myogenic lineages [31]. There are some potential concerns that may limit the clinical applicability of bone marrow-derived MSCs for applications in large-volume reconstruction. In particular, the process of harvesting the cells, typically from the ileac crest, is invasive and can be painful. Further, the yield of multipotent stem cells is often extremely low, with the MSCs representing only a very small percentage (0.001 - 0.01 %) of the total cells in the bone marrow [55]. Careful control of the culture conditions is required, including serum selection, and purification is necessary to remove contaminating populations, including a variety of blood cells [56].

Osiris Therapeutics Inc. holds the patent invented by Dr. A. I. Caplan and Dr. S.E. Haynesworth pertaining to the use of MSCs derived from human bone marrow for the regeneration of connective tissue defects [57]. The patent includes methods for isolating, purifying, culturing, and characterizing the MSCs from human marrow. The cells may be administered in combination with differentiation factors, a cell carrier, and/or a prosthetic device that facilitates cell attachment. The differentiation of MSCs specifically along the adipogenic lineage is the main focus of the patents "Adipogenic differentiation of human mesenchymal stem cells " [58-60], invented by Dr. M.F. Pittenger with contributions from Dr. S.C. Beck, which are also held by Osiris. The patent describes the use of adipogenic inducing factors, such as glucocorticoids, compounds that elevate intracellular cAMP, and insulin, to promote the differentiation of the multipotent MSCs, and also includes methods for isolating the differentiated cell population. In addition, Osiris Therapeutics Inc. is the assignee on the US-patent entitled "Human Mesenchymal Stem Cells" [61], which describes the use of $SH3^{+}/CD45^{+}$ MSCs for regenerative cell therapies, including the treatment of connective tissue disorders.

In the patent application "Mesenchymal stem cells and uses therefore" [62], Dr. S. Aggarwal *et al.* disclose methods of treating inflammatory conditions, including cuts, burns

and other wounds, in mammals with autologous, allogenic or xenogenic MSCs derived from adherent marrow or periosteal cells. In particular, the investigators have shown that the MSCs mediate regeneration through cell interactions (i.e. with dendritic cells, T-cells and natural killers cells) and the secretion of factors, such as interleukins, that regulate the immune response. Moreover, the MSCs have an immuno-privileged status *in vivo*, as they do not express major histo-compatibility (MHC) class II antigens, making them a promising source for either autologous or allogenic applications in cell-based therapies.

Embryonic Stem Cells

Pluripotent ESCs derived from the inner cell mass of the blastocyst are another potential stem cell source for new approaches in regenerative medicine [26]. ESCs have great potential, and can be stimulated to differentiate into any cell type derived from the ectoderm, mesoderm, or endoderm. However, ESCs present ethical issues and safety concerns, including the potential for teratoma formation, which may limit their clinical application. Overall, the patenting of intellectual property pertaining to human ESCs is a complex scenario, influenced not only by science, but also by morals, ethics, religion, and law [63, 64]. Some of the key issues related to the patenting of ESC-based technologies have been the focus of other recent review papers [65-68]. A full discussion of ESCs is beyond the scope of the current paper, but below we highlight examples of recent patents or applications related to the specific use of ESCs in adipogenesis and soft tissue reconstruction.

In the patent application "Methods for differentiation of embryonic stem cells" [69], Dr. F. Kreamer *et al.* describe methods through which ESCs can differentiate *in vitro* into cells of the mesenchymal lineage, and more specifically, into osteoblasts and adipocytes. The methodology includes culturing ESCs to form embryoid bodies (EBs), plating these aggregates on a substrate, and treating the EBs with an inductive differentiation medium. Adipogenesis can be initiated through the application of all trans retinoic acid (ATRA), and the adipogenic medium may contain insulin, triiodothyronine (T3; thyroid hormone), isobutylmethylxanthine (a phosphodiesterase inhibitor that prevents cAMP degradation), and dexamethasone (a glucocorticoid). The resulting differentiated adipocytes can be used for tissue reconstruction, and the cells can also be genetically modified in order to enhance cell viability, proliferation, and/or lineage-specific differentiation.

Dr. D. Aberdam and Dr. C Coraux are co-inventors on the US-patent "Keratinocytes obtained from embryonic stem cells of mammals" [70]. The patent describes methods for differentiating ESCs into keratinocytes, with applications in the production of artificial skin grafts for the treatment of severe epidermal injuries, including burns and ulcers. The differentiation process involves culturing the ESCs on a cell-secreted extracellular matrix, including components such as laminin-5, collagen type IV, collagen type I, and/or fibronectins. Differentiation can be induced in the absence of leukemia inhibitory factor (LIF), and the differentiated population may be cultured at the air-fluid interface to promote stratification and the formation of a more mature artificial epidermis. The ESCs may be genetically modified prior to differentiation, and the inventors suggest the possibility of altering the expression of MHC genes, so that the resulting keratinocytes will not initiate a strong immune response.

Alternative Stem Cell Sources

Adult stem cells have been isolated from a diverse range of tissues in a variety of species, including humans, and many have demonstrated adipogenic potential *in vitro* and/or *in vivo*. Under the aspect of stem cell retrieval, recent patent applications have been filed describing the isolation and culture of stem cell/progenitor populations from urine [71], wisdom teeth [72], and fetal blood [73]. In addition, multipotent stem cells derived from the placenta [74], as well as perivascular progenitor cells extracted from the Wharton's jelly of the umbilical cord [75], may be potential cell sources for new cell-based strategies in adipose tissue-engineering and wound-healing applications.

Stem Cell Products

The use of "stem cell products" (SCPs) for regenerative purposes is described in the patent "Soft tissue repair and regeneration using stem cells products" [76], filed through the World Intellectual Property Organization (WIPO) and held by Johnson & Johnson. SCPs, including combinations of soluble and insoluble cell fractions, cell lysates, supernatants, and membrane-containing fractions, may be applied to support cells of a soft tissue lineage. More specifically, SCPs may provide trophic support for soft tissue repair, augmentation, or reconstruction. Preparation techniques may include osmotic, mechanical, ultrasonic, enzymatic, or chemical disruption. SCPs may be administered alone, in combination with cells, or embedded within a tissue-engineered scaffold.

1.2. Use of Adult Cells for Tissue Regeneration

Differentiated adult cells, including adipocytes, fibroblasts, epidermal, and endothelial cells have also been investigated in the development of cell-based strategies for soft tissue regeneration [13]. In MSCs, lineage specification is controlled by key transcription factors from each of the lineages, which cross-regulate one another to maintain the undifferentiated state [77]. Further, differentiation is associated with precise patterns of gene expression. Some studies have suggested that stem cells may be able transdifferentiate into alternative lineages through the activation and/or deactivation of specific regulatory genes, although there is debate about the role of transdifferentiation in tissue regeneration processes [78, 79]. Interestingly, studies have demonstrated that populations of differentiated adult cells may also have the potential to transdifferentiate, and can be genetically reprogrammed to have a very large differentiation potential, in the form of induced pluripotent stem cells (iPSCs) [80-82]. Recent patents related to iPSCs are beyond the scope of the current review, and are summarized elsewhere [83, 84].

Dr. S. Cinti *et al.* patented [85] a method for extracting mature adipocytes without altering their gene expression patterns, and while preserving their plasticity, for use in cosmetic procedures aimed at correcting tissue defects, including of the lips, face, or breasts. The plasticity of the cells in adipose tissue is well recognized, with the de-differentiation of mature adipocytes and the re-differentiation of endoge-

nous ASCs and more-committed adipogenic progenitors occurring naturally when there are changes in body fat content [86]. Further, during breast feeding the transdifferentiation of mature adipocytes results in a dramatic increase of epithelial lobulo-alveolar structures and ducts for milk production [87].

Dr. M. Leek and Dr. P. Kemp have filed patents through the WIPO [88, 89] pertaining to the isolation and injection of autologous or allogenic dermal fibroblasts for the repair of dermal and subcutaneous defects, with a focus on cosmetic applications. The fibroblasts may be derived from non-immunogenic neonatal foreskin, or from tissues extracted during mammoplasty, abdominoplasty, or from patients with polydactylism. Methods are provided for extracting and culturing the fibroblasts, and their subsequent injection is described to have long-term augmentation effects (4-6 months to a year), with the desired tissue volume obtained within 1 – 2 weeks of administration.

A similar approach involving suspensions of keratinocytes and fibroblasts for the *in situ* production of autologous cell transplants is described in the patent "Two constituent compositions for the *in situ* production of cell transplants that comprise fibroblasts and keratinocytes" [90], with applications in the healing of ulcers, burns or traumatic injuries. Similarly, Dr. L. Kemény *et al.* filed a patent [91] through the WIPO pertaining to methods for cultivating autologous keratinocytes, melanocytes, and fibroblasts *in vitro* for the preparation of skin grafts for the treatment of various pathological conditions, including burns and post-traumatic defects. In particular, to overcome the issue of senescence when culturing keratinocytes, the inventors identified a rich source of undifferentiated cells in the outer root sheaths (ORS) and/or bulge of the hair follicles in the scalp. An advantage of this source is that it also contains melanocytes and fibroblasts. The co-cultivation of melanocytes with the keratinocytes overcomes the problem of graft pigmentation. Using the methods, the cells can also be cultured separately, and later mixed in a desired ratio, depending on factors including the wound depth and anatomical location.

In the WIPO patent "Activated fibroblasts for treating tissue and/or organ damage" [92], Dr. J. Bizik *et al.* describe methods for culturing fibroblasts or bone marrow stromal cells in the form of cell-aggregates, or spheroids, that can be transplanted into damaged tissues or organs to promote regeneration, using methods similar to those described by Dr. A. Katz *et al.* [41] for ASCs. The culturing methods enhance the production of growth factors, such as hepatocyte growth factor (HGF), to stimulate cell proliferation, angiogenesis, and wound healing. The inventors demonstrate that the cells aggregate when deprived of a cell-adhesive substrate, to form activated multicellular spheroids, which secrete mitogens and angiogenic factors when implanted in patients with severe burn injuries. Autologous or allogenic fibroblasts can be applied, as the cells are believed to primarily mediate healing through the secretion of paracrine factors, rather than through direct integration into regenerating tissues.

2. BIOCOMPATIBLE TISSUE SUBSTITUTES

With the advancement of knowledge in the fields of chemical and materials engineering, new improved biocompatible tissue substitutes for large-volume soft tissue regeneration are being developed and characterized. In order to better control the 3-D shape and volume of the newly-forming tissues, and to also provide the infiltrating cells with the appropriate mechanical and/or biological cues, the implementation of biocompatible scaffolds is a promising approach in regenerative medicine. The optimal scaffold is soft and compliant, mouldable to meet the needs of each individual patient, non-immunogenic, mechanically and thermally stable, and has non-toxic degradation products [13]. Porosity is an important parameter to consider, and a balance must be achieved in terms of facilitating cell infiltration, while providing sufficient mechanical integrity and stability. Ideally, healthy host soft tissues would replace the scaffold as it biodegrades, to facilitate complete regeneration without scarring. To minimize fibrosis, the mechanical properties of the scaffold should closely mimic the native soft tissues, and the scaffolding should fully restore the 3-D shape and volume of the defect(s), to maintain the normal body contours [13, 93, 94]. In adipose tissue-engineering studies to date, many different scaffolding materials have been investigated, including both synthetic (i.e. polylactic-co-glycolic acid [6, 95], polyglycolic acid [96], polyethylene glycol diacrylate [97]) and naturally-derived (i.e. collagen [98], fibrin [19], derivatives of hyaluronan [99], silk protein [100]) carriers, and the ideal biomaterials to facilitate stable adipogenesis *in vivo* remain unresolved. Many of these materials have been investigated in combination with cells with adipogenic potential, such as ASCs or bone marrow-derived MSCs, to maximize the regenerative effects [13], as discussed in the next section.

In cosmetic surgery, injectable materials present numerous advantages for the correction of small-volume defects, as they are more easily administered with minimal pain and surgical requirements [101]. Moving towards developing new injectable materials for soft tissue augmentation, many different approaches have been tested including synthetic polymers, and natural materials derived from autografts, allografts, and xenografts. However, each of these approaches has associated limitations, and to date, the clinically-available strategies support only temporary bulking, with repeated treatments required to maintain the desired tissue volume.

Many patents have been filed pertaining to synthetic and naturally-derived polymers that could be used as acellular tissue substitutes for temporary or long-term soft tissue augmentation in cosmetic and reconstructive plastic surgery. While the focus of the current review is on cell-based strategies for soft tissue regeneration, we will briefly summarize some patents related to biomaterials developed specifically for volume-augmentation applications. For example, breast augmentation materials and techniques have been patented, including the use of implantable textured or smooth polymer envelopes that can be filled with gel or fluid to achieve a desired volume enhancement [102, 103]. In terms of injectable strategies, Dr. A. Ashman has patented [104] a soft tissue

substitute comprised of biocompatible, non-resorbable, polymer particles, which may be porous. The particles may be combined with extracellular matrix constituents, and implanted or injected to facilitate the permanent correction of soft tissue defects. Similarly, BioForm™ Medical markets products including dermal fillers (Radiesse®, FDA approved for the treatment of moderate to severe wrinkles and folds), as well as bulking materials for the treatment of urinary incontinence (Coaptite® Injectable Implant), based on patented technology related to injectable calcium hydroxylapatite microspheres, which can be encapsulated and delivered in an aqueous gel [105]. Hydrogels are also being investigated, including by Genzyme, who are affiliated with a recent patent application entitled "Methods of Augmenting or Repairing Soft Tissue" [106], which describes biodegradable, water-soluble, polymerizable macromers, including modified polyethylene glycol hydrogels, for the repair or augmentation of soft tissue defects. As an example of an approach involving 3-D polymer scaffolds, Tepha Inc. currently holds the patent "Bioabsorbable, Biocompatible Polymers for Tissue Engineering", invented by Dr. S.F. Simons [107]. This patent describes biocompatible polyhydroxyalkanoate scaffolds with tuneable mechanical properties that can be engineered to match the characteristics of specific tissues of interest, and used as tissue-engineering devices for applications in wound healing and guided tissue regeneration, including of musculo skeletal tissues and skin.

There are a variety of patents or applications that describe methods for the decellularization of cells [108, 109], tissues [110-112], or organs [113], to fabricate primarily acellular scaffolds that can be used to promote wound healing and soft tissue regeneration. The general objective in tissue decellularization is to remove the cellular components and antigenic materials that would initiate an immune response, while preserving the native structure and composition of the extracellular matrix, as much as possible [114]. Studies support the potential of this approach, showing that depending on the processing methods, decellularized matrices are well tolerated when implanted, initiating minimal immune responses and facilitating healing, through the promotion of normal cell-matrix interactions that can enhance the cell response in terms of viability, proliferation, migration, and/or differentiation [114-116].

In our lab, we have recently filed a preliminary patent application [117] describing detergent-free decellularization methods to isolate intact extracellular matrix from mammalian adipose tissue, as a means to engineer novel bioscaffolds for soft tissue engineering that closely replicate the ASC niche, with potential applications in plastic and reconstructive surgery [118]. Discarded fat from procedures such as panniculectomy and abdominoplasty, can be collected and treated with the described methods (mechanical disruption, enzymatic digestion, and/or polar solvent extraction), to yield a scaffolding material that can be easily manipulated and moulded into a variety of 3-D shapes to correct soft tissue defects. Preliminary *in vitro* experiments with the decellularized adipose tissue (DAT) seeded with human ASCs demonstrate the potential of our approach. More specifically, the master regulatory genes of adipogenesis (PPARγ and C/EBPα) were expressed by the ASCs seeded on the DAT scaffolds, without the need for inductive differentiation factors. Further, the highest levels of expression of adipogenic genes and proteins were observed in the ASCs on the DAT scaffolds cultured in adipogenic differentiation medium, relative to two-dimensional (2-D) tissue culture controls and cell aggregate cultures devoid of exogenous matrix, suggesting that the DAT scaffolds provided an inductive microenvironment for adipogenesis.

Similarly, Dr. J. Choi *et al*. [119] have developed novel biomaterials derived from processed adipose tissue obtained by liposuction. In brief, human lipoaspirates were treated to remove blood and oil, homogenized, freeze-dried, and milled to form a powder, which can be used as a scaffold base material. The extracellular matrix powders facilitated human ASC adhesion and proliferation *in vitro*, and when injected into nude mice, demonstrated stable soft tissue formation at 8 weeks, with blood vessel formation and intracellular lipid accumulation, in both the ASC-seeded and unseeded grafts. Dr. G. Rossen *et al*. [120] have also filed a WIPO patent application pertaining to the use of biocompatible biomaterials derived from adipose tissue, which may be prepared through acid treatment and/or non-ionic detergent extraction.

3. COMBINATION APPROACHES: CELL-SEEDED BIOCOMPATIBLE SCAFFOLDS

The combination approach, involving cell-seeded biomaterials, combines the biological advantages of the cell-based therapies with the 3-D, volume-augmentation capabilities of the biocompatible tissue substitute approaches. Ideally, the cell-seeded scaffold should facilitate the establishment of a regenerative milieu, including an inductive microenvironment to promote the differentiation of seeded and host-derived stem cells towards the required soft tissue lineages. Ideally, the constructs should be non-immunogenic, and should enhance tissue regeneration by secreting factors that promote angiogenesis, mediate the inflammatory response, and recruit host stem cells to the target site. The optimal scaffold properties described in the previous section translate to these combined approaches.

3.1. Stem-Cell Seeded Scaffolds

ASC-Seeded Scaffolds

As previously discussed, Dr. A. Katz *et al.* are inventors on the relevant patent entitled "Adipose derived stem cells and lattices" [35]. This patent includes cell-seeded, scaffold-based approaches for soft tissue engineering, in which 3-D lattices are seeded with ASCs to form complex tissue structures. The lattices are preferably biodegradable, and may be derived from the acellular portion of adipose tissue, or alternatively, from synthetic polymers, proteins, or polysaccharides, amongst other materials. The inventors describe methods by which different layers can be populated by ASCs differentiating towards different target lineages, to form composite tissue substitutes. The prepared construct may be implanted directly into the target site or, alternatively, it may be positioned in a temporary regenerative site to promote new tissue formation, and then moved to its final location.

Dr. J. Gimble, Dr. D. Kaplan, and Dr. J. Mauney are inventors on a patent [121] pertaining to the use of silk scaffolds seeded with ASCs for the regeneration of soft tissue

defects. The silk may be functionalized with molecules that influence cell adhesion, intracellular signalling pathways, and differentiation, such as cell-adhesive peptides, hormones, and growth factors. Moreover, the scaffolds may be cultured *in vitro* in medium that will induce the adipogenic differentiation of the seeded ASCs. Alternatively, the cell-seeded scaffolds may be directly implanted *in vivo* to facilitate *in situ* regeneration. Methods for fabricating porous silk scaffolds incorporating the ASCs are also described.

MSC-Seeded Scaffolds

As discussed previously, the patents held by Osiris Therapeutics Inc. [57-61] include the implementation of MSCs in regenerative therapies in combination with cellular carriers, such as 3-D scaffolds.

In the patent application "Shape and dimension maintenance of soft tissue grafts by stem cells" [122] methods and compositions are described to facilitate *de novo* synthesis of soft tissues *in vivo*, using biocompatible scaffolds seeded with MSCs, such as bone marrow-derived MSCs. More specifically, the MSCs are encapsulated within scaffolds of defined shape and volume for the treatment of soft tissue defects. A variety of synthetic- and naturally-derived polymers are included as possible cell delivery vehicles, and the constructs may be implanted in multiple forms, including as a solid, liquid, gel, mesh or sponge. A specific focus is placed on polyethylene glycol diacrylate as a hydrogel of interest for soft tissue regeneration. The cells may be exposed to adipogenic-inducing supplements that promote differentiation prior to implantation. Adipocytes and fibroblasts can be found in the newly-formed tissue substitutes, which work in synergy with the host system to promote the healing process.

3.2. Directed Stem Cell Recruitment

All of the previous inventions involving cell-based therapies, either with or without biomaterials, require the collection of cells from a donor site or external source, and potentially, isolating, expanding, and/or differentiating the cells *in vitro*. As an alternative, a cutting-edge approach in regenerative medicine would be to use the natural resources and capabilities of the human body to facilitate regeneration by endogenous populations. The general theory is that the human organism possesses the innate means and mechanisms for tissue repair and regeneration via recruitment of host stem cells to the site of injury, thereby reducing or eliminating the need for donor cell procurement. In a recent WIPO patent application entitled "Directed stem cell recruitment" [123], methods are provided for promoting host stem cell recruitment and tissue regeneration at a target site. The inventors describe that natural cell migration following the introduction of a foreign body includes not only inflammatory and fibroblast-like cells, but also cells with multilineage differentiation potential, including along the adipogenic, osteogenic, myogenic, and endothelial lineages. Further, a hyperbaric environment may promote the infiltration of native pluripotent cells into the target tissues, using an implanted biocompatible scaffold (i.e. porous matrix or hydrogel). Both natural and synthetic polymers that can be shaped according to the defect site may be implemented, and implanted or injected via simple surgical procedures. Growth factors and cytokines, selected based on the organ or tissues to be reconstructed or augmented, can be added to promote graft healing and new tissue formation. Anti-inflammatory agents may be utilized to control collagen deposition and fibrosis, and to thereby facilitate the regenerative processes initiated by the infiltrating cells.

3.3. Control of Cell Multipotentiality in Three-dimensional Matrices

A similar approach to profit from the natural environment of the human body has been patented by Dr. C.E. Semino *et al.* from the Massachusetts Institute of Technology (MIT) [124]. The inventors observed that when adult-cells or stem cells are cultured under particular conditions in a 3-D matrix, the differentiated cells assume a phenotype more similar to progenitor cells, and there is enhanced maintenance of the stem cell capacity to differentiate in the seeded stem cells (even in absence of maintenance factors, such as LIF). The matrices may include peptide scaffolds or hydrogels, polysaccharides, or specific glycosaminoglycans or proteins. The invention describes methods to culture fibroblasts within these 3-D scaffolds either *in vitro* or *in vivo* in the presence of anti-inflammatory factors, to yield a stem cell phenotype in the cultured cells. These induced multipotent cells can differentiate along several lineages, including the adipogenic lineage, and can contribute to healing and tissue regeneration in wounds or burns. Additionally, different matrix compositions can be used to modulate the lineage specification process. During culturing, gene therapy procedures may be performed. The inventors also claim that to optimize the healing process, various strategies can be utilized before or after implantation, such as reducing the blood flow to the treated site to decrease the immune response, or limiting oxygen exposure by covering the wound with a semi-permeable membrane.

LICENSING AND COMMERCIALIZATION

While patenting intellectual property is important, the most valuable patents will ultimately be licensed to interested companies for the development of new regenerative therapies. There are multiple biotechnology, biomedical engineering, and pharmaceutical companies from around the world that hold patents covering inventions in the field of soft tissue engineering. More importantly, many of these companies are testing or already producing commercial products derived from these inventions, some of which have already been discussed. Further testing will facilitate the development of safer, more effective, and more affordable strategies for soft tissue reconstruction in plastic and reconstructive surgery.

Artecel Inc., founded in 2000, was one of the first companies involved in ASC research, with initiatives in autologous applications in cosmetic surgery. In addition to serving as an ASC bank, they are also developing therapeutic products based on autologous and allogenic ASCs. This company holds patents related to methods for applying adipo-differentiated adipose-derived stromal cells in regenerative therapies for the treatment of a broad range of soft tissue disorders [125]. More specifically, they state that their specific cell population is defined by the secretion of a significantly greater quantity of extracellular matrix, such as collagen, and

smaller intracellular lipid droplets in a more multilocular morphology, as compared to mature adipocytes. The resulting adipocytic differentiated population has improved biochemical and mechanical properties for applications in tissue engineering, relative to isolated mature fat cells. Moreover, these cells are claimed to be more resistant to mechanical stresses, such as those that may be associated with harvesting and implantation procedures.

CryoLife is developing BioFoam®, a hydrogel-like biomaterial indicated for the treatment of wounds and for use as a hemostatic agent, as well as potentially having applications in soft tissue augmentation. The product is composed of two separate components that initiate a foaming reaction when mixed, as described by the inventors Dr. U. K. Yuskel *et al.* in the patent "Expandable foam-like biomaterials and methods" [126]. The composition is formed by an aqueous proteinaceous solution, supplemented with bicarbonate and a cross-linking solution, which can be mixed during injection to yield a 3-D scaffold *in situ*.

As previously discussed, Cytori is developing systems for processing the ASCs. More specifically, their core product is the Celution® system, a cell processing device, sold together with the necessary consumables and reagents, to separate, concentrate, and deliver regenerative cells isolated from fat, including lipoaspirate materials, in order to yield cosmetic benefits. The intellectual property of the technology is protected by patents that describe both the device and the methods of employing the isolated cell population [46, 47, 127].

Integra® is a line of products for skin grafting and wound healing, produced and commercialized by Integra Life Sciences Corporation. These products, as well as certain methods, are covered by US and international patents. Integra® Dermal Regeneration Template [128] is a two-layer material for skin regeneration in burns and reconstructive surgery. The inner layer is composed of cross-linked fibers of collagen and glycosaminoglycans, and the outer layer is a silicone barrier to protect against infections. Ultimately, the outer layer can be replaced by an epidermal skin graft, once the host cells have begun the regeneration process. Similarly, Integra™ Flowable Wound Matrix [129] is a collagen-glycosaminoglycan scaffold that can facilitate host cell migration and proliferation in full-thickness burns, surgical wounds, and diabetic ulcers.

Additional products used for the treatment of burns include Transcyte® [130], Dermagraft® [131-133], Isolagen™ [134, 135], Biobrane™ [136, 137], and Apligraf™ [138-140]. The first two skin substitutes are currently produced by Advanced Biohealing (Transcyte® was initially developed by Smith & Nephew), and the others by Isolagen Technologies, Smith & Nephew, and Organogenesis, respectively. Biobrane™ is an acellular biosynthetic wound dressing composed of a nylon fabric coated in bovine collagen type I that is partially embedded into a film of silicone, which acts as a protective barrier against infection, while allowing moisture permeation. Transcyte®, Dermagraft®, Isolagen™, and Apligraf™ are all based on fibroblastic production of extracellular matrix proteins and growth factors to support the regeneration of host populations. Although the underlying technology of these products is similar, the cell source and/or scaffolds vary. Transcyte®, Apligraf™ and Dermagraft® all use fibroblasts isolated from neonatal tissues, while the Isolagen procedure involves the isolation and *ex vivo* culturing of autologous fibroblasts, followed by the direct injection of this suspension back into the subject for dermal tissue repair. In terms of cell substrates, similar to Biobrane™, Transcyte® incorporates collagen-coated nylon mesh with a semi-permeable silicone membrane, while Dermagraft® is comprised of a polyglactin scaffold, and Apligraf™ is based on a bovine collagen type I gel, which can ultimately be seeded with autologous keratinocytes.

Alloderm™ and Strattice® Reconstructive Tissue Matrix are both artificial tissue matrices produced by LifeCell, and they are covered by multiple US and foreign patents [141-143]. The potential applications of these engineered constructs include breast reconstruction following mastectomy, abdominal wall reconstruction, and skin grafting. The primary difference between the two substitutes is the specific source of the dermal base material. More specifically, Alloderm™ is obtained from decellularized human cadaveric tissues, while the main component of Strattice® is decellularized porcine dermis.

A number of hyaluronic acid (HA)-based materials are also available for cosmetic augmentation for the treatment of wrinkles and small to medium-sized facial defects [144]. Chemical cross-linking is generally required to stabilize these injectable hydrogels to prevent rapid *in vivo* degradation. Restylane® is a patented injectable material that is widely used in the clinic, which is based on bacterially-produced HA [145]. Similarly, Hylaform® is a somewhat stronger HA-based product that is derived from rooster combs [146]. In general, these dermal fillers will have temporary augmentation effects lasting between 3 – 12 months [147].

CURRENT AND FUTURE DEVELOPMENTS

Our knowledge of stem cells and regenerative medicine is continually advancing as research in the field progresses. As such, it is anticipated that there will continue to be many new patent applications related to soft tissue engineering for plastic and reconstructive surgery. While our listing of relevant patents is incomplete due to the extensive nature of the field, we have tried to effectively summarize examples of the most relevant intellectual property, which is representative of the broad array of promising new approaches being investigated for the surgical reconstruction of soft tissue defects.

In the future, we anticipate that major advancements will involve multidisciplinary efforts that bring together biologists, materials scientists, clinicians, engineers, physiologists, and computer scientists, amongst others, to collaborate effectively in new and interesting ways. The combination cell-seeded, scaffold-based approaches are potentially the most promising for the regeneration of large-volume defects, as they integrate the beneficial properties of cell-based therapies with the supportive mechanical characteristics of 3-D scaffolds, to help stabilize and maintain the integrity of the deficits as healing progresses, thereby preventing scarring and contracture. Using this methodology, future developments will likely lie at the interface of science and technology. In our opinion, ongoing efforts to apply cell and mo-

lecular biology techniques to characterize tissue-engineering systems will be integral to the process of rational construct design, and will help to elucidate the key parameters that will ultimately facilitate the stable regeneration of functional soft tissues, including fat.

While there are multiple potential regenerative cell sources, ASCs are a particularly promising source of stem cells for adipose tissue regeneration, as they can be readily isolated from a uniquely expendable tissue source, and have the potential to be applied in either autologous or allogenic therapies. In terms of cell delivery vehicles, there are a wide variety of possible materials and methods, depending in part on the nature and site of the defect(s) to be corrected. Injectable materials are well-suited for applications in small-volume cosmetic augmentation, while the reconstruction of larger defects will likely require an implantable, 3-D scaffold. In the latter strategy, the implementation of bioreactor systems may help to promote *in vitro* tissue formation by overcoming the limitations of traditional static culture methods [148, 149]. Many groups are investigating new approaches with naturally-derived and synthetic scaffolds seeded with human ASCs for soft tissue engineering. Both classifications of materials have associated strengths and limitations, and it is possible that a combination biosynthetic approach may ultimately be most successful, as it could combine the favourable cell interactions of scaffolds derived from the extracellular matrix, with the tunability and flexibility of polymer systems.

Overall, the outlook for the development of safe, effective and clinically-applicable regenerative therapies for cosmetic and reconstructive plastic surgery is very promising, but efforts will likely require a balanced perspective on the scientific, legal, and ethical ramifications of the potential approaches.

ACKNOWLEDGEMENTS

The authors would like to thank Mr. Christopher McNevin for assistance with information gathering on recent patents in the field.

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