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(54) **Title:** STEM CELL-BASED PREPARATIONS AND METHODS FOR DERMAL AND CONNECTIVE TISSUE RECONSTRUCTION AND REJUVENATION

(57) **Abstract:** Grafts, systems, methods and kits are provided for treating a subject having a defect using adult stem cells that are characterized in that the cells do not substantially induce a T cell or natural kill (NK) cell-mediated immune response in the subject. The systems, methods and kits optionally further include exogenous biological factors and energy sources such as an ultrasound device for stimulating healing of the target tissue and tissue defect and/or tissue formation.

Stem cell-based preparations and methods for Dermal and connective tissue reconstruction and rejuvenation

Related application

This application claims the benefit of U.S. provisional application serial number 61/861,292 filed August 1, 2013 entitled, "Stem Cell-Based Preparations and Methods for Dermal and connective tissue Reconstruction and Rejuvenation", which is incorporated by reference herein in its entirety.

Field of the invention

Systems, methods and kits are provided for treating a subject with a tissue defect, by administering grafts.

Background

Tissue grafting is used to treat a diverse group of defects and disorders that present serious medical problems for patients. Tissue grafts stimulate re-growth of Organs and tissues including the skin, liver, cornea, cartilage, and bone. Dermal skin grafts are applied to distinct types of skin defects caused by trauma such as burns, abrasions, and cuts, diseases such as pemphigus foliaceus and pemphigus vulgaris, and conditions such as venous stasis ulcers.

Success of a tissue graft is influenced by factors including the techniques used to collect, apply, and treat of the graft. Sufficient uniform donor cells or tissues (e.g., depth and size) are collected, and graft material is applied to a defect site to stimulate formation and re-integration of tissue.

Once the dermal graft is collected and applied to the defect site rejection of the transplanted cells or tissue can result in the recipient subject by an immune response involving T-cells or B-cells. Immune responses against donor (i.e., "non-self or "allogeneic") antigens are the primary cause of rejection of tissue graft and graft failure. Strategies for avoiding rejection include minimizing antigenic differences between donor and recipient by matching Human Leukocyte Antigens (HLA; also known as Major Histocompatibility or MHC antigens) and subjecting the transplant recipient to potent immunosuppression.

Perfect HLA typing is difficult to achieve due to limited numbers of donors and the complexity of HLA alleles. Rejection of well-matched grafts may occur due to unknown factors. Administration of immunosuppressive drugs to the recipient has been found to enhance graft survival, however these drugs reduce activation or efficacy of the immune system in the recipient and increase likelihood of infection and toxicity. Accordingly, thousands of graft rejections occur each year resulting in millions of hospitalizations, increased costs to patients and insurers, and death.

Therapeutic ultrasound therapy has been used for treatment of some wounds, specifically to accelerate the process of healing. However many of these ultrasound procedures require significant amounts of time, extensive physiotherapy, and even further surgery.

Thus, there is a medical need for systems, methods and kits for regenerating tissue and promoting healing of defects with agents that provoke little or no immune response in the recipient subject.

Summary

An embodiment of the invention provides stem cell based therapeutic preparations for treating a subject having a dermal or subdermal defect with a population of synovial fluid-derived stem cells, which promotes tissue ingrowth into the defect site and induce tissue formation in the defect site. In various embodiments the defect is an injury, an ulcer, a blister, a lesion, a burn, a sore, a boil, an abrasion, a laceration, an excision, an malformation, a discoloration, a wound, a cut, and wrinkle and the resultant tissue formation includes: dermal, epithelial, skin, connective tissue, muscle, fat, neural, and bone tissues. In one embodiment, an ultrasound device or other means of external energy are applied to the treatment site of the subject such that the energy enhances functional healing, ELA cell and other ASC differentiation and an aesthetically pleasing outcome.

In related embodiments, the synovial fluid derived adult stem cells include at least one type of cell selected from the group of: early lineage adult (ELA) stem cells, mesenchymal stem cells (MSC), hematopoietic stem cells (HSC), multipotent adult progenitor cells (MAPC), and mesenchymal precursor cells (MPC). In related embodiments the cells have been cultured to expand the populations and increase the number of cells.

In certain embodiments, the ultrasound device includes at least one selected from of: a transducer, a sensor, a display, a handle, and a housing. In related embodiments, the

transducer is at least one selected from: a piezoelectric crystal transducer, a PZT (lead zirconium titanate) transducer, an array transducer, a phase transducer, a polyvinylidene fluoride (PVDF) transducer, and a magnetostrictive transducer. In a related embodiment, the ultrasound device is a handheld device that is positioned on, over or adjacent to the defect. In a related embodiment, the ultrasound device focuses ultrasound waves to a portion of the defect or to an entirety of the defect. For example, the ultrasound waves are focused to an area needing increased vascularization.

In a related embodiment, the ultrasound device includes a pad for contacting the defect or the subject. For example, the pad is a conventional packing or dressing such as gauze or cotton. In related embodiments, the pad is at least one selected from: sterile, porous, biocompatible, and layered. In a related embodiment, the pad includes a sensor, for example a sensor that detects temperature or a presence of a composition or molecule. In a related embodiment, the pad includes at least one bioresorbable composition. In a related embodiment, the ultrasound device includes a fastener for securing the device to the defect or the subject. In a related embodiment, the ultrasound device includes a transducer, a power source, or power adapter.

In another embodiment, the invention includes a composition that enhances the operation of the ultrasound device that may optionally contain ELA stem cells, extracts or preparations of secreted factors. For example, the composition that enhances the operation is a gel, for example the gel is contacted to the pad or the subject. In various embodiments, the composition is at least one selected from: a fluid, an oil (e.g., a mineral oil, a synthetic oil, and a vegetable oil), a hydrogel, a polymer, a viscous material, and a plastic.

In related embodiments, the device generates an ultrasound frequency of about 10 kilohertz (kHz) to about 10 megahertz (MHz), for example 10 kHz to about 20 kHz; 20 kHz to about 30 kHz; 30 kHz to about 50 kHz; about 50 kHz to about 100 kHz; about 100 kHz to about 250 kHz; about 250 kHz to about 500 kHz; about 500 kHz to about 750 kHz; about 750 kHz to about 2 megahertz (MHz); about 2 MHz to about 4 MHz; about 4 MHz to about 6 MHz; about 6 MHz to about 8 MHz; or about 8 MHz to about 10 MHz.

In related embodiments, the device in the system generates a low frequency modulating signal of at least: about I kHz, 2 kHz, about 5 kHz, about 7 kHz, 10 kHz, about 12 kHz, about 14 kHz, about 18 kHz, or about 20 kHz.

In a related embodiment, the device in the system generates and an intensity of at least about: 0.5 milliwatts/cm ^{2}, 1 milliwatts/cm ^{2}, 2 milliwatts/cm ^{2}, 5 milliwatts/cm ^{2}, 10

milliwatts/cm ^{2}, 20 milliwatts/cm ^{2}, 40 milliwatts/cm ^{2}, 50 milliwatts/cm ^{2}, 60 milliwatts/cm^2, 70 milliwatts/cm ^{2}, 80 milliwatts/cm ^{2}, 90 milliwatts/cm ^{2}, 100 milliwatts/cm ^{2}, 110 milliwatts/cm ^{2}, about 120 milliwatts/cm^2, 130 milliwatts/cm ^{2}, 140 milliwatts/cm ^{2}, or about 150 milliwatts/cm ^{2}.

In a related embodiment of the system, the graft further includes an autograft or allograft tissue from the subject or a donor, such as a skin graft. In a related embodiment, the adult stem cells in the system are an allograft to the subject. For example, the allograft is not MHC matched to the subject. Alternatively, the allograft is about 50% MHC matched, 60% MHC matched, about 70% MHC matched, about 80% MHC matched, about 90% MHC matched, about 95% MHC matched, or about 99% MHC matched.

In a related embodiment, the adult stem cells in the system are substantially free of erythrocyte cells. In a related embodiment, substantially free is about 90% free of erythrocyte cells, about 95% free of erythrocyte cells, or about 99% free of erythrocyte cells compared to a control set of adult stem cells.

In a related embodiment, the graft further includes at least one selected from: a plasma for example Platelet Rich Plasma, a buffer, cell culture medium, a preservative, an antibacterial agent, an antifungal agent, a conditioning agent, a cryogenic agent, a pharmaceutically acceptable salt, a growth factor, a vitamin, a hormone, and a therapeutic agent.

In a related embodiment, the population of stem cells further includes a carrier matrix that conforms substantially to the defect site such that the matrix is a structurally stable, three dimensional surface that retains, binds, infiltrates and attaches to the graft and supports ingrowth of stem cells into the matrix at the defect site. In an embodiment, the carrier matrix includes a plurality of pores or holes, for example the plurality of pores or holes is found on at least one of a top surface and a bottom surface of the carrier matrix. The carrier matrix is various embodiments is accllular or contains cells. In various embodiments the carrier matrix includes at least one selected from the group of: a scaffold, a bone material, a synthetic material, a mesh, a plastic, a polymer, and a membrane.

In a related embodiment of the system, the defect includes at least one from the group of: a scar, a surgical wound, an excisional wound, a blister, an ulcer, a lesion, an abrasion, an erosion, a laceration, a boil, a cut, a sore, a wrinkle, a discoloration, a blemish, and a burn.

In a related embodiment, the population of adult stem cells includes cryogenically frozen cells. For example, the cryogenically frozen cells are obtained from a stem cell bank.

In related embodiments, the population of synovial fluid-derived stem cells includes at least about 100 stem cells, about 10^3 stem cells, about 10^4 stem cells, about 10^5 stem cells, about 10^6 stem cells, about 10^7 stem cells or about 10^8. In an embodiment, the population of adult stem cells is obtained from a stem cell bank.

In a related embodiment, the system further includes a pressure modulation device and/or a cutting device. For example the pressure modulation device is a negative pressure device or a hyperbaric device. In a related embodiment, the cutting device excises tissue and for example includes at least one selected from the group of: a cutting component; a component for holding or retaining the excised tissue such as a filter or a reservoir; and a dispenser.

An embodiment of the invention provides a method for treating a subject having a defect, the method including: contacting a graft including tissue and a population of adult stem cells to the defect, wherein the stem cells do not substantially induce a T cell or natural kill (NK) cell-mediated immune response in the subject; and, performing ultrasound therapy on the defect using an ultrasound device, thereby stimulating healing of the skin defect.

An embodiment of the invention provides a method for treating a subject having a tissue defect, the method including: contacting the tissue defect with a stem-cell based therapeutic comprising a population of 10^2 to 10^7 cells ELA stem cells in a formulation including a matrix component supporting ingrowth of mesenchymal lineage differentiated cells of the subject. In a related embodiment, the adult stem cells include at least one type of cell selected from: early lineage adult (ELA) stem cells, mesenchymal stem cells (MSC), hematopoietic stem cells (HSC), multipotent adult progenitor cells (MAPC), and mesenchymal precursor cells (MPC).

In various embodiments of the method, at least one of the graft, the defect, and the tissue includes at least one from the group of: dermal, epithelial, skin, connective tissue, muscle, fat, neural, and bone.

In related embodiments of the method, contacting the graft further includes contacting the stems cells or defect with at least one selected from: a plasma such as Platelet Rich Plasma (PRP), a buffer, cell culture medium, a preservative, an anti-bacterial agent, an anti-fungal agent, a conditioning agent, a cryogenic agent, a pharmaceutically acceptable salt, a growth factor for example epidermal growth factor, a bioactive factor for example stem cell factor, a vitamin, a hormone, and a therapeutic agent.

In related embodiments, contacting further includes applying to the graft at least one selected from: a bone morphogenetic factor selected from the group consisting of: bone morphogeneic proteins BMP-2, BMP-3, BMP-4, BMP-6, and BMP-7; platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), interleukins selected from the group consisting of IL-3, IL-4 and IL-1, insulin-like growth factor-1 (IGF-1), leukemia inhibitory factor (LIF), vascular endothelial growth factor (VEGF), erythropoietin (EPO), GDF-5, transforming growth factor β-3 (TGF- β3), granulocyte colony stimulatory factor (G-CSF), granulocyte-macrophage colony stimulatory factor (GM-CSF), Flt-3 ligand, stem cell factor (SCF), IL-3 receptor agonists, Daniplestim; thrombopoietin agonists, chimeric cytokines, leridistim, progenipoietin-1, peg-filgrastim, SDF-1 antagonists, AMD 3100; and chemotherapeutic agents selected from the group consisting of cyclophosphamide, iphosphamide, carboplatin, etoposide (ICE), etoposide, methylprednisolone, ara-c and cisplatin.

In a related embodiment, performing the ultrasound therapy on the defect using the ultrasound device includes generating an ultrasound frequency of about 10 kHz to about 10 MHz, for example 10 kHz to about 20 kHz; 20 kHz to about 30 kHz; 30 kHz to about 50 kHz; about 50 kHz to about 100 kHz; about 100 kHz to about 250 kHz; about 250 kHz to about 500 kHz; about 500 kHz to about 750 kHz; about 750 kHz to about 2 MHz; about 2 MHz to about 4 MHz; about 4 MHz to about 6 MHz; about 6 MHz to about 8 MHz; or about 8 MHz to about 10 MHz.

In a related embodiment, performing the ultrasound therapy on the defect using the ultrasound device includes generating a low frequency modulating signal of at least about 1 kHz, 2 kHz, about 5 kHz, about 7 kHz, 10 kHz, about 12 kHz, about 14 kHz, about 18 kHz, or about 20 kHz.

In a related embodiment, performing the ultrasound therapy on the defect using the ultrasound device includes generating power at an intensity of at least about 0.1 to 10 milliwatts/cm ^{2}, 20 milliwatts/cm ^{2}, 40 milliwatts/cm ^{2}, 50 milliwatts/cm ^{2}, 60 milliwatts/cm ^{2}, 70 milliwatts/cm ^{2}, 80 milliwatts/cm ^{2}, 90 milliwatts/cm ^{2}, 100 milliwatts/cm ^{2}, 110 milliwatts/cm ^{2}, about 120 milliwatts/cm ^{2}, 130 milliwatts/cm ^{2}, 140 milliwatts/cm ^{2}, or about 150 milliwatts/cm ^{2}.

In a related embodiment, performing the ultrasound therapy on the defect using the ultrasound device includes treating the defect for seconds, minutes or days, for example for at least about: 30 seconds per day to about 2 minutes per day, about 2 minutes per day to about

5 minutes per day, about 5 minutes per day to about 10 minutes per day, about 10 minutes per day to about 20 minutes per day, or about 20 minutes per day to about 40 minutes per day.

In a related embodiment, performing the ultrasound therapy on the defect using the ultrasound device includes regulating a width of each pulse, for example the width of each pulse is about 2 microseconds to about 10 microseconds, 10 microseconds to about 100 microseconds, about 100 microseconds to about 500 microseconds, about 500 microseconds to about 1,000 microseconds, and about 1,000 microseconds to about 2,000 microseconds.

In a related embodiment, the cells and the subject are autologous. In a related embodiment, the cells and the subject are allogeneic. In a related embodiment, the cells and the site of the subject are xenogeneic. In a related embodiment, the cells are not MHC-matched to the recipient subject.

In a related embodiment of the method, contacting the graft to the defect includes at least one route selected from the group consisting of: injecting, infusing, pouring; and spraying. In a related embodiment, contacting the graft to the defect includes using an applicator. In various embodiments of the method, using the applicator includes for example using at least one selected from: a syringe, a sprayer, a sponge, a gel a strip, a tape, a catheter, a hook, a board, a knife, a chisel, a mesher, and a bandage.

An embodiment of the invention provides a kit for treating a subject having a defect, the kit including: a graft including a population of adult stem cells that do not substantially induce a T cell or natural kill (NK) cell-mediated immune response in a transplant recipient; an ultrasound device for stimulating the graft contacted to a defect to form tissue, such that at least one of the graft and the defect comprises at least one of: dermal, epithelial, skin, connective, muscle, fat, neural, and bone; and, instructions for use. In related embodiments, the graft further includes tissue from a donor.

in a related embodiment, adult stem cells include at least one type of cell selected from: early lineage adult (ELA) stem cells, mesenchymal stem cells (MSG), hematopoietic stem cells (HSC), multipotent adult progenitor cells (MAPC), and mesenchymal precursor cells (MPC). For example, the ELA stem cells express Oct-4, Nanog, and Sox9, and do not detectibly express CD34, CD45 and CD90.

In a related embodiment, the method further includes an ELA cell carrier matrix, for example the carrier matrix includes at least one selected from: a gel, a connective tissue that is substantially depleted of living cells, a polymer scaffold, calcium triphosphate, demineralized bone, collagen, hyaluronic acid (HA) and cellulose.

In a related embodiment of the kit, the instructions for use include instructions for stimulating the tissue using the ultrasound device. In a related embodiment, the instructions for use include instructions for treating a subject having a defect.

In a related embodiment, the ultrasound device includes at least one selected from the group consisting of: a transducer, a sensor, a display, a handle, and a housing. For example, the ultrasound device is a handheld device that is positioned over or adjacent to the defect. In a related embodiment, the ultrasound device focuses ultrasound waves to an entire or a portion area of the defect.

In a related embodiment, the ultrasound device includes a pad that contacts the defect or the subject. For example the pad is biocompatible or sterile. In a related embodiment, the pad is coated with composition, for example the pad is coated with antibiotic compositions for enhancing healing of the defect or wound.

In a related embodiment, the ultrasound device includes a fastener for directly securing the device to the defect or the subject.

In a related embodiment, the kit further includes at least one selected from: PRP or another plasma, a buffer, cell culture medium, a preservative, an anti-bacterial agent, an anti-fungal agent, a conditioning agent, a cryogenic agent, a pharmaceutically acceptable salt, a growth factor, a vitamin, a hormone, and a therapeutic agent. For example, the therapeutic agent is an amino acid, a protein, a nucleic acid, a lipid, a chemotherapeutic agent, a lipid, a carbohydrate, or a sugar.

In a related embodiment, the kit further includes at least one selected from: a bone morphogenetic factor selected from the group consisting of: bone morphogeneic proteins BMP-2, BMP-3, BMP-4, BMP-6, and BMP-7; platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), interleukins selected from the group consisting of IL-3, IL-4 and IL-1, insulin-like growth factor- 1 (IGF-1), leukemia inhibitory factor (LIF), vascular endothelial growth factor (VEGF), erythropoietin (EPO), GDF-5, transforming growth factor [3-3 (TGF- β3), granulocyte colony stimulatory factor (G-CSF), granulocyte-macrophage colony stimulatory factor (GM-CSF), Flt-3 ligand, stem cell factor (SCF), IL-3 receptor agonists, Daniplestim; thrombopoietin agonists, chimeric cytokines, leridistim, progenipoietin- 1, peg-filgrastim, SDF-1 antagonists, AMD 3 100; and chemotherapeutic agents selected from the group consisting of cyclophosphamide, iphosphamide, carboplatin, etoposide (ICE), etoposide, methylprednisolone, ara-c and cisplatin.

In related embodiments, the transducer is at least one selected from: a piezoelectric crystal transducer, a PZT (lead zirconium titanate) transducer, an array transducer, a phase transducer, a polyvinylidene fluoride (PVDF) transducer, and a magnetostrictive transducer.

In related embodiments, the device generates an ultrasound frequency of about 10 kHz to about 10 MHz, for example 10 kHz to about 20 kHz; 20 kHz to about 30 kHz; 30 kHz to about 50 kHz; about 50 kHz to about 100 kHz; about 100 kHz to about 250 kHz; about 250 kHz to about 500 kHz; about 500 kHz to about 750 kHz; about 750 kHz to about 2 MHz; about 2 MHz to about 4 MHz; about 4 MHz to about 6 MHz; about 6 MHz to about 8 MHz; or about 8 MHz to about 10 MHz.

In related embodiments, the device in the kit generates a low frequency modulating signal of at least about 1 kHz, 2 kHz, about 5 kHz, about 7 kHz, 10 kHz, about 12 kHz, about 14 kHz, about 18 kHz, or about 20 kHz. In certain embodiments, the low frequency modulating signal is at least about 25 kHz or at least about 30 kHz.

In a related embodiment, the device generates and an intensity of at least about: 0.2 milliwatts/cm^2, 5 milliwatts/cm^2, 7 milliwatts/cm^2, 10 milliwatts/cm^2, 20 milliwatts/cm^2, 40 milliwatts/cm^2, 50 milliwatts/cm^2, 60 milliwatts/cm^2, 70 milliwatts/cm^2, 80 milliwatts/cm^2, 90 milliwatts/cm^2, 100 milliwatts/cm^2, 110 milliwatts/cm^2, about 120 milliwatts/cm^2, 130 milliwatts/cm^2, 140 milliwatts/cm^2, or about 150 milliwatts/cm^2.

Detailed description

Early lineage adult stem cells (ELA) are adult progenitor cells that have extravasational and tissue regenerative properties. ELA stem cells are capable of differentiating into tissues of endodermal, mesodermal and ectodermal origin. The source, isolation, characterization and certain uses and formulations of ELA cells are described in patent applications: U.S. provisional application serial numbers: 60/927,596, 61/247,236, 61/247,242, 61/249,172, and 61/501,846, as well as U.S. patent application serial number 12/598,047 and PCT applications serial numbers PCT/US2008/005742 and PCT/US201 0/050288. Each of these applications is incorporated herein by reference in their entirety.

The ELA cells are described by their expression of stem cell specific pluripotency genes (e.g., Oct-4, KFL-4, Nanog, Sox-2, Rex-1, GDF-3 and Stella). ELA cells are distinct from embryonic stem cells (ESC) and other types of early lineage adult cells such as MSC, VSEL, MAPC and cord blood derived progenitor cells because the ELA stem cells do not

appear to detectibly express the various stem cell markers CD34, CD44, CD45, CD49a, CD66A, CD73, CD90, CD 105, CXCR4, SSEA, or MHC class 1or MHC class II structures, found on other cell types.

Defects are treated by the methods herein using grafts and/or transplants that include synovial fluid derived adult stem cells such as ELA stem cells. These are first isolated and purified from synovial fluid to remove inhibitory factors and undesirable cells (e.g., immunoreactive and immunogenic cells). Typically, the stem cells are used at an application number of about 10^2 to about 10^7 cells in volumes of from about 0.5 ml to about 1.0 ml, to about 5 ml, or to about 10 ml. The volume and cell number depends on the defect size, and the use of exogenous materials such as scaffolds and growth and differentiation factors. Growth and differentiation factors are provided by the use of a medium conditioned ELA cellular cytokine suspension (or ECCS) by growth of ELA cells, lysates from devitalized ELA cells and ELA cell derived products from primary, expanded or transgene expressing ELA cells. Methods of expressing transgene products from transfected ELA cells are described in PCT application serial number PCT/US2008/005742. These ELA cell extracts and isolates are used from about 0.01 , 0.1, 1% to 10% w/v.

A dermal filler, such as injectable fillers comprised of hyaluronic acid (with Restylane being a specific example of a non-animal sourced product) is combinable with synovial fluid derived fresh or expanded stem cells, such as 10^2 to 10^7 stem cells in volumes of from 0.25 ml to about 1 ml containing from about 5%-95% dermal filler by volume. Such preparations are useful for lip enhancement (volume and contouring), to diminish wrinkles and aging lines of the face such as the nasolabial folds (nose to mouth lines), melomental folds (sad mouth corners), "crow's feet" and forehead wrinkles (frown lines), for filling aging-related facial hollows and "orbital troughs" (under and around the eyes), for enhancing cheek volume and contouring of the chin, forehead and nose, and to revitalize the skin by increasing skin elasticity structure, with or without adding substantial volume. The dosage of the synovial fluid derived stem cells is increased relative to the volume of dermal filler in this regard.

The grafts and/or grafts including ELA stem cells are further treated with various types of stimuli in various embodiments to further promote or enhance tissue formation and wound healing. For example the treated defect is stimulated with vibrational energy, biochemical agents, or by physical manipulation of the defect site or body. The vibrational energy may be ultrasonic energy, for example produced by transducers such as a piezoelectric crystal, a PZT (lead zirconium titanate) or other piezoelectric transducer or transducer array,

or a magnetostrictive transducer operating at the desired power and vibrating at ultrasonic frequency, and configured to couple ultrasound into the affected tissue, or into a nearby tissue structure (such as bone or cartilage) to stimulate the targeted tissue. The transducer may be a sheet transducer, a button transducer, or a combination thereof, and may be operated to apply local vibration, or to focus its energy at a depth through the surface tissue. The application of ultrasonic energy may stimulate cells or cellular components, may locally apply or enhance contact or activity between the cells and nearby material, or otherwise stimulate the microstructure of the target tissue or its neighbors, without causing gross physical movement oflayers that could disrupt the processes of tissue anchoring, growth, or healing processes. In particular, the application of ultrasonic energy enhances the release of, or the rate of diffusion of cytokines, proteins and other cell signaling molecules that are instrumental in development and differentiation of the ELA cells, thus modulating the interaction of the ELA cells with the surrounding or adjacent tissues. The exogenous application of ultrasonic vibration to target biological tissue that itself possesses piezoelectric properties (such as bone), stimulates the targeted tissue to develop a locally charged surface, which is a fact in the mechanism of local bone or tissue growth.

Methods and apparati for treating wounds include using ultrasound waves are shown in Huckle et al. U.S. patent number 7,789,841 issued September 7, 2010, which is incorporated by reference in its entirety. Ultrasound stimulates the ELA stem cells by altering expression of genes or proteins (e.g., growth factors) and enhances dermal healing processes. Thus, ultrasound therapy as described herein refers to a non-invasive application of ultrasonic energy to a skin defect or area or tissue in a subject, to enhance and/or accelerate wound healing and dermal graft incorporation. The therapeutic ultrasound therapy in certain embodiments includes applying ultrasonic pulses using an ultrasonic device placed on or adjacent to a tissue defect, e.g., skin, bone, and cartilage. See Duarte U.S. patent number 7, 628,764 issued December 8, 2009, and may include special configuration known, for example from industrial nondestructive testing or monitoring technology for optimally coupling, converting shear or compression waves, and for aiming, concentrating or scanning the coupled energy.

Ultrasound devices and systems in embodiments of devices and methods herein include an ultrasound transducer that generates sound waves in an ultrasonic range having a frequency greater than about 10 kHz near the upper limit of human hearing using electrical drive signals of appropriate energy to generate the sound. The ultrasound waves generated

have a frequency for example greater than about 18,000 hertz (Hz) to about 20,000 Hz. Greater frequencies generated by radio frequencies (RF) are used to drive signals in embodiments for generating the ultrasound and ultrasonic power density levels. The duration of each ultrasonic pulse, ultrasonic pulse frequencies, and duration of daily treatment may differ at stages of graft application and healing, and are varied according to embodiments of the method. See Duarte U.S. patent number 7,628,764.

The frequency of the ultrasound is for example about 20 kilohertz (kHz) to about 30 kHz; 30 kHz to about 50 kHz; about 50 kHz to about 100 kHz; about 100 kHz to about 250 kHz; about 250 kHz to about 500 kHz; about 500 kHz to about 750 kHz; about 750 kHz to about 1 megahertz (MHz; $1x10^6$ hertz); about 1 MHz to about 5 MHz; about 5 MHz to about 10 MFlz. For example the frequency is about 1.3MHz to 2 MHz. The width of each pulse is for example about 2 microseconds to about 10 microseconds, 10 microseconds to about 100 microseconds, about 100 microseconds to about 500 microseconds, about 500 microseconds to about 1,000 microseconds, and about 1,000 microseconds to about 2,000 microseconds. The pulse repetition rate is for example about 5 Hz to about 100 Hz; about 100 Hz to about 250 Hz; about 250 Hz to about 500 Hz; about 500 Hz to about 1,000 Hz; about 1,000 Hz to about 2,000 Hz; about 2,000 Hz to about 5,000 Hz; about 5,000 Hz to about 7,5000 Hz; or about 7,500 Hz to about 10,000 Hz.

The power level of the ultrasound is maintained for example less than: about 150 milliwatts per square centimeter (milliwatts/cm^2), about 125 milliwatts/cm^2, about 100 milliwatts/cm^2, about 90 milliwatts/cm^2, about 80 milliwatts/cm^2, about 70 milliwatts/cm^2, about 60 milliwatts/cm^2, about 50 milliwatts/cm^2, about 40 milliwatts/cm^2, about 30 milliwatts/cm^2, about 20 milliwatts/cm^2, about 10 milliwatts/cm^2, or about 5 milliwatts/cm^2. See Duarte U.S. patent number 4,530,360 issued July 23, 1985 and Heckman et al. 1994 JBJS 76 (1): 26-34. Greater power levels of the ultrasound may be applied as appropriate for certain treatment modes.

The ultrasonic therapy in certain embodiments includes an ultrasound delivery system in which both an RF generator and transducer are part of a modular ultrasound device that is placed at the location of the skin defect. Talish et al. U.S. patent numbers 5,003,965 issued April 2, 1991 and 5,186,162 issued February 16, 1993. The signals controlling the duration of ultrasonic pulses and the pulse repetition frequency in certain embodiments are controlled by the user who operates the ultrasound device, and the device may include a simple controller and appropriate signal generation circuitry for setting ultrasound frequency, pulse duration,

power level, pulse repetition rate and other parameters of a treatment regimen. A fixing apparatus (e.g., a strap, a band, a fastener, or sleeve) may be provided to attach the ultrasound device to a skin location such as the dermal graft applied to the skin defect, or an area of healthy skin. The ultrasound device is mounted on an uncovered skin area, alternatively the ultrasound device is attached to a dressing covering the skin, including a cast, or a pad through which the signal may be propagated. See Talish et al. U.S. patent number 5,21 1,160 issued May 18, 1993. The ultrasound device includes a hand-held device or portable device for localized treatment of the skin defect. See Babaev U.S. patent publication number 20080051693 filed August 31, 2007. The ultrasound device may include a transducer housing that contains the ultrasound transducer and/or RF generator, or the transducer may be integrally fabricated in the device, for example in a PZT circuit-board type construction.

The methods of ultrasound therapy herein are performed using ultrasound devices including in various embodiments: ultrasound generators, applicators, and/or transducers. See Duarte et al. U.S. patent number 7.628,764 issued December 8, 2009. For example, a low intensity pulsed ultrasound device (LIPUS) is applied to or adjacent to a skin defect to stimulate wound healing incorporation of the dermal graft with ELA cells. See El-Bialy et al. U.S. patent publication 2008/0021327 filed May 10, 2007. For example, the LIPUS device is applied at about 0.75-1.5 MHz frequency pulses, with a pulse width of about 50-550 microseconds (μs), repeated at about 0.5-1.5 kHz, at an intensity of about 15-100 mW/cm^2. Further the LIPUS device therapy is applied at least: about 5 minutes/day, about 10 minutes/day , about 20 minutes/day, about 30 minutes/day, about 45 minutes/day. about 1 hour/day, about 1.5 hours/day, about 2 hours/day, or about 3 hours/day. The ultrasound therapy is used for cavitational ultrasound wound cleaning, debridement of wounds, removal of dead tissue, and for maintenance or for preparation of wounds for other dermal grafts. Ultrasound devices and therapies are commercially available, for example ultrasound systems and devices include: Ultrasound Bone Healing System by Exogen Inc. (Piscataway, NJ); Qoustic Wound Therapy System™ by Arabella Medical, LLC (Minnetonka, MN); Sonoca 180 system by Soring Inc. (North Richland Hills, TX); and MIST Therapy® System by Celleration, Inc. (Eden Prairie, MN). The Qoustic Wound Therapy System™ for example includes a sterile saline solution that directs ultrasound energy to the wound producing tiny vibrating gas bubbles that separate dead and diseased skin from the healthy tissue. A high energy cavitation mode may be provided to produce short bursts during a cleaning, disruption or stimulation phase of the healing process.

Methods of treating a surface wound or skin defect in embodiments of the invention herein include placing a packing or dressing, for example of a conventional material such as cotton, gauze, or other bandage material directly in contact with the subject's wound or defect. The defect or wound is treated further with an ultrasound device or apparatus that applies an ultrasonic energy through the packing to promote healing. Alternatively, the pad is manufactured from a bioresorbable or biocompatible material. One or more additional therapeutic agents may be added to the packing for example an anti-fungal agent, an anti-bacterial agent, an anti-viral agent, a growth factor, a sugar such as a polymer for example hyaluronan, a hemostatic agent, an antioxidant, or a nitric oxide promoter.

The ultrasound device in various embodiments of the method and system includes the contact pad which can be disposable or can be sterilized and re-used. Alternatively, other embodiments of the device include an ultrasound source or a connector to an ultrasound device, so that the contact pad or dressing is connected to a specific source of the ultrasound. The ultrasound source for example is a device that generates ultrasonic energy at the defect site, the ultrasonic energy being distributed by a connector to the contact pad or dressing on the skin defect. The pores or channels of the contact pad uniformly distribute the ultrasonic energy in a distributed array over the skin defect. The contact pad contains a perimeter area with an adhesive or sealant that seals the pad to the skin defect, particularly to an area of the skin surrounding the site of the skin defect, such that the surrounding area is healthy tissue. Pads are manufactured in geometrical shapes suitable to conveniently contact a skin defect of a variety of sizes and shapes, and thus the methods are not limited by size or shape.

Without being limited by any particular theory or mechanism of action, it is here envisioned that ultrasound therapy after ELA stem cells dermal graft transplantation to a skin defect promotes integrity of dermis and epithelium layers, enhances cell-cell migration, and integration of the dermal graft having ELA stem cells to underlying or surrounding tissues. The ultrasound therapy enhances the natural processes of wound healing including the acute inflammatory phase with the deposition of provisional tissue, re-epithelialization, collagen synthesis and deposition, fibroblast proliferation, and neovascularization. Further, the ultrasound therapy enhances anchoring and differentiation of dermal grafts having ELA stem cells, and interaction of the graft and the cells with surrounding tissue environments to aid healing.

One of ordinary skill in the art of wound healing knows how to adjust an amount of ultrasound therapy (e.g., duration and frequency) to effectively treat skin defects described

herein. Methods are adjusted to treat patients and subjects according to factors for example such as weight, age, and condition. Ultrasound therapy is administered for example hours or days after a dermal graft to allow the graft to begin to incorporate into the skin defect, for example, so that cells and tissues in the graft adjust to ambient conditions of the skin defect, then are exposed to ultrasound to improve conditions for the cells and tissues. Alternatively in other embodiments, ultrasound is applied within minutes or hours of application of the graft.

The ultrasound methods herein include, prior to applying the ultrasound, using a disposable handheld device for harvesting dermal tissue. For example the cutting device includes a housing or frame, a horizontally extending handle, and cutting component or blades located within the housing, and/or slits in the housing for the cutting components to protrude from the bottom of the housing and cut the donor tissue. The cutting components or blades perform a first cut in one direction and make a second cut at a 90° angle to the first cut. See Seegert et al. U.S. patent number 7,666,192 issued February 23, 2010.

Tissue grafts, transplants and treatments ELA stem cells described herein are administered to repair a tissue defect by improving function and appearance of a tissue having a defect. The defect in various embodiments is a naturally occurring condition in the tissue for example a condition caused by aging or maturation, or is an irregularity or condition that arose in the tissue because of an accident, injury, disease or surgery. The defect in various embodiments is a malformation, a birthmark, a wrinkle, a discoloration, an excision, a surgery incision scar or mark, a burn, a burn scar, a cut, or a depression. In various embodiments the defect includes one tissue (e.g., skin, muscle, cartilage, bone and fat) or a plurality of tissues.

Without being limited by a particular theory or mechanism, it is here envisioned that the adult stem cells described herein promote wound healing and tissue formation (e.g., skin, muscle bone, nerve, fat, bone, and cartilage), thereby improving the function and/or physical appearance and characteristics of the tissue. For example, the adult stem cells are used prior to, during or after a surgery or treatment of a tissue or organ (e.g., skin, breast, legs, stomach etc.) to promote tissue formation and/or to improve the aesthetic or appearance of the subject such that appearance of the tissue after healing is improved in comparison to the tissue prior to the surgery or the treatment. For example the ELA stem cells are applied to a skin burn to re-epithelialize a tissue to form an uniform skin surface that functions as a barrier and/or to improve the appearance of the skin tissue. Adult stem cells are administered for example to

promote functional strength or integrity in the tissue or organ and also to impart and maintain a regular uninterrupted physical appearance. It is here envisioned that grafts and transplants that include ELA stem cells described herein differentiate into tissues or organs that mesh with and match the structural and functional characteristics of the natural tissue or organ of the subject. These matched characteristics are not currently achieved by grafts and transplants alone because of widespread occurrence of variations in the proteins that control and result in phenotypes, for example color and texture of skin and hair or size or proportions of muscles or bone.

The compositions, methods and kits include adult stem cells described herein to treat defects and/or wounds caused by disease, injury, surgery, and natural aging and maturation etc. The ELA stem cells herein are a component of the various embodiments of transplants, grafts and compositions (e.g., injections and patches) to promote wound healing including re-epithelialization and improved physical appearance and aesthetic qualities of a tissue defect in a subject. Examples of skin defects include an ulcer, acne vulgaris, actinic keratosis, and a sarcoma. Acne vulgaris, referred to commonly as acne, is characterized by inflammation and infection of a sebaceous gland of the skin. Methods of treating acne include applying creams, lotions, masks, or washes to the affected skin to clean clogged pores or glands, inhibit growth of bacteria, moisturize, and to reduce inflammation and irritation (Bernstein U.S. patent number 7,371,367 issued May 13, 2008). Acne treatments include microdermabrasion and exfoliation procedures (e.g., peels) using an alpha-hydroxy acid such as glycolic acid to remove surface layers of skin (Sefton U.S. patent number 6,262,117 issued July 1, 2001; Perricone U.S. patent number 6,743,433 issued June 1, 2004; Ashely U.S. patent number 7,014,858 issued March 21, 2006; and Voorhees et al. U.S. patent number 7,268,148 issued September 11, 2007, each of which is incorporated herein by reference in its entirety).

Re-epithelialization of wounds is an essential mechanism in treating skin conditions associated with age, disease and infection. Repair of mammalian tissues involves an orderly, controlled cellular response involving an initial acute inflammatory phase, followed by re-epithelialization, collagen synthesis and deposition, fibroblast proliferation, and neovascularization (Panjwani U.S. patent number 9,697,021 issued November 22, 2005 which is incorporated herein by reference in its entirety). The failure of epithelial cells to migrate over a wound surface and then to remain adherent to the substratum are one of the major causes of debilitating clinical conditions such as persistent epithelial defects, e.g., acne,

blisters, ulcers, and erosions (Cell Adhesion and Human Disease Ed. by Marsh et al., Ciba Foundation Symposium, Vol. 189, John Wiley & Sons, New York, NY, 1995).

Without being limited by a particular theory or mechanism of action, it is here envisioned that grafts or transplants containing adult stem cells are used to treat a skin tissue of a subject, such that the tissue is enhanced both functionally and aesthetically. For example an ELA stem cell graft applied to a burn injury is effective to re-epithelialize the skin defect and replace damaged or missing skin, and also to provide a more natural matching appearance of grafted skin relative to the surrounding undamaged skin. In another embodiment, the adult stem cells are contacted (e.g., by injection or topical application) to an area of the neck, back, and/or face having acne to improve the appearance of the skin, or after a surgical procedure to improve healing and appearance of the skin. For example the surgery is an abdominoplasty, a tissue lift (e.g., arm, brow, face, and breast), an eyelid surgery, a botox treatment, or a breast surgery such as a breast augmentation or reduction.

The adult stem cells described herein are pluripotent cells that are used to treat and repair various types of defects (e.g., muscle, bone, cartilage and skin) and thus improve or alter the functional or physical characteristics of the defect and surrounding tissues. For example adult stem cells are administered to a subject to promote muscle tissue formation, for example in a cachexia patient, and thus effectively improve the strength and integrity of a tissue or organ and also promote supporting the overlying tissue and resulting in a smooth and unwrinkled skin having for example a more youthful appearance. In another embodiment, the adult stem cell grafts are used to promote fat, cartilage or bone tissue formation, and also to promote a natural appearance to the tissues surrounding, underlying or overlying the grafts. In various embodiments the adult stem cells are administered before, during or after a surgical procedure such as a fat grafting to the face or neck (Fraser et al. U.S. patent number 7,887,795 issued February 15, 2011), or auricular cartilage grafting to the nose (Pavesio et al. U.S. patent number 7,968,111 issued June 28, 2011; Zhang et al. U.S. patent number 7,842,669 issued November 30, 2010; and Hart et al. U.S. patent number 7,491,384 issued February 17, 2009), or bone grafting to the mouth and/or extremities (Knaack et al. U.S. patent number 7,959,941 issued June 14, 2011; and Moseley et al. U.S. patent number 7,754,246 issued July 13, 2010, each of which is incorporated herein by reference in its entirety). Methods involving administering a graft including ELA stem cells are effective to repair tissue irregularities in the subject and also to improve the appearance

and physical characteristics of the contacted area or defect, and also to tissues adjacent, contiguous or nearby to the stem cells.

The stem cells or a tissue adjacent to the stem cells in various embodiments are stimulated to promote differentiation and tissue formation of the stem cells. The stimulus used to promote stem cell differentiation include chemical, cellular and external, for example the stimulus includes contacting the stem cells or adjacent tissue with genetic material, protein, drug, pharmaceutical composition, carbohydrate, or a sugar. In various embodiments, the stimulus is pressure modulation therapy, heat, tissue manipulation, or ultrasound therapy as described herein. Ultrasound therapy for example stimulates healing of the skin defect by increasing blood flow to the site of the defect and/or graft.

Preparation of grafts

Grafts are provided herein for treating a wide range of injuries or defects (e.g., skin, dermal, muscle, bone, and fat) including skin and/or dermal defects with split-thickness dermal grafts, full thickness dermal grafts, or composite dermal grafts. Split-thickness dermal grafts contain epidermis and an amount of dermis. Full thickness grafts contain epidermis and the entire dermis. Split-thickness dermal grafts and full thickness grafts are collected from an area of the body having healthy skin (i.e., lacking a skin defect). Dermal grafts are obtained or harvested for example from tissue of the head including the face, the leg including foot, shin, or calf, thigh, chest, abdomen, back, shoulder, or arm. Composite grafts contain portions of skin or dermal tissue and at least one other type of tissue such as bone, fat, cartilage, neural, and muscle. Composite grafts that include cartilage are collected for example from the ear or nose. The tissues thus collected for these types of dermal grafts are contacted with the ELA stem cells as described herein and the grafts are applied to a skin defect site to stimulate tissue formation without inducing an immune response.

Methods for collecting the donor tissues involve for example using cutting devices such as dermatomes and other skin harvesting devices. A cutting device such as a dermatome removes sections of tissue from a donor site and then a different device minces or cuts the tissue into micron sized pieces referred to as "micrografts". Alternatively, the cutting device cuts the tissue into micrografts directly from the donor site. Devices and methods for collecting skin grafts and applying the skin as grafts to a skin defect are shown in VanBeek et al. U.S. patent number 5,21 1,644 issued May 18, 1993; Ysebaert et al. U.S. patent number 6,248,1 11 B1 issued January 19, 2001; Mishra et al. U.S. patent application 2007/01 84032 published August 9, 2007; Mishra et al. U.S. patent number 7,651,507 B2 issued January 26,

2010; Seegert et al. U.S. patent number 7,666,192 B2 issued February 23, 2010; and Eriksson et al. U.S. patent number 7,708,746 B2 issued May 4, 2010, each of which is incorporated herein by reference in its entirety.

Dermatomes and cutting devices for cutting skin are commercially available from Zimmer Inc. (Warsaw, IN; Product number 00-8801-000-00) and Robbins Instrumental Inc. (Chatham, NJ; model 16-1992). Cutting devices in certain embodiments include suction pumps and filters or reservoirs for collecting the excised skin. Methods herein include using enclosed devices having cutting components and adapters that vary the size of the donor tissue collected and micrografts produced. For example, the size of the harvested tissue produced into micrografts includes about 50 micrometers to about 1500 micrometers, or about 100 micrometers to about 2000 micrometers in size. In alternative embodiments, the dermal tissue and/or micrografts are collected within the devices in a chamber or reservoir or are directed to a separate container outside the device.

The dermal tissue or skin tissue is contacted by methods herein to ELA stem cells to form a dermal graft. The graft material is prepared within the cutting device, for example the cutting device is used to excise skin tissues and includes a filter or reservoir to collect the cut or excised pieces of tissue. The skin tissues are then contacted with adult stem cells such as ELA stem cells, MSC cells or MAPC cells in the reservoir to form the dermal graft. Alternatively, the skin tissue and adult stem cells are contacted on the skin defect or in a tube, receptacle, or container and then applied to the skin defect. Alternatively, the dermal graft is applied to the skin defect and then ELA cells are contacted to donor tissue previously applied to the skin defect.

The dermal tissues before or after addition of ELA cells in certain embodiments are treated by a process referred to as graft meshing. This technique is designed to incise and "mesh" a piece of skin to expand the graft to cover a larger area than that of the donor site. See Rosenberg U.S. patent number 6,063,094 issued May 16, 2000. For example a 4cm^2 dermal tissue is meshed and is expanded to cover a skin defect site having a size of 6 cm^2, 8 cm^2, 10 cm^2, 12 cm^2, 16 cm^2, 20 cm^2, or 24 cm^2. A donor graft is expanded or meshed to an extent having a range of ratios of harvested skin to defect site of about 1.5:1 to about 3:1, about 3:1 to about 5:1, about 5:1 to about 10:1, or about 10:1 to about 12:1.

Methods and devices for collecting and applying dermal grafts are shown in Mishra et al. U.S. patent number 7,651,507 B2 issued January 26, 2010 and U.S. patent application number 2007/0184032 published August 9, 2007; and Seegert et al. U.S. patent number

7,666,192 issued February 23, 2010. Methods of meshing grafts can decrease the rate of dermal formation and re-epithelialization and result in an unnatural appearance to the resulting skin, referred to as "crocodile skin" or "checkerboard skin". Examples herein show-that meshed ELA stem cells-dermal grafts differentiate into dermal tissue and stimulate tissue formation in the skin defect without resulting in an unnatural appearance. Methods herein using dermal grafts with ELA stem cells are effective for cosmetically treating dermal skin defect by reducing extent of scarring in skin grown at the defect site.

The dermal graft with ELA stem cells described herein is in certain embodiments activated prior or after graft transplantation to induce and expedite differentiation into skin (e.g., dermis, and epithelia) by factors, including mechanical, cellular, and biochemical stimuli.

Examples herein include methods for preparing and applying effective dermal grafts to defects including underlying skeletal disorders and/or other connective tissue disorders. Methods and composite dermal grafts herein are used for treating skin defects and defects of underlying support and skeletal and connective tissues including bone, cartilage, tendon, ligament, muscle, adipose, and marrow stroma.

The amount or dose of the autologous or allogeneic ELA stem cells in the dermal graft is adapted by one skilled in the art of transplantation based on the specifics and needs of the recipient subject. For example, the amount of ELA stem cells contacted to donor tissue to form the dermal graft is based on the size of the donor tissue and the size of the skin defect, the weight and condition of the recipient, and other variables known to those of skill in the art, e.g., depth and severity of the skin defect.

The number of synovial fluid derived stem cells contacted to the donor tissue to form the dermal graft is for example at least about 100 stem cells to about 500 stem cells, about 500 stem cells to about 1,000 stem cells; about 1,000 stem cells to about 10,000 stem cells; about 10,000 stem cells to about 75,000 stem cells; about 75,000 stem cells to 150,000 stem cells; about 150,000 stem cells to about 1,000,000 stem cells; about 1,000,000 stem cells to about 5,000,000 stem cells, or at least about 5,000,000 stem cells to about 10,000,000 stem cells, or at least about 50,000,000 stem cells to about 100,000,000 stem cells, depending on size of the graft and other activities.

The dermal graft containing the ELA stem cells is administered by a route that is suitable for the particular tissue or organ to be treated. In general, the graft is topically administered directly to the entire dermal site having the defect or injury, or are administered

to a section or portion of the site. For example the particular dermal site having the defect or injury is located on a portion of the body such as head, neck, hand, arm, face, ear, shoulder, chest, abdomen, knee, thigh, back, vagina, shin, calf, foot etc. The dermal grafts in other embodiments are administered for example by surgical implant or by injection, such as implantation of ELA stem cells directly into tissue underlying the skin defect or into surrounding, underlying, adjoining cells, e.g., subcutaneous skin cells, fat cells, and muscle cells.

Dermal grafts herein having ELA stem cells for application to skin defects are treated in various examples herein with various other types of stimuli to promote tissue formation and wound healing. For example the skin defect with dermal graft is stimulated with vibrational energy, biochemical agents, or physical manipulation of the defect site or body. Methods and apparati for treating wounds using ultrasound waves are shown for example in Huckle et al. U.S. patent number 7,789,841 issued September 7, 2010, which is incorporated by reference in its entirety.

Stem cells **suitable** for treating defects using **ultrasound** therapy

Stem cells are distinguished by ability to differentiate into different types of cells and tissues. A totipotent or pluripotent hematopoietic stem cell for example generates different types of blood cells and is therefore of early lineage, in contrast to leukopoietic and erythropoietic stem cells that are more differentiated and are restricted to generating white blood cells and red blood cells, respectively. Zygotes are totipotent cells that are capable of generating all tissues of an adult organism, and early embryonic stages of development contain cells that retain pluripotency or totipotency. While embryonic stem cells are typically totipotent, these cells are not suitable candidates for therapeutic use because bio-ethical questions arise concerning creating embryos and recent data indicating that these cells may be tumorigenic.

Earlier theories concerning stem cells stated a number of incorrect conclusions including that: adult organisms contain only fully committed cells specialized for each tissue, mitotic replication generates only similarly differentiated cells; only embryos contain totipotent and pluripotent cells. In fact researchers have discovered another source of totipotent or pluripotent cells beyond those found in embryos, specifically adult stem cells.

Numerous embryonic cell genetic markers associated with adult stem cells are transcription factors, for example Oct-4, Nanog, Sox-2, Rex-1, GDF-3 and Stella. Adult stem cell surface proteins markers include for example CD13 which encodes an aminopeptidase N

and is a marker of mesenchymal stem cells; CD34 which encodes sialomucin transmembrane protein (adhesion molecule) and is a hematopoietic marker; CD44 which encodes hyaluronic acid-binding receptor and is a marker of mesenchymal stem cells and hematopoietic progenitor cells; CD45 which encodes protein tyrosine phosphatase receptor type C and is a hematopoietic marker; CD73 which encodes ecto-5'-nucleotidase and is a T and B cell marker; CD90 which encodes Thy-1 and is a mononuclear stem cells marker; and CD105 encodes endoglin and is a mesenchymal stem cell and endothelial marker.

Adult stem cells are pluripotent and regenerate a variety of tissues and express embryonic markers. Very small embryonic-like (VSEL) stem cells are a type of adult stem cells that are isolated from bone marrow, and express CD34 (Ratajczak, MS et al. 2008 Stem Cell Rev 4:89-99; Kucia, M. et al. 2005 Leukemia 19(7): l 118-1 127). MAPC ceils are isolated from bone marrow express CD13 and CD90 (Zeng et al. 2006 Stem Cells 24(1 1): 2355-66; Jiang et al. 2002 Nature 418(6893): 41-9; and Aranguren et al. 2007 Blood 109(6): 2634-2642). Muscle stem cells express CD34 (Wada, M. et al. 2002 Dev 129: 2987-2995; Fukada, S. et al. 2007 Stem Cells 25(10): 2448-2459). Certain adult stem cells express major histocompatibility antigens, for example, AFS cells isolated from amnion express MHC class I and MHC class II proteins, as well as CD44, CD73, CD90, and CD 105. See De Coppi et al. 2007 Nat. Biotechnol 25(1): 100-1 06.

Effective comparison of stem cell data is problematic because researchers use different criteria to identify and name stem cells. Ambiguities arise from the phrase "multipotent mesenchymal stromal cells" or MSC, specifically as various MSG stem cells express clearly distinctive proteins (Dominici ct al. 2006 Cyotherapy 8(4): 315-317). Minimal standards for referring to MSC have been proposed including that the stem cells be evaluated for expression of CD 105, CD73 and CD90, and lack of expression of CD45, CD34, CD 14 or CD *lib,* CD79a or CD 19 and HLA-DR surface molecules.

MSC express surface proteins that induce graft rejection in a recipient subject, as these markers are immunogenic antigens. Thus, there is a clear need for adult stem cells of an early lineage that are pluripotent and that express few or no immunogenic determinants and few or no tissue antigen markers.

Examples herein describe methods of making and using grafts and systems, and kits that include a population of early lineage adult (ELA) stem cells, which are pluripotent, and which express few immunogenic determinants or tissue antigen markers. For example, the adult stem cells express at least one of Oct-4, Nanog, and Sox9, and do not detectibly express

CD34, CD45 and CD90. Grafts or transplants containing these adult stem cells do not substantially induce T cell or NK cell mediated immune response in a graft recipient. The dermal grafts with adult stem cells herein differentiate into a dermal tissue, and methods herein are suitable for making adult stem cell-dermal grafts that differentiate into other tissue types, including glandular, connective, muscle, neural, or bone tissue.

Without being limited by any particular theory or mechanism of action, it is here envisioned that the ELA stem cells described herein are "invisible" to the immune system. Normally, co-culturing cells from different individuals (allogeneic cells) results in T cell proliferation, manifested as a mixed lymphocyte reaction (MLR). However, when human ELA stem cells are contacted with allogeneic T lymphocytes, *in vitro*, they do not generate an immune response by the T cells. Thus, the T cells are not responsive to allogeneic ELA stem cells. The ELA stem cells described herein actively reduce the allogeneic T cell response in mixed lymphocyte reactions in a dose dependent manner. Data show that ELA stem cells also attenuate NK cell killing. Examples herein show that grafts having ELA stem cells do not have be MHC matched to a target cell population. The ELA stem cells in grafts reduce NK cell mediated cytotoxicity and the proliferative response of alloreactive T cells.

The ELA stem cells, and cell populations that include these adult stem cells and that are derived from or expanded from ELA cells into a population, are initially isolated from synovial fluid, blood, bone marrow and other tissues in the fetus, newborn, child or adult body. These cells are embryonic in character and prior to culture do not present the surface markers generally associated with other adult stem cells, even after days in culture. More specifically, the ELA stem cells do not express the surface markers generally associated with other adult stem cells, including but not limited to the mesenchymal stem cell (MSC), multipotent adult stem cells (MASC) and very small embryonic like stem cell (VSEL). Multipotent adult progenitor cells (MAPC) for example express CD13 and Rexl (Jiang 2002 Nature 418(6893): 41-49; and Zeng 2006 Stem Cells 24(11): 2355-2366).

ELA stem cells also express key embryonic transcription factors within a few days of isolation, in contrast to other adult stem cells, which require longer periods of culture before such expression. Further, these cells can differentiate into at least two of the three germ layers (mesoderm, ectoderm, and endoderm) and do not form teratoma bodies in vivo. This discovery allows for a non-controversial supply of easily attainable embryonic-like adult stem cells.

The ELA stem cell is a quiescent adult stem cell having embryonic stem cell characteristics and the capacity to differentiate into at least two of ectoderm, mesoderm and endoderm, and having low immunogenic potential, as it does not express cell surface markers including MHC class I, MHC class II, CD44, CD45, CD34, CD49c, CD73, CD66b, CD105 and CD90.

The quiescent ELA stem cell is in the resting phase of the cell cycle, GapO (GO), and therefore, may not exhibit proliferative characteristics, such as expression of the transcription factor Oct-4. This stem cell can be found in bodily fluids such as blood, bone marrow and synovial fluid or tissue. The blood may be mobilized or non-mobilized blood.

Upon activation, the quiescent stem cell becomes proliferative, and expresses genes including Oct-4 (Lau et al. 2006 Adv. Anat. Pathol. 13:76-79), Nanog (Pan et al. 2005 J. Biol. Chem. 280: 1401-1407), Sox2 (Lee et al. 2006 Cell 125:301-313), GDF3 (Hexige et al. 2005 Neurosci. Lett. 389: 83-87), P16INK4 (Gray-Schopfer et al. 2006 Br. J. Cancer; 95:496-505), BMI (Itahana, K. 2003 Mol. Cell. Biol. 23:389-401), Notch (Chiang et al. 2006 Mol. Cell. Biol.; 26:6261-6271), HDAC4 (Zeremski et al. 2003 Genesis 35: 31-38), TERT (Middleman et al. 2006 Mol. Cell. Biol. 26:2146-2159), Rex-1 (Zhang et al. 2006 Stem Cells; 24:2669-2676), and TWIST (Guenou et al. 2005 Hum. Mol. Genet. 14:1429-1439), and retains low immunogenic potential, as it does not express cell surface markers including MHC class I, MHC class II, CD44, CD45, CD34, CD49c, CD66b, CD73, CD105 and CD90. The size of the cell has been observed to include about two microns to about six microns, about six microns to about 15 micros, or about 15 μη to about 20 μη in size.

The proliferative ELA stem cells are uniquely suited for large scale use. The stem cells are proliferative after less than about ten days in culture, e.g., about one day, three days, or seven days in culture, and do not require expansion to achieve an activated and/or proliferative state. From a population of about 500,000-20,000,000 mobilized cells, about 5% to about 30% to about 90% proliferative stem cells are obtained without expansion in vitro.

The ELA stem cells are multipotent and differentiate into a cell lineage of at least two of the three germ layers (e.g., ectoderm, mesoderm, and endoderm) and do not form teratomas in vivo. Upon further differentiation, the stem cells upon contact with appropriate stimulus differentiate into cell types including without limitation neurons, chondroblasts, osteoblasts, adipocytes, hepatocytes, smooth muscle cells, skeletal muscle cells, cardiac cells, pancreatic cells, pulmonary cells, and endothelial cells. Methods for the differentiation of

stem cells include culturing the stem cells in the presence of differentiation-specific agents, which promote lineage commitment. Differentiation-specific agents and conditions include, for example, PDGF (e.g., about 10 ng/ml) and TGF-βI (e.g., about 5 ng/ml) for the formation of muscle cells; bFGF (e.g., aboutlOO ng/ml), FGF-8 (e.g., about 10 ng/mL), SHH (e.g., about 100 ng/ml) and BDNF (e.g., about 10 ng/ml) for the formation of neurons; hepatocyte growth factor (HGF) and FGF-4 for the formation of hepatocytes; dexamethasone, 3-isobutyl-l-methylxanthine (IBMX), insulin and indomethacin for the formation of adipocytes; VEGF (e.g., about 100 ng/ml of VEGF-165) for the formation of endothelial cells; and any of bone morphogenic proteins , 2, 4, 6, 7 (BMP2, BMP4, BMP6, BMP7) (e.g., about at least or at most 10 ng/ml), VEGF, bFGF, stem cell factor (SCF), Flt3L, hyper IL6, thrombopoietin (TPO), and erythropoietin (EPO) for the formation of hematopoietic cells.

The ELA stem cells under appropriate conditions form embryoid bodies (e.g., colony forming units) within two days, four days, five days, seven days or ten days in culture. The cells forming the embryoid bodies, express KLF-4 or Myc and embryonic transcription factors described herein. Culture conditions for embryoid body formation are described in Crawford et al. U.S. patent publication number 20]1/0070205 filed September 24, 2010.

The terms "transplant" and "graft" as used herein refer to tissue or organ transplanted from a donor to a recipient. Transplants or grafts described herein include dermal donor tissue and ELA stem cells. The ELA stem cells are obtained and purified from bodily fluids, such as synovial fluid or peripheral blood, or from tissues, such as bone marrow. Methods and systems for isolating and purifying ELA stem cell populations, e.g., through centrifugation of synovial fluid, described herein and include isolation of ELA stem cells as shown in international application serial number PCT/US20 10/50288 filed September 24, 2010; U.S. utility application serial number 2010/0291042 filed October 29, 2009; international application PCT/US2008/005742 filed May 5, 2008; U.S. provisional application serial number 60/927,596 filed May 3, 2007; U.S. provisional applications serial numbers 61/247,236 filed September 30, 2009; 61/247,242 filed September 30, 2009; 61/249,172 filed October 6, 2009; and 61/501 ,846 filed August 20, 2010; each of which is hereby incorporated by reference herein in its entirety.

The ELA stem cells are capable of proliferating and differentiating into distinct types of tissue (e.g., ectoderm, mesoderm, and endoderm) and express and do not express certain markers or proteins. The ELA stem cells express Oct-4, KFL-4, Nanog, Sox-2, Rex-1, GDF-3 and/or Stella, but do not detectibly express CD45, CD90, CD34. The ELA stem cells further

do not detectibly express the proteins which are identified as MHC class I, MHC class II, CD44, Cdl05, CD49a, CD73, CD66A, CSCR4 or an SSEA. The ELA stem cells in grafts herein do not detectibly express antigenic proteins or markers (e.g., CD34 and HLA-DR), and thus are suitable to be used as therapeutic agents as they do not induce an immune response in a recipient subject.

ELA stem cells described herein may be contacted to or seeded on a matrix or scaffold and to incorporate and to grow on the matrix or scaffold. The matrix for example is an acelluiar matrix, such as Strattice® (LifeCell, Branchburg, NJ), SurgiMend® (TEI Biosciences, Boston, Mass), GraftJacket® (Wright Medical Technologies, lnc, Arlington, Tenn), NeoForm® (Mentor Corporation, Santa Barbara, Calif), and DermaMatrix® (Synthes, Inc, West Chester, Penn). See also PCT/US201 0/50288 filed September 24, 201 0. Methods of making an acelluiar tissue matrix including cryofracturing a dry acelluiar tissue matrix are described for example in Griffey et al. U.S. patent number 6,933,326 issued August 23, 2005; and Griffey et al. U.S. patent number 7,358,284 issued April 15, 2008, each of which is incorporated herein by reference in its entirety.

Without being limited by any particular theory or mechanism of action, it is here envisioned that vectors are used to transfect ELA cells of the dermal grafts to express specific proteins or molecules that are therapeutic to the skin defect and to the recipient subject. Delivery of the transformed cells is effected through various modes including topical or direct application or injection into dermal, epithelial, muscle and subcutaneous sites. See McLachlin et al. 1990 Progress in Nucleic Acid Research and Molecular Biology 38: 91-135; and Gerson et al. U.S. patent number 5,591,625 issued January 7, 1997.

Dermal grafts envisioned herein include allogeneic, isogeneic or autogeneic populations of synovial fluid derived stem cells, which may be culture expanded, admixed or otherwise applied to or in a hydrogel filler, administered by an injection. Cells are present in a therapeutically effective amount e.g., from $1 O^2$ to 10^6 or more adult stem cells per ml or per dose. The stem-cell based therapeutic may include one or more of plasma such as platelet rich plasma, conditioned medium, or al least one growth factor. These additions are present at a vol/vol amount of 0.1% to 10% w/w in the hydrogel/cell formulation.

The following examples and claims are illustrative and are not meant to be further limiting. Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are within the scope of the present invention and claims. The contents of all

references including published references, issued patents and published patent applications cited in this application are hereby incorporated by reference.

Examples

Example 1: Materials and Methods

ELA® cells which are an early lineage adult stem cell that exists in an undifferentiated and highly plastic state, are recovered and purified from synovial fluid, and cultured in TheraPEAK™ MSCGM-CD™ Mesenchymal Stem Cell Medium, (Lonza Ltd.) chemically defined with 1% Fetal Bovine Serum (FBS) or in serum-free medium (GIBCO™ AJM V Medium liquid; Invitrogen, Carlsbad, CA), or in Dulbecco's Modified Essential Medium (DMEM) with low glucose (DMEM-LG) and chick fibroblast basal medium (MCDB 201) containing insulin-transferrin-selenium (ITS), linoleic acid-bovine serum albumin (LA+BSA), dextrose, L-ascorbic acid, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-growth factor (IGF- 1), penicillin/streptomycin, and 2% fetal bovine serum (FBS). Cell-impermeant viability indicator ethdium homodimer-1 (EthD-1) which emits red fluorescence and stains non-living cells is obtained from Setareh Biotech. Cell viability fluorescent indicator Calcein AM that stains living cells is obtained from Life Technologies. Trichrome Stain (Masson) Kit to stain collagen fibers is obtained from Sigma-Aldrich. Modified Verhoeff Van Gieson Elastic Stain Kit is obtained from Sigma-Aldrich. qPCR kit for detecting Collagen 1and III and Elastin is obtained from Qiagen. RNA extraction kit is obtained from Invitrogen.

Example 2: Animals

Athymic homozygous nude mice (4-5 weeks old, 20-25 g,) or other suitable strains of subjects having reduced immune response are purchased from Harlan (Chicago, IL, USA). Mice are housed and fed under standard conditions. Mice are used according to animal protocols that comply with the Guide for the Care and Use of Laboratory Animals in the USA.

Example 3: Purification of ELA stem cells

ELA stem cells are obtained from blood of mammalian subjects which is harvested, diluted in serum-free medium (GIBCO™ AIM V Medium liquid; Invitrogen, Carlsbad, CA),

and centrifuged three times at 200 g for ten minutes at room temperature. The cells are then re-suspended in serum-free medium, and then are layered over a combined gradient of Ficoll-Paque and Stem Cells Technologies Granulocyte gradient commercially available from ROSETTE SEP DM-M; Stem Cell Technologies, Vancouver, British Columbia. In this combined gradient granulocytes are prevented from entering the gradient and red blood cells and stem cells are obtained in to pellet. The cells are centrifuged at 500 g for 30 minutes at room temperature.

The Ficoll-Stem Cell Technology gradients separate the cells into a buffy layer, an intermediate layer, and pelleted layer. The desired stem cell population is isolated from the pelleted layer, washed in phosphate-buffered saline, and resuspended in culture medium. See U.S. utility application serial number 12/598,047 filed October 29, 2009, publication US 2010/0291042 published November 18, 2010.

Example 4: Culture and analysis of ELA stem cells

The stem cell preparation containing ELA stem cells are inoculated in culture vessels (5,000 cells/cm^2 per vessel) containing culture medium, for example Dulbecco's Modified Essential Medium (DMEM) with low glucose (DMEM-LG) and chick fibroblast basal medium (MCDB 201) containing insulin-transferrin-selenium (ITS), linoleic acid-bovine serum albumin (LA+BSA), dextrose, L-ascorbic acid, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-growth factor (IGF-1), penicillin/streptomycin, and 2% fetal bovine serum (FBS). Cells are grown to confluence and multiple expansion cultures are also obtained. See PCT/US2010/50288 filed September 24, 2010.

The ELA stem cells are analyzed for expression of adult stem cell markers using quantitative polymerase chain reaction (QPCR) analysis. The data show that the ELA cells do not express MHC class I, MHC class II, CD44, CD45, CD34, CD49c, CD73, CD105, and CD90 cell surface markers. These cell surface markers induce or are associated with an immune response in subjects, thus the ELA stem cells herein are not immunogenic stem cells.

Example 5: ELA stem cells differentiate into epithelial and dermal tissue

An amount of the ELA stems cells is contacted with a growth factor that stimulates skin tissue growth and the cells are applied to an acellular matrix to form dermal grafts. The grafts are then applied to a recipient subject having a skin defect. Data obtained after a period

of days, weeks or months show that the ELA stem cell grafts effectively incorporate the cells into the underlying and surrounding tissue of the skin defect.

Serum samples are collected after application of the dermal graft and characteristics are compared to a control serum obtained prior to transplant. Data show no increase in graft rejection markers such as amounts of T cells or B ceils in the serum samples of the graft recipient, compared to serum samples obtained from recipients prior to grafting. Thus, the ELA cells are observed to promote tissue formation and not produce an immune response in the recipient subject.

Example 6: Collection of donor tissue and preparation of allografts

Skin tissues grafts are collected from a donor subject using a hand-held skin harvesting device that is commercially available Robbins Instrumental Inc. (Chatham, NJ; model 16-1992). Blood samples are also collected and analyzed for HLA typing. The tissue collected includes dermal layers and epithelial layers about 25 micrometers to about 1000 micrometers in size. Donor subjects are treated after tissue excision to reduce infection and excess blood loss.

The collected tissues are divided into samples; one set of sample is contacted in a cell culture reservoir with a population of ELA stem cells as shown in examples herein, another set of samples is contacted in the reservoir with buffer. The grafts are then meshed to expand the coverage area.

Example 7: Ultrasound therapy stimulates tissue formation and prevents graft rejection of ELA stem cell dermal grafts

To analyze effects of ultrasound treatment on dermal grafts having ELA stem cells contacted with donor tissue as described in examples herein, healing is compared to control skin defects treated with ELA stem cells with and without ultrasound, and to untreated skin defects.

Recipient non-human mammalian subjects are treated by removal of dermal tissues by excision from several parts of the body to produce a set of comparable skin defects. Blood samples are collected from the recipient animal subjects. A set of skin defects is contacted with dermal grafts having ELA stem cells and donor tissue, and a similar set is contacted with dermal grafts having donor tissue and control buffer only. Control subjects with skin defects are untreated, i.e., receive no dermal grafts.

After application of the dermal grafts to skin defects of the treated subjects, ultrasound therapy is performed using the Ultrasound Bone Healing System from Exogen Inc. (Piscataway, NJ). The skin defects are treated with an ultrasound frequency of 50 kHz or 2 MHz, a low frequency modulating signal of about 10 kHz, and an intensity of 100 milliwatts/cm ^{2}.

The dermal graft areas are analyzed daily for effective incorporation or for signs of graft rejection, e.g., increased body temperature, excessive inflammation, or discomfort. Blood analysis is also performed on the recipient analysis for rejection markers including MHC proteins, B cells, and T cells. The subjects are sacrificed weeks or months later and tissue samples are analyzed.

Subjects treated with dermal grafts having ELA stem cells show extensive, uniform tissue formation across the entire skin defect site independent of the ultrasound frequency applied. The tissue formation for these subjects treated with dermal grafts having ELA stem cells is greater than the tissue formation results of subjects applied dermal grafts with control buffer alone (i.e., without ELA cells). Data show that control subjects having skin defects and not contacted with a dermal grafts have little or no tissue formation, or substantially slower tissue formation. Most important, ultrasound is observed to increase rate of tissue formation both in dermal grafted and untreated subjects. Ultrasound and presence of ELA cells are observed have a surprising synergistic effect.

Little or no immune response is observed to be associated with graft rejection in recipient subjects treated with dermal grafts having ELA stem cells which are not HLA matched to the donor subjects. Further, the extent of tissue formation in these subjects is observed to be independent of extent of HLA matching between the donor and the recipient. Surprisingly, little or no indicia of graft rejection are observed even though no immunosuppression agents are administered to the subject prior to or after the graft transplantation.

Thus, the systems, methods and kits herein using dermal grafts having ELA stem cells, donor tissue, and ultrasound therapy are found to be effective for preparing and applying a dermal graft that stimulates tissue formation.

Examples herein show that dermal grafts having ELA stem cells stimulate tissue formation and do not induce graft rejection. Recipient subjects treated with dermal grafts described herein and then are given ultrasound therapy show no indicia of graft rejection including elevated amounts of B cells and T cells. Thus, the dermal grafts with ELA cells

stimulate tissue formation at the site of a skin defect without activating an immune response that could result in rejection of the graft. Thus, the dermal grafts and methods of preparing the dermal grafts herein are effective for treating dermal defects.

Example 8: Analysis of ultrasound effects with dermal grafts ex vivo

Dermal tissue is collected, ELA stem cells are added as described in examples herein, attached to culture plates in presence of culture medium, and cultures are established for the purpose of determining an optimal range of ultrasound for growth of the ELA stem cells and skin tissue.

The ELA stem cell dermal grafts are layered on hMatrix™ (Bacterin International Holdings), an artificial matrix that supports cellular ingrowth. The matrix is a cell lineage-inducing environment that enhances the differentiation of dermal graft having ELA stem cells to dermal tissue. See PCT/US20 10/50288 filed September 24, 2010.

Areas of each graft (such as a quadrant) is exposed to ultrasound energy having a frequency of 100 kHz, a width of each pulse of 1 second, a pulse repetition of 100 Hz, and the power level is varied. The power level of the ultrasound applied is: 150 milliwatts per square centimeter (milliwatts/cm^2), 100 milliwatts/cm^2, 80 milliwatts/cm^2, 40 milliwatts/cm^2, 20 milliwatts/cm^2, 10 milliwatts/cm^2, or 5 milliwatts/cm^2.

The exposed quadrants of the dermal graft sections are microscopically examined daily for an incubation period of at least four weeks and for additional months for effective ingrowth of the dermal grafts having the ELA stem cells and for signs of graft deterioration or tissue disintegration. Exposed quadrants are also stained for presence of proteins indicating dermal graft incorporation and lack of graft rejection. Data is obtained to determine the range of power levels of ultrasound energy that induce ingrowth and formation of the dermal grafts without disruption of the dermal graft.

Example 9: Analysis of ultrasound duration with dermal grafts ex vivo

Cultures are established as in Example 6 for the purpose of determining an optimal treatment duration of ultrasound for growth of the ELA stem cells and skin tissue.

The ELA stem cell dermal grafts are layered on the artificial matrix to promote cellular ingrowth. Areas of each graft (such as a quadrant) is exposed to ultrasound energy having a frequency of 100 kHz, a width of each pulse of 1 second, a pulse repetition of 100 Hz, and the optimal power level as determined in Example 6. The areas of each graft are

treated with ultrasound energy for 2 minutes per day (min/day), 5 min/day, 10 min/day, or 20 min/day.

The exposed quadrants of the dermal graft sections are microscopically examined daily for an incubation period of at least four weeks and for additional months for effective ingrowth of the dermal grafts having the ELA stem cells and for signs of graft deterioration or tissue disintegration. Data is obtained to determine the optimal duration of ultrasound treatment that induces ingrowth and formation of the dermal grafts without disruption of the dermal graft.

Example 10: Dermal filler Hydrogel

Commercially available hydrogels or dermal fillers tested include: HyStem C, JLiP, PRP, collagen , elastin, Restylane, Juvederm, Artecoll and Perlane. HyStem-C® Hydrogel Kit is obtained from Advanced BioMatrix. Juverderm is obtained from Allergan, Platelet-rich Plasma (PRP) is obtained from an autologous donor. Perlane® is obtained from Medicis Pharmaceutical Corporation.

Example 11: Collagen staining

Weigert's Iron Hematoxylin solution and Phosphotungstic-Phosphomolybdic Acid solution is prepared. The tissue sections are deparaffinized and hydrated by deionized water., then incubated in Bouin's solution at 56 °C for 15 minutes and washed in running tap water until removal of yellow color. The sections are then stained in working Weigert's Iron Hematoxylin solution for five minutes, washed in running tap water for 5 minutes and rinsed in deionized water. Next, the sections are stained in Biebrich Scarlet Acid Fuchsin solution for five minutes, rinsed in deionized water and incubated in Phosphotungstic-Phosphomolybdic Acid solution for five minutes. The sections are then stained in Aniline Blue solution for 5 minutes, incubated in 1% Acetic Acid solution for 2 minutes, rinsed, dehydrated by alcohol, cleared in xylene and mounted. The nuclei in the sections are stained black, muscle fibers are stained red, collagen is stained blue and cytoplasm is stained red.

Example 12: Elastin staining

Elastic Stain solution is prepared by adding 20 mL of Hematoxylin solution, three mL of Ferric Chloride solution, eight mL of Weigert's Iodine solution and five mL of Deionized Water. Ferric Chloride solution is prepared by adding three mL of Ferric Chloride solution to

37 mL deionized water. The tissue sections are deparaffinized and hydrated by deionized water. The sections are incubated in Elastic Stain for 10 minutes and rinsed in deionized water. Each slide is individually incubated in Ferric Chloride solution and rinsed in tap water. The sections are then rinsed in 95% alcohol to remove iodine and rinsed in deionized water. The sections are stained in Van Gieson solution for 1-3 minutes, rinsed in 95% alcohol, dehydrated in alcohol, cleared in xylene and mounted. Nuclei in the sections are stained blue to black, Elastin fibers are stained black, collagen fibers are stained red and other muscle and muscle fibers are stained yellow.

Example 13: ELA cells adheres to dermal filler hydrogel

Human ELA cells are cultured in multiwell plates at a concentration of about 10^3 to about 10^6 cells per well. The cells were contacted with each of different hydrogel preparations and control samples are treated with PBS. The cells are then incubated for a period of time such as 24 hours, 36 hours, 48 hours or 72 hours. Media is discarded and the cells are stained with EthD-1 and Calcein AM to analyze the cell-impermeant non-viability and cell viability using manufacturers' protocols. The plates are imaged, the fluorescence intensity is recorded, and the data is analyzed.

The data indicate significant adherence and maintenance of cell viability.

Example 14: Increase in collagen and elastin production by injected ELA cells

ELA cells are mixed with hydrogel selected as described in examples herein to test ELA cells production of collagen and elastin fibers. A set of subjects such as nude mice is divided in two groups. One group of mice is injected 10^5 to 10^7 ELA cells mixed with hydrogel subcutaneously. The control group of mice are injected hydrogel alone. The mice are sacrificed and soft tissue surrounding the injection site is harvested, paraffin fixed and sectioned. The paraffin sections are stained with Trichrome Stain Masson to stain collagen fibers and Modified Verhoeff Van Gieson Stain to stain for elastin fibers according to procedure described herein. The sections are then imaged and analyzed.

The staining for collagen and elastin in mice treated with the combination of ELA cells and hydrogel is envisioned to be significantly greater than that in control mice. Therefore, ELA cells increase production of collagen and elastin in soft tissue.

Example 15: Culture-expanded cells derived from populations that contain ELA cells stimulate synthesis of Collagen and Elastin

Stem cells or culture-expanded cells derived from populations that contain ELA cells are cultured and cell-free conditioned media are collected after an approximate period of cell growth. Cells are harvested by centrifugation at 72 hours, counted and supernatants are centrifuged at 14,000x g for 5 min to remove cellular debris. The supernatants are mixed with hydrogel as selected in examples herein to obtain a treatment solution.

Cell line of interest is cultured in appropriate media in multi-well plates. The wells are divided into four groups. Two groups are investigation sample groups and two groups are control sample groups. The four groups are treated with the treatment solution. For control samples, the cell line of interest is treated with chemical inhibitors of TGFb and IGF-1 prior to treatment with the treatment solution. The cells are allowed to culture for an approximate period of cell growth. One investigation sample group and one control sample group is stained for collagen and elastin using manufacturer protocol. The cells from other investigation sample group and control sample group are harvested after appropriate period of cell growth and RNA is extracted according to manufacturer protocol. qPCR is performed on the RNA obtained and probed for TGFb and IGF-1 . The qPCR data is collected and analyzed.

The staining for collagen and elastin in investigation sample group is envisioned to be significantly greater than control sample group. The RNA levels of TGFb and IGF-1 in investigation sample group are envisioned to be significantly greater than control sample group.

it is here proposed that stem cells or culture-expanded cells derived from populations that contain *ELA* cells increase production of collagen and elastin by increasing synthesis of TGFb and IGF-1 .

What is claimed is:

1. A stem-cell based therapeutic comprising:

a population of 10^2 to 10^7 ELA stem cells in a formulation including a matrix component that supports ingrowth of mesenchymal lineage differentiated cells.

2. The stem-cell based therapeutic of claim 1, wherein the matrix component is selected from: a gel, a connective tissue that is substantially depleted of living cells, a connective tissue containing living cells, a polymer scaffold, calcium triphosphate, demineralized bone, collagen, and cellulose; the matrix component optionally substantially conforming in vivo to an implant site.

3. The stem-cell based therapeutic of claim 2, wherein the matrix component includes a dermal filler such as hyaluronic acid.

4. The stem-cell based therapeutic of claim 1, further comprising plasma such as platelet rich plasma, or at least one growth factor.

5. The stem-cell based therapeutic of claim 1, further comprising a chondrocyte differentiating agent.

6. The stem-cell based therapeutic of claim 1, further comprising mesenchymally committed or differentiated populations of ELA stem cells.

7. The stem-cell based therapeutic of claim 1, further comprising a fibroblast differentiating agent.

8. The stem-cell based therapeutic of claim 3, further comprising: a population of 10^2 to 10^7 cells ELA stem cells in a formulation including a dermal filler from about 5%-95% dermal filler by volume.

9. A method of reconstructing or rejuvenating a target tissue in a subject comprising: administering to a subject at the target tissue site by injection, a preparation of a dermal filler having 10^2 to 10^7 cells ELA stem cells or mesenchymally committed or differentiated

populations thereof, thereby adding volume and a cellular therapy product to the target tissue, which reconstructs and/or rejuvenates the target tissue.

10. The method of claim 9, wherein reconstructing or rejuvenating a target tissue includes: lip enhancement (volume and contouring), diminishment in the visible appearance of wrinkles and aging lines of the face such as the nasolabial folds, melomental folds, crow's feet and forehead wrinkles, frown lines, filling aging-related facial hollows and orbital troughs under and around the eyes, enhancing check volume and contouring of the chin, forehead and nose, and revitalizing the skin by increasing skin elasticity structure.

11. A method for treating a subject having a tissue defect, the method comprising: contacting the tissue defect with a stem-cell based therapeutic comprising a population of 10^2 to 10^7 cells ELA stem cells in a formulation including a matrix component supporting ingrowth of mesenchymal lineage differentiated cells of the subject.

12. The method of claim 11, wherein the matrix component is a gel, a connective tissue that is substantially depleted of living cells, a connective tissue containing living cells, a polymer scaffold, calcium triphosphate, demineralized bone, collagen, and cellulose, and optionally the matrix component substantially conforms in vivo to an implant site.

13. The method of claim 11, wherein the tissue defect is further contacted with a dermal tissue graft.

14. The method according to claim 11, wherein the wherein the tissue defect is further contacted with a connective tissue graft.

15. The method according to claim 11, wherein the tissue defect is contacted with at least one selected from the group consisting of: plasma, a buffer, cell culture medium, a preservative, an anti-bacterial agent, an anti-fungal agent, a conditioning agent, a cryogenic agent, a pharmaceutically acceptable salt, a growth factor for example epidermal growth factor, a bioactive factor for example stem cell factor, a vitamin, a hormone, a therapeutic agent, a bone morphogenetic factor such as bone morphogeneic proteins BMP-2, BMP-3, BMP-4, BMP-6, and BMP-7; platelet-derived growth factor (PDGF), platelet rich plasma.

epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), interleukins selected from the group consisting of IL-3, IL-4 and IL-1, insulin-like growth factor-1 (TGF-1), leukemia inhibitory factor (L1F), vascular endothelial growth factor (VEGF), erythropoietin (EPO), GDF-5, transforming growth factor β-3 (TGF- β3), granulocyte colony stimulatory factor (G-CSF), granulocyte-macrophage colony stimulatory factor (GM-CSF), Fit-3 ligand, stem cell factor (SCF), IL-3 receptor agonists, Daniplestim; thrombopoietin agonists, chimeric cytokines, leridistim, progenipoietin-1, peg-filgrastim, SDF-1 antagonists, AMD 3100; and chemotherapeutic agents selected from the group consisting of cyclophosphamide, iphosphamide, carboplatin, etoposide (ICE), etoposide, methylprednisolone, ara-c and cisplatin.

16. The method according claim 9, further comprising: performing low energy ultrasound therapy on or near the target tissue or the tissue defect using an ultrasound device.

17. The method according claims 11, further comprising: performing ultrasound therapy on or near the target tissue or the tissue defect using an ultrasound device generating an ultrasound frequency of about 10 kilohertz to about 10 megahertz, generating power at an intensity of at least about 1.0 milliwatts/cm ^{2}.

18. The method according to claim 11, wherein contacting the tissue defect comprises at least one route selected from the group consisting of: injecting, infusing, co-culturing, mixing pouring, and spraying.

19. The method of claim 11, further comprising reducing the recovery time of the subject by administering the stem-cell based therapeutic at a cell dose greater than 1.5 x 10^2 ELA stem cells.

20. The method of claim 19, further comprising reducing the recovery time of the subject by administering the stem-cell based therapeutic at a cell dose greater than 10^4 synovial fluid derived adult stem cells.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 14/49395

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 5/00, 5/02, 2506/02, 2501/1 15 (2014.01)
CPC - A61K 9/0024, 9/0056, 9/0014
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C12N 5/00, 5/02, 2506/02, 2501/1 15 (2014.01)
CPC: A61K 9/0024, 9/0056, 9/0014; USPC: 424/484; 435/377, 375, 325

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C.B, DE-A, DE-T, DE-U, GB-A, FR-A); Google; Google Scholar; Dialog ProQuest; Entrez Pubmed; 'ELA stem cells', 'dermal filler", 'hyaluronic acid,' 'fibroblast differentiation,' 'chondrocyte differentiation', 'mesenchymal lineage cells', cosmetic, therapeutic, ultrasound, 'target tissue'

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2012/0230966 A1 (CRAWFORD, KD et al.) September 13, 2012; abstract; paragraphs [0010], [001 1], [0013], [0024], [0025], [0029], [0034], [0064], [0074], [0086], [0106], [01 12], [0200], [0202], [0226], [0250], [0294]	1-20
Y	US 2010/0185219 A1 (GERTZMAN, AA et al.) July 22, 2010; paragraphs [0017], [0019], [0035], [0073], [0078]	1-8, 11-15, 17-20
Y	US 2012/0189588 A1 (NAHAS, Z et al.) July 26, 2012; paragraphs [0009], [0044], [0057], [01 18], [0134], [0135], [0205]	3, 8-10, 16
Y	US 2012/0225484 A1 (BHATIA, M et al.) September 6, 2012; paragraphs [0008], [0009], [0191], [0194], [0198], [0199]	10
Y	WO 2013/048912 A2 (BARTHE, PG et al.) April 4, 2013; paragraphs [0009], [0010], [0012], [0120], [0249]	16, 17

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
03 November 2014 (03.1 1.2014)

Date of mailing of the international search report
12 NOV 2014

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