ADIPOSE TISSUE STORAGE AND ADIPOSE-DERIVED STEM CELLS: TODAY'S PATHWAYS TO TOMORROW'S THERAPIES

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Introduction: The discovery, characterization, and application of tissue-derived "adult" mesenchymal stem cells (MSCs) to the development of therapeutic interventions for a potentially vast array of pathological conditions have generated significant excitement for several years. Initially identified in the 1960's as fibroblast-like, colony-forming cells obtained from bone marrow and capable of differentiating into osteocytes (bone cells)[1-5], similar cells were later identified in liver[6], spleen[7], and thymus[8]. However, the full significance and multilineage potential of these cells were not appreciated until many years later[9], when they were shown definitively to be self-renewing entities capable of differentiating along several mesenchymal lineage pathways, including osteogenic, chondrogenic (cartilage-producing cells), and adipogenic (fat-storing cells) pathways. Obviously, these characteristics quickly prompted thoughts of therapeutic development, and the discovery that MSCs with identical or nearly identical properties are also resident within umbilical cord blood[10], placenta[11-15], and amniotic fluid[16] provided therapeutic strategists many options. However, cells derived from cord blood, placenta, and amniotic fluid offer limited autologous (cells and tissues derived from self) options, and their widespread applications would require the development and validation of allogeneic (cells and tissues derived from another person) strategies. As a result of the "self-healing-self" mentality as well as the elimination of tissue rejection issues, autologous therapeutic approaches face fewer hurdles from regulatory (i.e. FDA) and public acceptance standpoints, adding to the desire of many organizations to pursue autologous approaches. As a result of the aforementioned issues with cord blood, placental, and amniotic-derived MSCs, autologous therapeutics, would have to rely much more on bone marrow-derived MSCs, and the discomfort experienced during extraction makes that option unattractive to some. In addition, the MSC yield from bone marrow aspirate is fairly low, making it a less-than-ideal option. Thus, the excitement at the prospect of using MSCs for autologous therapeutics was rekindled and took a huge leap forward with the discovery that human adipose tissue is an extremely rich source of MSCs (adipose-derived stem cells, or ADSCs) which may be readily harvested and expanded and which have the ability to differentiate into osteocytes[17-20], chondrocytes (cartilage-producing cells)[17-18, 21-22], cardiomyocytes (heart muscle cells)[23-25], nervous tissue[26-28] , muscle tissue[29], and endothelial tissue (lining of blood vessels)[30]. This large array of potential tissues which could be created on an autologous basis has created a strong belief that regenerative medicine is poised to make huge strides in several areas, powered by the new found value of adipose-derived stem cells (ADSCs).

Gold From Garbage: Why Fatty Tissue Should Be Retained Earlier this year, it was announced that liposuction procedures have became the most common type of cosmetic surgical procedure performed in the US[31]. The lipoaspirates which are obtained from these

procedures may be broadly broken down into two main components: fatty tissue and stromal vascular fraction (SVF). The fatty tissue is largely comprised of adipocytes, while the SVF is a heterogeneous mixture of many cell types, including pre-adipocytes, tissue fibroblasts, endothelial progenitor cells, red and white blood cells, and ADSCs. With little or no processing of harvested tissue, lipoaspirates obtained from one location may be reimplanted in another location, a process known as autologous fat transfer, or AFT. However, reabsorption/regression rates of reimplanted tissue can be quite high, with reported rates varying between 25 and 80%[32], and this often creates a need for "followup" procedures or "touch ups" involving additional transfer procedures to create optimal aesthetics. A validated solution for the long-term storage of adipose tissue would alleviate the need for additional liposuction procedures to provide tissue for these "touch ups".

To meet this need, American CryoStem Corporation has developed validated and proven solutions for the collection, shipping, processing, and long-term cryopreservation of a client's lipoaspirate. This process begins with the CELLECT™ Collection Kit, which includes all the supplies necessary to perform an adipose tissue extraction procedure as well as a transport bag containing ACSelerate™ TR, an optimized transport medium designed to discourage microbial growth while providing nutritional support for a client's tissue cells (Figure 1). Validation tests have demonstrated that tissue suspended in ACSelerate™ TR retains good viability for up to 70 hours at ambient temperature, and experience has proven that delays in shipping are less problematic than they could potentially be in terms of tissue quality.

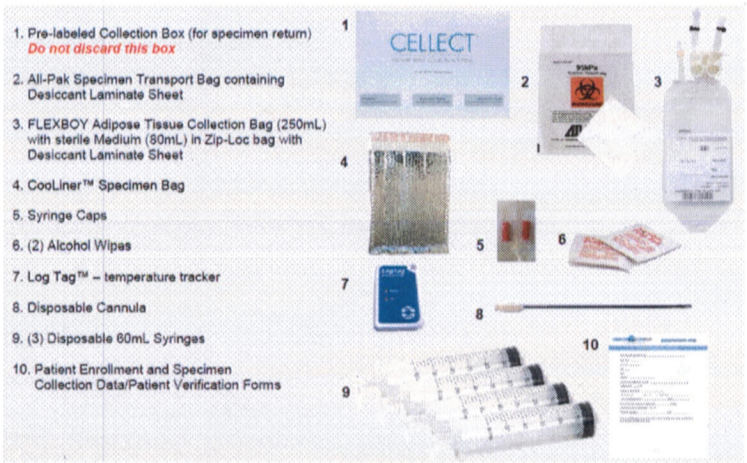

Figure 1: Collection and Transport of Lipoaspirate Made Simple. The CELLECT™ Collection Kit contains all the components necessary for the collection and safe shipment of a client's lipoaspirate material. This kit includes ACSelerate TR™ (in #3), a tested and validated medium designed to discoursage microbial growth within the sample while providing nutritional support for the tissue during transit. Tissue transported in ACSelerate TR™ has been shown to retain good viability for up to 70 hours following harvest.

Once tissue arrives at the American CryoStem Laboratory, a proprietary processing protocol is conducted. Termed "ATGRAFT™", this technique consists of a proprietary processing stage

during which oil, blood, and debris are washed from the lipoaspirate followed by incorporation of a proprietary cryopreservation medium, ACSelerate™ CP, optimized controlled-rate freezing, and long-term cryogenic storage. Validation studies have verified that tissue processed and stored according to this methodology retains good viability and consistency following thaw (Figure 2).

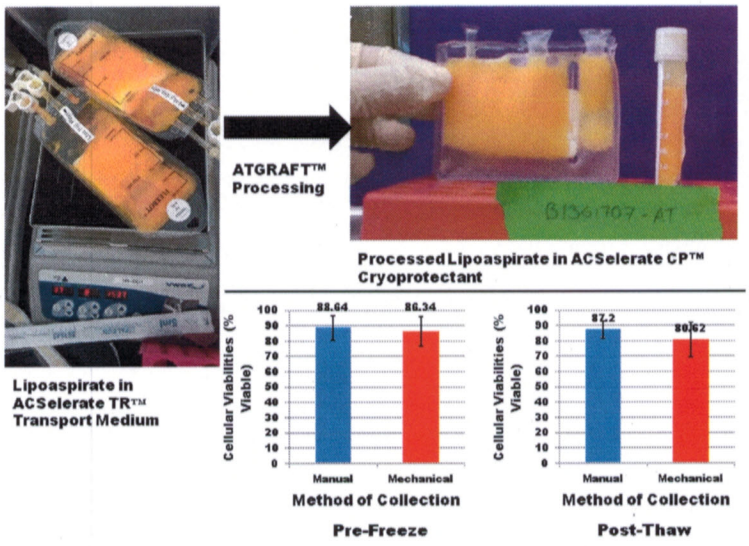

Figure 2: Processing and Storage of Adipose Tissue. Lipoaspirate is collected and incorporated into ACSelerate TR™ Transport Medium, a proprietary formulation optimized to discourage microbial growth within samples while providing nutritional support for cells (Upper Left). Following processing using the ATGRAFT™ methodology, during which ACSelerate CP™ CryoProtectant Medium is incorporated into the tissue, tissue is dispensed into client-specified storage configurations, which may include bags or smaller vials (Upper Right). Adipose tissue with incorporated ACSelerate CP™ is non-toxic and may be directly used for cosmetic procedures without the need for tissue washing. As shown (Lower Right), cellular viabilities are maintained during cryopreservation.

Taken as a source of MSCs, the SVF fraction of lipoaspirates has been shown to include substantial numbers of ADSCs, with between 1% and 10% of the total numbers of nucleated cells falling into that category[33-35]. When compared with bone marrow aspirates, where only 0.001-0.01% of those are MSCs[9], adipose tissue offers a much more rich source of MSCs which could be used for personalized therapeutics. To put this another way, it is estimated that 1 gram of lipoaspirate contains roughly 500 times the number of MSCs than 1 gram of bone marrow aspirate[36-37], and more cells can translate into greater therapeutic options at a patient's disposal.

Stromal Vascular Fraction: The Cream from the Crop? As explained, harvested lipoaspirates can be broadly divided into two predominant fractions: the fatty tissue fraction and the stromal vascular fraction, or SVF. The SVF is a heterogeneous mixture of multiple cells types, including preadipocytes, endothelial progenitor cells, T-regulatory lymphocytes, monocytes/macrophages, and, of course, adipose-derived mesenchymal stem cells[38]. The incorporation of SVF into lipoaspirate, creating an SVF-supercharged fatty lipoaspirate, or "Turbo Fat", has also been seen to increase the survival of fatty tissue following implantation,

potentially alleviating the need for "touch up" procedures through the improvement of graft survival and retention[39-41]. However, this procedure requires the on-site extraction of SVF from a portion of a patient's lipoaspirate, and this realization has led to multiple technologies designed to facilitate same-day SVF procedures. For example, Cytori has marketed their "Celution" device, which has been shown to be able to create SVF identical to that produced from manual procedures[42], and Cytori has launched several clinical trials aimed at investigating the safety and efficacy of Celution-produced SVF in the treatment of a number of pathological conditions[43-47]. Indeed, in addition to these studies, SVF has been used for other autologous therapies in the clinic and been shown to be safe in treatment studies of multiple sclerosis[38] and rheumatoid arthritis[48], and, in the first case, improvements in clinical symptoms were seen, so SVF may turn out to be a reasonable therapeutic. However, in a study where the efficacy of SVF was been compared directly with ADSCs cultivated and expanded from SVF in the treatment of sufferers of Crohn's disease with enterocutaneous fistulas, a 3-fold greater rate of cure was achieved in patients treated with ADSCs expanded from SVF when compared with patients treated with SVF, alone (75% cure as opposed to 25% cure)[49], which might indicate that pure cultures of ADSCs possess greater therapeutic potential than SVF, alone. Furthermore, a key problem of same-day procedures, such as those involving SVF extraction and immediate readministration, lies in the lack of sterility testing and/or quality control of the SVF to be readministered, while expanded cells may be well-controlled and assessed prior to administration. It cannot be overstated that, if the field of autologous cellular therapeutics is to move forward in a meaningful way, safety and adequate quality control must be maintained, something that would prove challenging for same-day procedures.

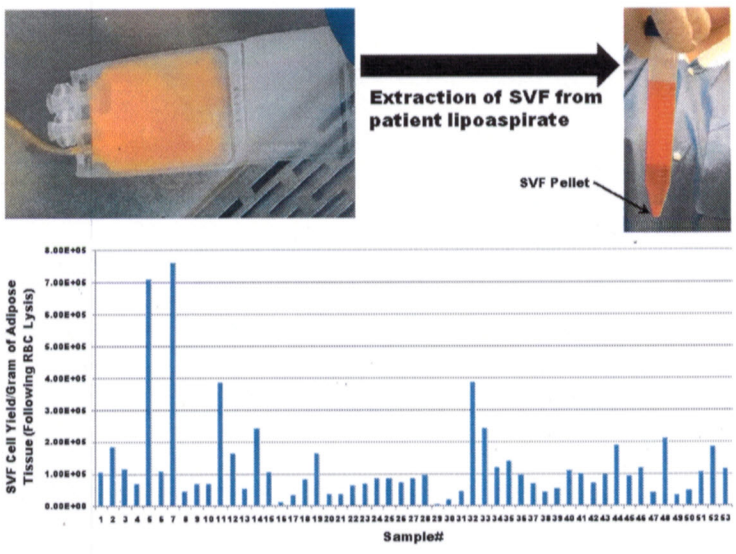

Figure 3: Stromal Vascular Fraction (SVF) May Be Isolated From Client Lipoaspirate. American CryoStem Laboratories is able to isolate the SVF (Upper Right) from client lipoaspirates (Upper Left), to remove the red blood cells via lysis, and provide cryogenic storage services for as long as a client desires. Following removal of red blood cells, typical SVF samples contain between 3.8×10^3 and 7.6×10^5 viable cells/gram of adipose tissue, depending largely on initial sample quality (Lower Panel).

To meet the growing therapeutic interest in SVF, American CryoStem Corporation is proud to offer client-based services for the isolation and long-term cryopreservation of SVF. The quantities of SVF which can be obtained by an adipose tissue sample are quite variable, being greatly affected by the quality of the tissue sample (Figure 3). These stored SVF samples may be used at any time for the creation, expansion, and banking of client-specific ADSC cell lines cultivated under optimal culture conditions. In-house validation studies have confirmed that the cell yields for SVF derived from cryopreserved tissue are statistically identical to those from fresh adipose tissue, and multiple client-specific ADSC cells lines have now been created from stored tissue samples.

Adipose-Derived Stem Cells (ADSCs): The Crème de La Crème? Studies have indicated that adipose-derived stem cells (ADSCs) represent between 2% and 10% of the cells found in SVF[50]. It is believed that, within the human body, these cells adopt a perivascular distribution[51-53], lying outside of the endothelial layer. Combining this finding with the high degree of vascularity seen in adipose tissue explains the high abundance of mesenchymal stem cells which can be isolated from lipoaspirates as it is likely that these MSCs are perivascularly distributed throughout the human body, a feature which renders them able to interact closely with the bloodstream, with blood-borne signals, and with the immune system.

Autoimmune responses are responsible for a number of relatively common, well-known, widespread pathological disorders, including Type 1 Diabetes, Discoid (chronic cutaneous) and Systemic Lupus Erythematosus, Crohn's Disease, Multiple Sclerosis, Psoriasis, and Rheumatoid Arthritis[54]. For many autoimmune disorders, treatment options are centered around immunosuppressive drug regimens, which can have significant side effects. For example, long-term use of prednisone, a very commonly-used glucocorticoid steroid, is known to lead to glucocorticoid-induced osteoarthritis[55] as well as degradation of articular cartilage[56]. Immunomodulatory approaches with fewer side effects would potentially have applications in the treatment of multiple pathologies, and this is a current area where MSCs are showing great promise. Critical components of this regulation include specific cytokines and trophic factors secreted by the MSCs, and, interestingly, no significant differences have been seen between ADSCs and other MSC types in terms of immune-regulatory secreted factors, which have been shown to include Tumor Necrosis Factor-Alpha (TNFα), Interferon Gamma (IFNγ), Indoleamine 2,3-Dioxygenase (IDO), and IL-17[57]. Preclinical studies have shown that ADSCs are capable of taming the immune responses associated with allergic airway inflammation[58], autoimmune colitis[59], and induced autoimmune encephalomyelitis, a model system for multiple sclerosis[60,61]. Currently, multiple NIH-registered clinical trials are being started to explore the therapeutic potentials of ADSCs in the treatment of congestive heart failure[62,63], chronic obstructive pulmonary disease (COPD) [64,65], regeneration of chronic wounds[66], Type II Diabetes[67], critical limb ischemia[68], Parkinson's disease[69], osteoarthritis[70], and many other illnesses. Based upon the enthusiasm and substantial activity focused around the use of ADSCs, it is not unreasonable to speculate that personalized, autologous therapeutics for a variety of conditions will be widely-available in the very near future.

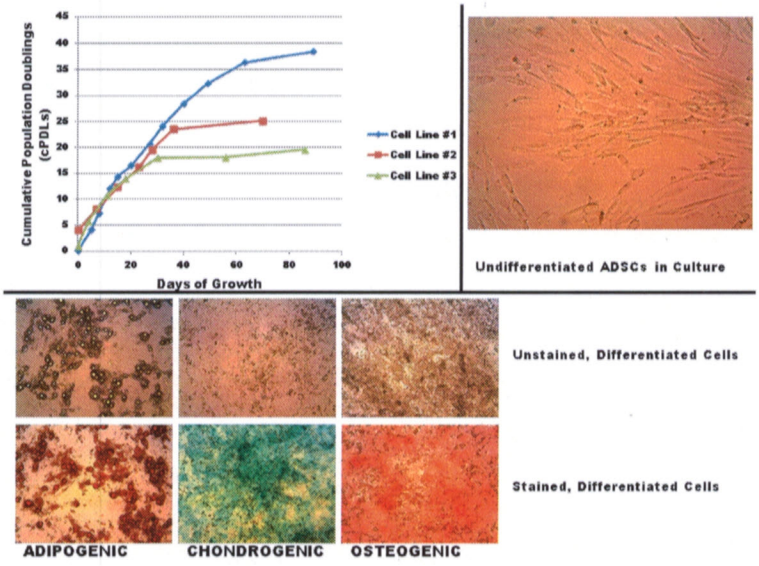

Figure 4: Adipose-Derived Stem Cells (ADSCs) May Be Expanded from Client SVF and Readily Differentiated to Adipogenic, Chondrogenic, and Osteogenic Lineages. Once a client's SVF is isolated from their lipoaspirate, the ADSCs may be isolated and expanded using ACSelerate SF-M™ xeno-free growth medium (Upper Panels) and differentiated to fat-storing adipocytes, cartilage-producing chondrocytes, and bone-forming osteocytes using ACSelerate AD™, ACSelerate CY™, and ACSelerate OB™ differentiation media, respectively (Lower Panels).

To meet the growing desire of clients for banked ADSC stocks suitable for these next-generation therapeutics, American CryoStem Corporation is able to expand a client's ADSCs under carefully optimized conditions and bank the resulting cell stocks for future use. These optimized conditions include expansion with American CryoStem's proprietary ACSelerate™ SF-M growth medium, a xeno-free growth medium carefully optimized to sustain a high growth rate while maintaining the full multipotent differentiation potentials characteristic of these cells, as well as culturing under hypoxic conditions, which is important for optimal cell production. In multiple studies, ACSelerate SF-M™ medium has proven capable of sustaining substantial cellular expansions of 12-38 population doublings, depending upon the client, while maintaining multipotency (Figure 4), and differentiations to the adipogenic, chondrogenic, and osteogenic lineages may be rapidly accomplished with a full line of lineage-specific differentiation media.

In addition, American CryoStem is able to provide researchers with stocks of donated and expanded ADSCs according to specific age and gender categories, and this product is termed "ATCELL™". Undifferentiated and expanded cells from ATCELL™ stocks have been demonstrated to exhibit a distinctive phenotypic profile, expressing the cell surface markers CD29, CD44, CD49d, CD73, CD90, CD105, and CD146 while being verified as negative for expression of the surface markers CD14, CD19, CD31, CD34, and CD45. This phenotypic profile is consistently maintained in culture and defines ATCELLs™ as being true ADSCs, a finding that is supported by the demonstration that ATCELLs™ are fully tripotent and able to be differentiated into the adipogenic, chondrogenic, and osteogenic lineages using the ACSelerate™ AD, CY, and OB formulations, respectively (Table 1, Figure 5).

Phenotypic Marker	Expected in ADSCs?	Found in Early Passages (P1-P4)?	Found in Later Passages (≥P7)?
CD14	NO	NO	NO
CD19	NO	NO	NO
CD29	YES	YES	YES
CD31	NO	NO	NO
CD34	NO	NO	NO
CD44	YES	YES	YES
CD45	NO	NO	NO
CD49d	YES	YES	YES
CD73	YES	YES	YES
CD90	YES	YES	YES
CD105	YES	YES	YES
CD146	YES	YES	YES

Table 1: ATCELLs™ Have Been Verified as ADSCs. Using a 12-parameter panel of cell surface markers known to be expressed or to specifically not be expressed on ADSCs, ATCELLs™ from

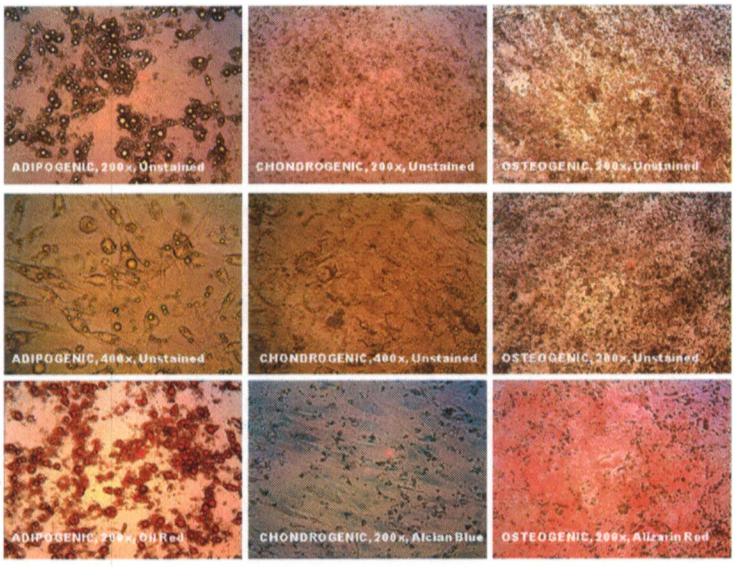

Figure 5: ATCELLs™ Are Multipotent, and Can Be Differentiated Along The Adipogenic, Chondrogenic, and Osteogenic Lineages. At P7 and beyond, ATCELLs have been shown to be multipotent and capable of becoming fat-storing adipocytes (Left Column), cartilage-producing chondrocytes (Central Column), and bone-producing osteocytes (Right Column) when treated with ACSelerate AD™, ACSelerate CY™, and ACSelerate OB™ differentiation media, respectively.

Realistic Culturing Conditions for Realistic Outcomes Recent works have pointed out that the oxygen concentrations present is most human tissues are substantially lower than the typical ambient oxygen concentrations (21%) used in modern cell culture facilities. In fact, the reported oxygen concentration range for adipose tissue is between 2% and 8%[71]. Fortunately, studies aimed at evaluating the effects of hypoxic culture conditions have revealed that the cells tend to behave similarly under both normoxic and hypoxic conditions, although

proliferation rates tend to be higher under hypoxic conditions[72,73]. Although the standard trilineage (adipogenic, chondrogenic, and osteogenic) multipotentiality appears to be maintained under hypoxic conditions, there have been reports indicating that chondrogenic differentiation potentials may be enhanced by lower oxygen, with concomitant decreases in adipogenic and osteogenic potentials[73], although other conflicting reports have seen adipogenic potential increased by lower O2[74]. Significantly, Oct3/4 and Nanog, well-characterized markers of "stemness", have been reported to have increased expressions under hypoxia[75], possibly implying that realization of the full differentiation potential abilities of ADSCs may require hypoxic growth. Furthermore, multiple aspects of ADSC-facilitated wound healing are increased by hypoxia, including cellular proliferation, cellular migratory ability, and cellular secretion of basic fibroblast growth factor (bFGF, or FGF2) and vascular endothelial growth factor (VEGF)[76-78]. In fact, conditioned medium (CM) harvested from ADSCs cultured under hypoxic conditions has been shown to be capable of positively-impacting wound healing when compared with CM harvested from normoxic ADSC cultures and to better promote the migratory activity of dermal fibroblasts and synthesis of new collagen[76,78]. Furthermore, it has been demonstrated that a combination hypoxic conditions, VEGF, and leptin is sufficient to encourage the endothelial differentiation of ADSCs[79], implying that ADSCs cultured under hypoxic conditions may not only be able to encourage angiogenesis in wound healing (through VEGF secretion), but also may be capable of directly contributing to the pool of endothelial cells.

Secreted Gold, The Potential of ADSC-Conditioned Medium As mentioned, ADSCs cultured under hypoxic conditions secrete a variety of chemical factors which may have great potential for wound healing through the stimulation of proliferation, migratory activity, and secretory activities of dermal fibroblasts[76,78]. It has also been shown that similar activities in the keratinocytes of the epidermis are stimulated by hypoxic ADSC-conditioned medium[80], suggesting that conditioned medium from hypoxic ADSC cultures (hADSC-CM) has the capability of stimulating regenerative and healing activities in both the dermis and epidermis. Indeed, hADSC-CM appears to also have potential for the stimulation of hair regrowth[81], implying that topical application of hADSC-CM may have very profound and wide-ranging applications for both regenerative and cosmetic purposes.

To facilitate these applications, American CryoStem Corporation offers a customized solution for the creation of client-specific ADSC-conditioned medium, termed AutoKine CM™. This may be combined with a base compound to create "U Autologous™", a unique and personalized skin cream which has been shown in clinical studies to reduce facial lines, to improve skin texture, to increase filaggrin and elastin abundance in skin (Figure 6). In addition, gene expression data has indicated that most of the test subjects experienced substantial reductions in the activities of the enzymes collagenase and elastase, responsible for the degradation of collagen and elastin, respectively (Data Not Shown).

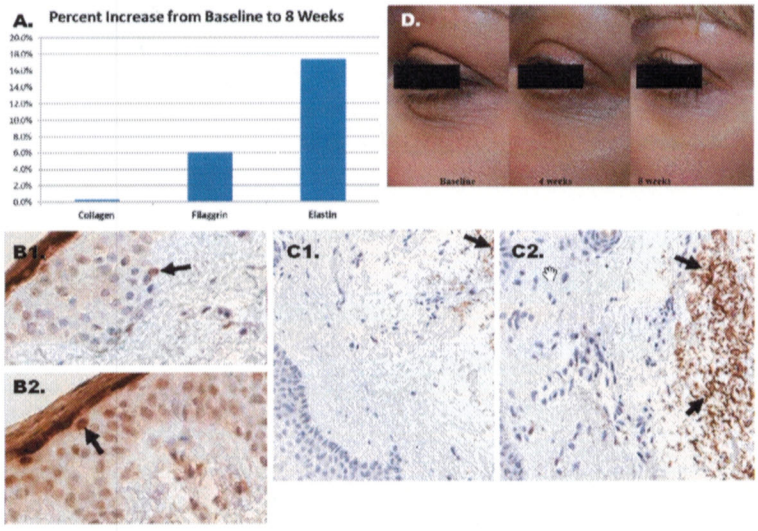

Figure 6: Topical Application of Autokine CM™-Based Skin Cream Has Pronounced Cosmetic Effects. Clinical studies to assess the effects of topical application of were performed at two separate and unrelated facilities on a total of 19 patients. For each patient, a mini-liposuction procedure was performed, followed by SVF extraction from lipoaspirate and expansion of ADSCs. Conditioned medium was produced from cultures of each patient's ADSCs and incorporated into unique U-Autologous™ formulations. As shown, topical application of the U-Autologous™ skin cream resulted in sharp increases in the abundance of the filaggrin (A, B1, and B2) and elastase (A, C1, and C2) proteins. In panels B1 (control) and B2 (treated), filaggrin is labeled in brown, while in panels C1 (control) and C2 (treated) elastase is also labeled in brown. Collagen expression (A), by contrast, was seen to not be greatly affected by U-Autologous™ application, although the expressions of the collagenase and elastase enzymes were seen to be strongly downregulated (Data not shown). The combinatorial effects of U-Autologous™ were seen to result in visible cosmetic improvements, including reductions in wrinkles and fine lines (D).

The Need for Banking, and the Biological Insurance Policy Studies have demonstrated that the immunoregulatory abilities of mesenchymal stem cells decline with age[82]. In addition, cells obtained from progressively older donors have been shown to have dramatic reductions in their proliferative and differentiation capacities, with the abilities to become chondrocytes and osteocytes being substantially impacted[83]. Thus, banking of ADSCs at an earlier age would be expected to be able to support more therapeutic and/or cosmetic options than those banked when one reaches older ages. This has given rise to the concept of the "Biological Insurance Policy" whereby an individual can store tissue, SVF, or ADSCs at a younger age and reap the benefits of those stored materials at later dates. A particularly attractive model would involve an individual storing multiple aliquots of tissue which could sequentially be used for a variety of applications upon demand, ranging from cosmetic or dermatological concerns to therapeutic applications depending upon that individual's life history and needs. Toward this end, American CryoStem Corporation can customize the configuration of sample types and volumes on a personalized, individual basis. These samples provide a lasting and reliable "Biological Insurance Policy" which can be used for multiple circumstances, even unforeseen ones, throughout a client's lifetime.

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