

re-generate or generate the essential multi-dimensional architecture required for cellular migration, differentiation, and tissue polarization. Without these cell-to-cell and cell-to-matrix interactions, remaining cellular entities within the wound bed, no matter their proliferative or lineage potential, are forced to provide primarily a barrier utility rather than develop a more complex, multi-tissue construct capable of recognizable "function." Consequently, the wound -- whether involving skin, muscle, fat, tendon, bone--becomes subsequently scarred, disorganized and dysfunctional.

Current applications in field of tissue engineering of cultured skin, cartilage, bone, muscle, blood vessels, nerves, lymphatics and related substitutes are largely based on a three part strategy: 1) acquiring a tissue source and harvesting cell suspension from such tissue; 2) applying these cells to a matrix or scaffold; and 3) grafting the construct onto or into a target site of a human or animal. However, in the absence of the above-identified inter-dependent, necessary components, tissue engineering applications, cell therapy applications, regenerative medicine applications, tissue healing applications and tissue transplant therapy applications do not possess the natural cellular micro-aggregate architecture needed to competently assemble functional polarized tissues. Thus, due to the lack of proper inter-dependency, progenitor cell mass and proper scaffolding prevent such constructs to be useful in therapeutic applications such as multi-compartment tissue regeneration and/or bone and muscle reconstruction.

Consequently, in part due to the foregoing, substantial efforts and resources have been directed by both industry and academics to developing synthetic tissue substitutes, autograft constructs, as well as patient-derived epidermal expansion autografts (i.e. EPICEL® from Vericel Corporation of Cambridge MA.). These products, although beneficial, are often expensive and do not provide the patient with a true multi-compartment tissue construct. For example, cultured epithelial autograft (CEA) remains unable to restore both epithelial and dermal compartments seen in native skin. But in view of the absence of interdependent functioning compartments, the cultured cells are left without an expanding localized stem cell population and the evolving tissue polarization needed to develop integument -- epidermis, dermis,

glands and hair -- which truly defines skin. This failure, in turn, leads to monolayer fragility, epithelial instability, barrier breakdown, and scar.

Alternatively, the more robust acellular matrices such as ALLODERM® from LifeCell Corporation, INTEGRA® from Integra LifeSciences Corporation and DERMATRIX® a product from Musculoskeletal Transplant Foundation, although excellent reconstructive options, lack those properly placed lineage specific stem cell populations which are necessary to develop functional native tissues.

The inventor herein has already written about the relatively recent recognition of LGR5 and LGR6 as markers of both intestinal and epidermal stem cells in mammals. In *Stimulation of the Follicular Bulge LGR5+ and LGR6+ Stem Cells with the Gut-Derived Human Alpha Defensin 5 Results in Decreased Bacterial Presence, Enhanced Wound Healing, and Hair Growth from Tissues Devoid of Adnexal Structures*, *Plast. Reconstr. Surg.* 132: 1159, 2013, Leucine-rich repeat-containing G-protein—coupled receptor (LGR) is a seven-pass transmembrane protein receptor with significant sequence and structural homology to the follicle-stimulating hormone, thyroid-stimulating hormone, and luteinizing hormone receptor families.

In that study, it was recognized that human alpha defensin 5 peptide significantly enhanced wound healing and reduced basal bacterial load compared with human beta defensin 1 and sulfadiazine. Human alpha defensin 5 was the only therapy to induce LGR stem cell migration into the wound bed. In addition, gene heat mapping showed significant mRNA up-regulation of key wound healing and Wnt pathway transcripts such as Wnt1 and Wisp1. So it was concluded that human alpha defensin 5 could be used for enhanced wound healing due to the observed increase of LGR stem cell migration into wound beds and associated bacterial reduction and hair production through the augmentation of key Wnt and wound healing transcripts. In short, this and other work led to the recognition of the potential for using LGR4+, LGR5+ and LGR6+ expressing epithelial stem cells in direct biomedical engineering soft tissue constructs.

Summary

The disclosure provides in a first embodiment a minimally polarized micro-

aggregate multi-cellular composition including isolated living LGR expressing cells and a multi-dimensional support selected from the group consisting of scaffolding, collagen, matrix, particle, and fiber.

5 In one aspect, there is provided a composition, comprising: (i) a multi-dimensional support selected from the group consisting of scaffolding, collagen, matrix, particle, and fiber; and (ii) living LGR-expressing epithelial stem cells; wherein said composition is a minimally polarized, micro-aggregate, multi-cellular composition.

10 The disclosure provides in a further embodiment to the previous embodiment a minimally polarized micro-aggregate multi-cellular composition including isolated living LGR expressing cells and a multi-dimensional support selected from the group consisting of scaffolding, collagen, matrix, particle, and fiber where the LGR expressing cells are supplemented with growth factors and where the LGR expressing cells are selected from the group consisting of LGR4, LGR5 and LGR6.

15 The disclosure provides in a further embodiment to any of the previous embodiments a minimally polarized micro-aggregate multi-cellular composition including isolated living LGR expressing cells and a multi-dimensional support selected from the group consisting of scaffolding, collagen, matrix, particle, and fiber where the LGR expressing cells are supplemented with migratory/recruiting analytes and the LGR expressing cells being selected from the group consisting of LGR4, LGR5 and LGR6.

20 The disclosure provides in a further embodiment to any of the previous embodiments a minimally polarized micro-aggregate multi-cellular composition including isolated living LGR expressing cells and a multi-dimensional support selected from the group consisting of scaffolding, collagen, matrix, particle, and fiber where the LGR expressing cells are supplemented with LGR specific binding elements selected from the group consisting of ligand families, R-spondin, EDGF, PDGF, Wnt, VEGF, and antimicrobial peptides and where the LGR expressing cells are selected from the group consisting of LGR4, LGR5 and LGR6.

25 The disclosure provides in a further embodiment to any of the previous embodiments a minimally polarized micro-aggregate multi-cellular composition including isolated living LGR expressing cells and a multi-dimensional support selected from the group consisting of scaffolding, collagen, matrix, particle, and fiber where the composition is used as a therapeutic construct for a select target consisting of a tissue region, wound, void, defect tissue, or blood for alteration of either surrounding adjacent tissues.

The disclosure provides in a further embodiment to any of the previous embodiments a minimally polarized micro-aggregate multi-cellular composition characterized by a isolated living LGR expressing cells transplanted to damaged tissue to accelerate healing thereof.

The disclosure provides in a further embodiment to any of the previous embodiments a minimally polarized micro-aggregate multi-cellular composition for tissue system repair or restoration throughout the body comprising a support scaffolding with isolated LGR containing cells secured thereto.

The disclosure provides a tissue graft comprising a composition described herein for application to ectodermal, mesodermal or endodermal-derived tissues systems throughout a mammalian body.

The disclosure provides a method for obtaining a minimally polarized micro-aggregate multi-cellular composition characterized by the steps of growing and isolating living LGR expressing cells for transplantation to a select mammalian target tissue.

In one aspect, there is provided a method of producing a minimally polarized micro-aggregate multi-cellular composition, the method comprising:

a) extracting at least one minimally polarized functional unit containing LGR-expressing cells from a mammalian tissue specimen;

b) processing of hypodermis and subdermal fat cellular components of the mammalian tissue specimen to provide a mesenchymal-derived cellular population;

c) adding at least a portion of the mesenchymal-derived cellular population to the at least one minimally polarized functional unit to create at least one epithelial stem cell functional singularity unit;

d) enriching the at least one epithelial stem cell functional singularity unit; and

e) adding the at least one enriched epithelial stem cell functional singularity unit to a delivery substrate.

The disclosure provides in a further embodiment to the foregoing method a method for obtaining a minimally polarized micro-aggregate multi-cellular composition characterized by the steps of growing and isolating living LGR expressing cells for transplantation to a select mammalian target tissue further characterized by the step of affixing the isolated living LGR expressing cells to a multi-dimensional support selected from the group consisting of scaffolding, collagen, matrix, particle, and fiber.

The disclosure is characterized in still another embodiment by a method for obtaining a minimally polarized micro-aggregate multi-cellular composition characterized by the steps of growing and isolating living LGR expressing cells for transplantation to a select mammalian target tissue further characterized by the step of selecting the LGR expressing cells from the group consisting of LGR4, LGR5 and LGR6.

The disclosure provides in a further embodiment to any of the previous method embodiments the step of applying the minimally polarized micro-aggregate multi-cellular to one of the group consisting of epithelial systems, glands, hair, nerves, bone, muscle, fat, tendons, blood vessels, fascia, ocular tissues and peptide secreting cellular elements employing delivery by a technique selected from the

group consisting of application, transplantation, implantation, directed seeding, directed migration, directed tracking, in setting, laminating and/or injection of the cellular element generating, regenerating, enhancing and healing.

5 The disclosure provides in a further embodiment to any of the previous method embodiments a method for obtaining a minimally polarized micro-aggregate multi-cellular composition characterized by the steps of growing and isolating living LGR expressing cells for transplantation to a select mammalian target tissue further characterized by the step of applying the minimally polarized micro-aggregate multi-cellular composition directly to a tissue *in vivo* for tissue
10 restoration.

 The disclosure provides in a further embodiment to any of the previous method embodiments a method for obtaining a minimally polarized micro-aggregate multi-cellular composition characterized by the steps of growing and isolating living LGR expressing cells for transplantation to a select mammalian
15 target tissue further characterized by the step indirectly applying the minimally polarized micro-aggregate multi-cellular composition via the blood stream for tissue restoration in a body.

 The disclosure provides a method for producing a minimally polarized micro-aggregate multi-cellular composition characterized by the steps of:

- 20 a) obtaining a tissue specimen;
- b) extracting minimally polarized functional units containing LGR expressing cells from the specimen;
- c) processing of hypodermis and subdermal fat cellular components from an appropriate source;
- 25 d) adding the processed hypodermis and subdermal fat components to the extracted minimally polarized functional units to create epithelial stem cell singularity units;
- e) enriching the epithelial stem cell singularity units;
- f) adding the epithelial stem cell singularity units to a construct scaffold; and
- 30 g) verifying the maintenance of minimum polarization of the obtained composition.

 The disclosure provides in a further embodiment to any of the previous

embodiments a media formulation used in obtaining minimally polarized micro-aggregate multi-cellular compositions using cell sustaining media composition for reducing the viability of micro-organisms during transport and processing of tissues, characterized by:

5 a) a mixture of epithelial cells and keratinocytes; b) at least one agent selected from the group consisting of penicillin, streptomycin, and amphotericin B; and c) fibrinogen.

The disclosure provides in a further embodiment to the previous embodiment of a cell sustaining media composition for reducing the viability of microorganisms during transport and processing of tissues, characterized by: a) a mixture of epithelial cells and keratinocytes; b) at least one agent selected from the group consisting of penicillin, streptomycin, and amphotericin B; and c) fibrinogen, where the fibrinogen is human and where the agent includes both an antibiotic and an antimycotic for stabilizing human tissues. The disclosure provides a cell sustaining media composition for reducing the viability of microorganisms during transport and processing of tissues, characterized by

10 a) a mixture of epithelial cells and keratinocytes,
15 b) at least one agent selected from the group consisting of penicillin, streptomycin, and amphotericin B, and
c) fibrinogen.

In the context of a first embodiment of the disclosure, it is characterized by LGR expressing cells being applied to scaffolding matrix, and/or fiber to thereby establish micro-aggregate multi-cellular grafts for tissue engineering applications, cell therapy applications, regenerative medicine applications, medical/therapeutic applications the grafts being directly applied to tissue or blood for improvement and or alteration of epithelial systems throughout the body.

20 In one aspect, there is provided a composition produced by the disclosed methods.

25 In one aspect, there is provided a composition of the disclosure for use in therapy.

In one aspect, there is provided a composition of the disclosure for use:

30 i) in the treatment of wounds, wherein the composition accelerates healing of the wounds; and/or
ii) in the treatment of tissue, wherein the composition repairs or restores tissue; and/or
iii) as a therapeutic construct for treating any one of a tissue region, wound, void, defect tissue, or blood; and/or
35 iv) as a therapeutic construct in a treatment to alter surrounding adjacent tissues.

A second embodiment of the disclosure is characterized by LGR expressing cells being applied to scaffolding, matrix, and/or fiber with/or without additional enhancing factors or analytes before or after either being applied to tissue or blood for improvement and or alteration of *in vivo* epithelial systems.

40 A further embodiment of the disclosure is characterized by LGR expressing cells altered by enhancing factors or analytes, being applied as targets within the body, tissue or blood for improvement and/or alteration of epithelial systems through local or distant migration throughout the body and/or to restore gland and hair growth.

45 A fourth embodiment of the disclosure is characterized by transplanting LGR expressing cells from tissue, blood or culture for alteration of surrounding adjacent tissues or distant tissues such as but not inclusive of LGR expressing cells applied to scaffolding, matrix, and fiber before or after either

being applied to tissue or blood for improvement and or alteration of ectodermal, mesodermal or endodermal- derived tissues systems throughout the body.

5 A fifth embodiment of the disclosure is characterized by directly applying LGR expressing cells to a delivery substrate vehicle selected from a group consisting of scaffolding, matrix, and fiber with/or without additional enhancing factors or analytes before or after either being applied to tissue or blood for improvement and or alteration of ectodermal, mesodermal or endodermal-derived tissues systems throughout the body.

10 Still another embodiment of the disclosure is characterized by combining LGR expressing cells altered by enhancing factors or analytes, with a delivery support substrate as targets within the body, tissue or blood for improvement and or alteration of ectodermal, mesodermal or endodermal-derived tissues systems throughout the body through local or distant migration throughout the
15 body.

A further embodiment of the disclosure is characterized by adhering LGR expressing cells to a support substrate for the delivery, application, transplantation, implantation, directed seeding, directed migration, directed tracking, in setting, laminating and/or injection of the cellular element
20 generating, regenerating, enhancing and/or healing epithelial systems, glands, hair, nerves, bone, muscle, fat, tendons, blood vessels, fascia, ocular tissues and peptide secreting cellular elements.

A final stated embodiment of the disclosure is to generate LGR expressing stem cells as micro-aggregate multi-cellular functional units
25 exhibiting minimal polarization for transplantation and direct application to a target within a mammalian body, tissue or blood to enhance and accelerate tissue generating, regenerating, enhancing and/or healing.

In most general terms, the disclosure herein contemplates the transplanting and/or delivery of isolated LGR expressing cells (Leucine-rich
30 repeat-containing G-protein coupled receptor) for the generation, regeneration, recruitment or enhancement of an epithelial system, hair, gland bone. The disclosure also contemplates application to both local/proximate and distant/remote tissue in clinical medicine, bioengineering and/or research

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constructs using a delivery vehicle in the form of scaffolding, matrix or fiber with or without the supplementation of growth factors migratory/recruiting analytes or LGR specific binding elements such as but not limited to ligand families, R-spondin, EDGF, PDGF, Wnt, VEGF, antimicrobial peptides.

5 Use of the LGR epithelial stem cells, particularly in conjunction with a formed scaffolding substrate, provides full thickness wounds and or voids in epithelial systems with a stem cell enriched tissue substitute. Moreover, the addition of this minimally polarized functional cell unit (MPFU) to an epithelial system is enhances/improves the status of that epithelium which includes the
10 growth, generation or regeneration of hair, glands, secreted anti-microbial peptides, growth factors and analytes generally required to maintain and promote the health and viability of the epithelium and local surrounding tissues elements.

 Recognizing that LGR4+, LGR5+ and LGR6+ stem cell and progenitor
15 cell proliferation kinetics remain high, especially when in contact with substrate scaffolding, complete epithelial turnover rates are typically less than 12 days (1cm inter-population distance spacing). This capacity to regenerate a sufficient tissue bi-layer and subsequent barrier function suggests a role for these cells as a type of evolving biologic dressing for complex full thickness
20 and multi- tissue wounds.

 Beyond a capacity to regenerate skin, muscle and bone quickly, the progenitors of the LGR4, LGR5 and LGR6 stem cells also have the ability to generate native anti-microbial peptides that not only reduce the basal level of microorganisms within the wound bed but also augment progenitor cells
25 amplification and differentiation, leading to a reduction in wound and peri-wound infections, faster wound closure, and hair follicle development.

 The disclosure herein describes the translational applicability of an LGR expressing epithelial stem cell -seeded scaffold in providing an immediate, deliverable and viable tissue barrier that is capable of maintaining a stem cell
30 colony focus with concomitant competent progeny. From these stem cell foci, progeny can undergo migratory proliferative-differentiation in order to stimulate epithelial tissue elements, healing and graft integration. It has been found that the LGR epithelial stem cells can be applied alone, with

described herein. The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention.

As used herein, the singular forms, “a”, “an” and “the” are intended to
5 include the plural forms as well, unless the context clearly indicates otherwise. It will be further understood that the root terms “include” and/or “have”, when used in this specification, specify the presence of stated features, steps, operations, elements, and/or components, but do not preclude the presence or addition of at least one other feature, step,
10 operation, element, component, and/or groups thereof.

As used herein Bone means the hard connective tissue consisting of cells embedded in a matrix of mineralized ground substance and collagen fibers. The fibers are impregnated with inorganic components, including crystals of calcium phosphate, such that using X-ray defraction, they are seen to be
15 organized in a hydroxyapatite pattern (calcium phosphate is 85% by weight) as well as calcium carbonate (10%), and magnesium; by weight, bone is composed of 65-75% inorganic and 25-35% organic material; a portion of osseous tissue of definite shape and size, forming a part of the animal skeleton; in humans there are approximately 200 distinct bones in the
20 skeleton, not including the auditory ossicles of the tympanic cavity or the sesamoid bones other than the two patellae. A bone is enveloped by a fibrous membrane, periosteum that covers the bone's entire surface except for the articular cartilage. Beneath the periosteum is a dense layer, compact bone, and beneath that a cancellous layer, spongy bone. The core of a long
25 bone is filled with marrow.

As used herein, the terms “comprises,” “comprising,” “includes,” “including,” “has,” “having” or any other variation thereof, are intended to cover a non-exclusive inclusion. For example, a process, method, article, or apparatus that comprises a list of features is not necessarily limited only to
30 those features but may include other features not expressly listed or inherent to such process, method, article, or apparatus.

As used herein Epithelium means the cellular layer covering all free surfaces, cutaneous, mucous, and serous, including the glands and other structures derived therefrom.

As used herein GMP means good manufacturing practices.

5 As used herein Integument means the enveloping membrane of the body; includes, in addition to the epidermis and dermis, all the derivatives of the epidermis, hairs, nails, sudoriferous and sebaceous glands, and mammary glands, as well as the subcutaneous tissue.

10 As used herein LGR4 means Leucine-Rich Repeat Containing G Protein-Coupled Receptor 4, G protein-coupled receptors (GPCRs) that play key roles in a variety of physiologic functions. Members of the leucine-rich GPCR (LGR) family, such as GPR48, have multiple N-terminal leucine-rich repeats (LRRs) and a 7-transmembrane domain. LGR4 (Leucine-Rich Repeat Containing G Protein-Coupled Receptor 4) is a Protein Coding gene.

15 Diseases associated with LGR4 include bone mineral density, low. Among its related pathways are Wnt signaling pathway (KEGG). GO annotations related to this gene include G-protein coupled receptor activity and transmembrane signaling receptor activity. An important paralog of this gene is LGR6.

20 Receptor for R-spondins that potentiates the canonical Wnt signaling pathway and is involved in the formation of various organs. Upon binding to R-spondins (RSPO1, RSPO2, RSPO3 or RSPO4), associates with phosphorylated LRP6 and frizzled receptors that are activated by extracellular Wnt receptors, triggering the canonical Wnt signaling pathway to increase expression of target genes.

25 In contrast to classical G-protein coupled receptors, LGR4 does not activate heterotrimeric G-proteins to transduce the signal. Its function as activator of the Wnt signaling pathway is required for the development of various organs, including liver, kidney, intestine, bone, reproductive tract and eye. LGR4 may also act as a receptor for norrin (NDP) and is required during

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cells that may differentiate into other cell types; a cell capable of maintaining its own number while exporting progeny to one or more cell lineages.

As used herein “substantially,” “generally,” and other words of degree are relative modifiers intended to indicate permissible variation from the characteristic so modified. It is not intended to be limited to the absolute value or characteristic which it modifies but rather possessing more of the physical or functional characteristic than its opposite, and preferably, approaching or approximating such a physical or functional characteristic.

As used herein Tissue means a collection of similar cells and the intercellular substances surrounding them. There are four basic kinds of tissue in the body: epithelium; connective tissues including adipose tissue, blood, bone, and cartilage; muscle tissue; and nerve tissue. The rind, capsule, or covering of any body or part.

In the following description, reference is made to the accompanying drawings which are provided for illustration purposes.

The following illustrated embodiment is described in sufficient detail to enable those skilled in the art to practice the invention. It is to be understood that other embodiments may be utilized and that structural changes based on presently known structural and/or functional equivalents may be made without departing from the scope of the invention.

Brief Description of the Drawings

Figure 1A depicts an example of location of said LGR expressing cells of cutaneous origin.

Figure 1B is a Fluorescent Activated Cell Sorting graph.

Figure 1C are photographs of a spectrum of various acellular supports contemplated for use in connection with the invention.

Figure 2A is photograph of a gross cellular construct/ de-cellularized collagen scaffold usable for seeding.

Figure 2B are immunofluorescent photomicrographs of a collagen construct following seeding with aggregates of partially digested cells.

Figure 3A - 3F present various images by different techniques of an array

of different LGR6+ epithelial stem cell seeded substrates.

Figure 4A depicts a time lapse *in vivo* healing progression of controls and an example of an LGR seeded matrix. Figure 4B is a graphical expression of Cytokeratin-17 transcript expression at day ten. Figures 4C-E depict controls and a matrix seeded with LGR ESC by bioluminescent imaging and scanning electron microscopy.

Figures 5A-E depict an example of a construct with LGR ECSs and stromal vascular fraction cellular isolate populations showing initial form of polarization accompanied by a graphic comparison.

Figures 6A-B depict an example of a construct containing LGR cells with and without stromal vascular fraction cellular entities and the relative production of growth factors.

Figures 7A-H illustrate a third degree wound bed induction and verification of the elimination of the LGR stem cell follicular bulge and adnexal structures.

Figures 8A-Q depict time progression of a wound/injury/void with DEFA5 as it relates to bacterial adhesion.

Figures 9A and B are comparative photographs of DEFA5 expressing cellular entities within a wound bed as it relates to augmented healing, tissue and appendage regeneration and subsequent hair growth in treated burn wounds devoid of adnexal structures.

Figures 10A-L illustrate the quantification of wound bed healing kinetics and LGR5 and LGR6 stem cell migration into burn tissue following treatment with topical focal agents.

Figures 11A and B illustrate RT-PCR quantification and gene heat mapping comparison of wound/injury/tissue voids treated with DEFA5 to SDZ as it relates to augmentation of pro-healing pathways.

Figures 12A-1 illustrate LGR6 expression of cells of the hair follicle and fluorescent activated cell sorting of co-expressing LGR6+, CD34+, CD73+ GFP labeled cells for culture expansion.

Figures 13A-D are photomicrographs by confocal microscopy and

Figure 2A is a photographic representation of an example of a gross construct without micro-aggregate multi-cellular functional units containing LGR expressing stem cell foci in accordance with the invention. Figure 2B depicts the construct following seeding of substrate with aggregates of partially digested cells.

Figure 3A, in columnar format, is an image series by differential interference contrast (DIC) confocal microscopy of LGR seeded substrates from different sources. Figure 3B is a corresponding column by immunofluorescent confocal microscopy at 20x magnification of LGR6+ ESC seeded matrices of respective constructs containing LGR expressing cells. The inset white boxes represent focal zoom regions indicated in the column Figure 3D while Figure 3C is a column depicting the Digital merge of the respective image of Figure 3A (DIC) and the immunofluorescent of Figure 3B indicating matrix contour and boundaries. The columns of Figures 3E and 3F respectively represent the bioluminescence measured in radiant efficiency of an acellular matrix control and a corresponding LGR6+ ESC seeded matrix at 72 hours post-seeding.

Figure 4A-E depict examples of said LGR containing construct placed into living mammalian system. Placement of an LGR6+ GFP ESC Seeded Matrix Augments Healing Hair Follicle Growth. Figure 4A is a 3x3 matrix of photomicrographs of 3mm full human de-cellularized dermis thickness burn wound beds at days 5, 8 and 10 containing no matrix (burn control), matrix (matrix control) and LGR6+ GFP ESC. Figure 4B graphically depicts the relative expression of Cytokeratin-17 transcript expression at day 10 of the wound beds depicted in Figure 4A. The percent wound bed healed was determined using quantification analysis of wound bed healing rates as a percent area function within the ImageJ NCBI application. Wound control contains burn wound bed only. Matrix control contains matrix only and LGR6+ GFP contains ADM seeded with LGR6+ GFP ESCs. Figure 4C is a photomicrograph of *in vivo* bioluminescent imaging in murine full thickness burn wound beds at day 5. Figure 4D are micrographs of human dermis at 100x of the controls and LGR6+ GFP containing dermis at 12 hours and 72 hours and after seeding with ESCs. The white arrow indicates the

presence of a dermal pore Figure 4E provides images of the controls and the construct of the invention containing human dermis seeded with ESCs with a silicone protective overlay to prevent desiccation. The LGR6+ GFP matrix image includes duplicate small black arrows that indicate nascent hair patches from the full thickness Nu/Nu murine wound bed.

Figures 5A-E depict an example of said construct with the effect of addition of Stromal vascular fraction (SVF) to LGR6+ ESC Seeded Matrices in promoting tissue polarization and a dual compartment skin-like System. Figure 5A is confocal 20x imaging of a 5×10^5 RFP expressing stromal vascular fraction cellular isolate population 24 hours after being seeded on to a representative Adrenomedullin (ADM) (such as that available from Integra LifeSciences Corporation under the name INTEGRA®). Figure 5B is a confocal 20x image of a 5×10^5 GFP expressing LGR6+ cellular isolate population 24 hours after being seeded on to a representative ADM (INTEGRA®). Figure 5C depicts confocal 20x imaging of a dual seeded representative ADM (INTEGRA®) with 5×10^5 RFP expressing SVF and 5×10^5 GFP expressing LGR6+ isolate populations 24 hours after being co-seeded in culture. Figure 5D is of a co-seeded matrix containing 5×10^5 RFP expressing SVF^{RFP} and 5×10^5 GFP expressing LGR6+ following 5 days of growth in culture. The dotted parallel lines indicate epithelial LGR6^{+GFP} lineage accumulating at the edge of the ADM substrate. The small bracket and large bracket indicate the relative locations of the two compartments in correlation with LGR6^{+GFP} and SVF^{RFP} abundance. The arrowed "U" shaped solid line indicates a region containing a pre-seeded pore induced by a 32 gauge sterile needle. Figure 5E is a graphical representation of the proliferation kinetics of a collagen substrate co-seeded with green LGR expressing cells and red SVF expressing cells.

Figures 6A and 6B depict an example of a construct containing LGR cells with and without supportive cellular entities and the relative production of growth factors. Correlative Expression Profiles of Pro-angiogenic Transcripts and Protein Analytes from LGR6^{+GFP} ESC and SVF^{RFP} Enriched Scaffolding Culture Constructs. Figure 6A graphs relative fold transcript expression ($\Delta\Delta CT$) of indicated gene element from total RNA: LGR6^{+GFP} ESC (black bar), SVF^{RFP}

(grey bar), and co-cultured LGR6^{+GFP} ESC + SVF^{RFP} (white box) on respective scaffold substrate. Significance above the x-axis (LGR6 + SVF) indicates the inter-comparison co-cultured LGR6^{+GFP} ESC + SVF^{RFP} expression vs. singular LGR6^{+GFP} ESC and SVF^{RFP} expression on indicated scaffolding. Ex. Average FGF-2 gene expression for co-cultured matrices was higher than the average expression of both singular systems (Scaffold+ LGR6 or scaffold +SVF) except for co-cultured INTEGRA® (INTEGRA®+ LGR6⁺SVF). Significance below the x-axis (LGR6) or (SVF) indicates the intra-comparison of substrates, while the cellular entity remains constant. Ex. VEGF-A gene expression for INTEGRA®+ LGR6^{+GFP} ESC only vs. DERMAMATRIX®+ LGR6^{+GFP} ESC only was nonsignificant (NS). Figure 6B graphically represents the relative densitometric unit (RDU) of indicated protein analyte from total protein isolates: LGR6^{+GFP} ESC (black bar), SVF^{RFP} (grey bar), and co-cultured LGR6^{+GFP} ESC + SVF^{RFP} (white box) on respective scaffold substrate. (*) indicates (p-value < 0.05), assays completed in triplicates, GAPDH housekeeping control.

Figures 7A-H illustrate a wound/injury/void receiving therapy example of enhanced LGR cell migration, proliferation and viability into a wound namely a third degree wound bed induction and verification of the elimination of the LGR stem cell follicular bulge and adnexal structures. Figure 7A depicts a wound bed template marks of 3mm diameter. Figure 7B depicts the wound bed structure at day 0 (the white scale bar being 1mm). Figure 7C illustrates an example of a 2x3 3mm wound bed grid. Figure 7D shows topical application of the re-suspended peptide at the wound site. Figure 7E is a photomicrograph of H&E stain of non-burned, intact Integument/skin with hair follicle and adnexal structures. The arrow indicates the location of the magnified follicle (inset image) where the white scale bar is 500µm. Figure 7F is an H&E stain of dorsal murine skin following high temperature cautery depicting removal of epidermal, dermal and hypodermal tissues including the follicular bulge. Figure 7G is DAPI/DNA stain (4',6-diamidino-2-phenylindole) of non-burned, intact skin with hair follicle and adnexal structures. The arrow indicates the magnified follicle with co-labeling of immunofluorescent LGR5 and LGR6 antibodies green and red respectively (inset image). Figure 7H DAPI/DNA stain of dorsal murine skin following high temperature cautery depicting removal of epidermal, dermal, and

expression of the LGR6^{+GFP} epithelial stem cells and Figure 14E is a merged image of Figures 14C and 14 D. The scale bar represents 20µm.

Figures 15A-E provide an example of LGR expressing cellular foci as it relates to a method of delivery through placement around and/or within wound/injury/tissue void. The three images of Figure 15A depict, respectively, an initial burn template; a full thickness burn on the dorsum on Nu/Nu mouse; and delivery of HYDROGEL® containing 10⁵ LGR6^{+GFP} epithelial stem cells at the base of the wound bed. The scale bar for Figure 15A is 1mm. Figure 15B is an immunofluorescence image of the injection pocket DNA/DAPI-BLUE at Day 0 Figure 15B is an immunofluorescence image of anti-LGR6/TRITC antibody labeling and Figure 15C the same for LGR6^{+GFP} epithelial stem cells. Figure 15 E is a merged image of Figures 15B-D and has a scale bar of 20µm. Figures 15A-E show full thickness burn wound bed induction and validation of LGR6+ stem cell engraftment into subsequent soft tissue defect.

Figures 16A-D depict an example of LGR containing stem cell focus as it relates to delivery into and around wounds via a deliverable vector and subsequent healing, regeneration of tissues and supporting structures including but not limited blood vessel angiogenesis and/or angiogenesis. Wound healing progression following LGR6+ epithelial stem cells transplantation into full thickness wounds. The progression of wound healing is depicted following the injection of HYDROGEL® from BD Biosciences, San Jose, Calif. (control) in Figure 16A compared with Figure 16B, LGR6^{+GFP} epithelial stem cells seeded HYDROGEL® over 15 days. The scale bar is 1mm. In Figure 16C, showing the implant pocket after day 15, the white arrow indicates presence of a remaining LGR6^{+GFP} epithelial stem cells population located within healing wound bed. In Figure 16D, the black arrow indicates the location of the burn wound base free of LGR6^{+GFP} epithelial stem cells.

Figures 17A-D depicts an example of LGR containing stem cell focus following delivery into and/or around wound with subsequent healing and regeneration of tissues and related appendages such as but not limited to hair follicle and related supportive structures. Figure 17A is a four panel matrix of confocal images of immunofluorescent labeled tissue specimen at day 10 following transplantation of LGR6+ epithelial stem cells migration into the wound

i. Slowly adding 1 part of chilled 10X PBS of 10X culture media to 8 parts of chilled collagen-based solution with gentle swirling. Adding ECM and viability proteins to the suspension;

ii. Adjusting the pH of mixture to 7.2-7.6 using sterile 0.1M NaOH and monitoring the pH adjustment carefully;

iii. Adjusting the final volume to a total of 10 parts with sterile molecular grade water;

iv. Maintaining temperature of mixture at 2 — 10°C to prevent gelation,

v. Forming a gel by warming to 37°C for approximately 90 to 120 minutes;

vi. Perforating the scaffold with a sterile micro-needle press (the scaffold can undergo freeze drying process if needed for storage).

It is also recommended that an additional material referred to as Pulse Rescue Media (PRM) be produced and be available prior to commencement of the LGR aggregate extraction procedures.

The PRM, in this embodiment which is direct to humans, is a cell sustaining, serum-free, media mixture Keratinocyte-SFM containing L-glutamine supplied with separately packaged prequalified human recombinant Epidermal Growth Factor 1-53 (EGF 1-53) and Bovine Pituitary Extract (BPE) sold as Keratinocyte-SFM (1X) from Thermo Fisher Scientific to which the antibiotic-antimycotic agents penicillin, streptomycin, and amphotericin B are added along with a GMP- fibrinogen: human. The agent used in one embodiment is GIBCO® Antibiotic-Antimycotic from Thermo Fisher Scientific, a solution containing 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of FUNGIZONE® Antimycotic. Because the PRM is used to transport human tissues, the supplemental reagents are utilized to stabilize the primary tissues and reduce the viability of micro-organisms during transport and processing.

The following relates specifically to the generation and preservation of LGR expressing epithelial-containing stem cell micro-aggregate functional units in accordance with an embodiment of the invention.

Example 1

Example 1 concerns a method for extraction of minimally polarized functional units in accordance with an embodiment of the invention. After

obtaining a specimen, it is removed from its associated transport container followed by:

- i. Placing the specimen into a sterile 50 ml conical tube containing pulse rescue media and placed on rocker for 5 minutes, repeat with fresh media and container for total of three times;
- ii. Removing and placing the specimen into a sterile culture dish containing pulse media and excise fat and hypodermal elements from the dermal and epidermal compartments carefully. Follicular units are left in place and are not overly dissected;
- iii. Placing excised hypodermal fat components into separate a 50ml conical tube containing PRM and place in +4°C on slow rocker.
- iv. Sectioning the remaining cutaneous elements containing epidermal, follicular and dermal compartments into minimal polarized functional units (MPFUs) using ultrafine WECPREP® Blades or some form of micro-16 lancet; and
- v. Placing the MPFUs components into separate a 50ml conical tube containing pulse media and place in +4°C.

The following relates to secondary processing where the primary cultures are established and functional tissue elements are prepared utilizing enzymatic preparation using conventional CLIA equipment and reagents meeting FDA and/or GMP certification:

Example 2

Example 2 is directed to processing of hypodermis and subdermal fat cellular components. Example 2 recites the following steps:

- i. Spraying 70% ethanol (EtOH) on the outer side of the tissue container and placing the tissue container into laminar air flow cabinet;
- ii. Sending a sample of the tissue or transfer medium for microbiological testing;
- iii. Placing the previously washed adipose and hypodermal tissue in 150mm sterile petri dish;
- iv. Washing the tissue two times with PRM;
- v. Trimming the tissue into small (3mm) pieces with sterile surgical instrument and place into sterile culture holding dish containing pulse media while the dissection is completed;

- vi. Aspirating media from holding dish and removing the specimen with sterile scoop or forceps followed by placing the specimen into 50 ml conical tube containing MSC Enzymatic Digestive Media, a pre-mixed digestive enzyme solution (collagenase and dispase-based), which is placed into a 37°C water bath or dry heat slow shaker and shaken for 30 minutes or until there are few particulate materials remaining;
- vii. Adding 37°C phosphate buffer saline (PBS) ethylenediamine tetra acetic acid (EDTA) (equal volume PBS-EDTA) to stop the digestion;
- viii. Centrifuging the suspension for 10 minutes to generate a “soft” pellet;
- ix. Discarding upper liquid portion and using a sterile pipette, separating the adipose population from stromal vascular fraction (SVF) in the saved mass;
- x. Re-suspending the SVF in phosphate buffer saline/EDTA, PBS-EDTA (1 mM of EDTA), and re-suspending adipocyte population in PRM in two separate conical tubes;
- xi. Using 100 μ m sterile, filter the suspension into new sterile conical tubes;
- xii. Washing the filter with PBS-EDTA;
- xiii. Spin filtering the suspension for 10 minutes at room temperature followed by aspiration of the media and replacing the aspirated media with a known volume of fresh media;
- xiv. Using a COUNTESS® automated cell counter (Thermo Fisher Scientific), count cell populations to determine viability;
- xv. Removing 20% of obtained cell population for cryopreservation with SYNTH-A-FREEZE® CTS™ (Cell Therapy Systems) from Thermo Fisher Scientific and subsequently cataloguing appropriately while using the remaining 80% population for construct assembly.

Example 3

- Example 3 is directed to addition of hypodermis and subdermal fat components to the example of a construct according to an embodiment of the invention. The illustrative component addition example involves:
- i. Placing a sterile NUNC® Skin Graft Cell Culture Dish or automated dish already containing the assembled and washed scaffold into a laminar flow hood and washing the scaffold again two times with pulse media prior to adding cells;

- ii. Inserting a label on each culture vessel with tracking number;
- iii. Transferring around 5×10^5 to 1×10^6 mixed SVF cells per dish system and 1×10^5 adipocytes per dish;
- iv. Adding a complete culture medium with or without autologous PRP as dictated by the particular requirements of a situation, to the loading reservoir;
- 5 v. Transferring the dishes into an incubator onto slow rocker for 1 hour followed by removal therefrom and resting flat for 48 hours in separate sentinel incubator;
- vi. Washing the culture medium after 48 hours, discarding the non-
10 adherent cells, and renewing the complete culture medium. Image with a cell imaging device such as an EVOS® (ThermoFisher Scientific) and store with the designated tracking number.
- vii. Every 72 hours replacing the culture medium;
- viii. At confluence, washing the culture with Dulbecco's phosphate-
15 buffered saline (DPBS) and replacing the culture media with fresh media.
- ix. Placing the epithelial stem cell functional singularity constructs (ESC FSUs) directly on the surface of the mesenchymal stem cell (MSC) construct, adding ESC media to cover both constructs, imaging the same and replacing the construct into the incubator.
- 20 x. Changing/replacing the construct media every 48 hours.

Example 4

- Example 4 concerns enrichment of the minimally polarized, epithelial stem cell singularity units. Following Example 1, the MPFUs is placed in pulse rescue media in a 15 ml conical tube and spin/centrifuged into a soft pellet. The
25 material is then subject to the following process of partial digestion:
- i. Obtaining a previously aliquoted frozen 10ml digestion buffer (collagenase and dispase-based), which has been brought to room temperature prior adding to MPFUs;
 - ii. Adding the digestion solution to the soft pellet of MPFUs and gently
30 mixing, by flicking, the tube to allow MPFUs to distribute throughout the solution;
 - iii. Placing the tube into 37°C water bath or dry incubator for 10 minutes;

- iv. Removing the tube from the bath/incubator, gently flicking tube and examining the content for string;
- v. Having observed string, centrifuging the content into a soft pellet;
- vi. Washing the cell pellet in 5-10 mL complete Defined Keratinocyte SFM medium (Keratinocyte-SFM (1X) from ThermoFisher Scientific) and centrifuging into soft pellet again;
- vii. Re-suspending the pellet of activated functional singularity units in 5 mL of complete the Keratinocyte-SFM medium; and
- viii. Determining the cell density of the units using a COUNTESS® Automated Cell Counter (ThermoFisher Scientific).

Example 5

Example 5 involves adding the epithelial stem cell functional singularities (ESC aFSUs) obtained from Example 4 to a construct/scaffold. The procedure entails:

- i. Placing an UPCELL™ Surface Skin Graft Cell Culture Dish already containing an assembled and washed scaffold, and to assure physiologic pH, washing the scaffold twice again with pulse media prior to adding the cells;
- ii. Labelling each culture vessel with a unique tracking number;
- iii. Transferring ESC aFSUs to the construct via disposable transfer pipette using complete Defined Keratinocyte SFM medium (additional autologous PRP is optional);
- iv. Adding the complete culture medium to a select loading reservoir and ensuring complete coverage of the construct;
- v. Transferring dishes into the incubator onto slow rocker for 1 hour. Then remove from rocker and allow to remain flat for 48 hours in separate sentinel incubator;
- vi. At 48 hours, aspirating the culture medium and adding fresh Keratinocyte SFM culture medium. Imaging the culture with EVOS and storing the culture with the assigned tracking number. Increasing the gingival fibroblasts (GF) population and viability protein and/or supplementing the PRP if a need is detected at this time.
- vii. Replacing the culture medium every 48-72 hours;

viii. Upon achieving confluence, washing the culture with DPBS and replacing the media. Reducing the temperature using temperature based system of the ESC construct scaffolding to facilitate release from the dish;

ix. Placing the ESC directly on the surface of the MSC construct and adding combined media to cover both constructs. Imaging the construct, placing it back into the incubator and changing the construct media every 48 hours.

x. To confirm polarization maintenance, imaging the construct daily and adding an appropriate Cornification (rind forming) medium following confirmation that polarization has been maintained for 48 hours;

xi. Washing the construct twice with pulse media at harvest and replacing the media with a defined transport media using CTSTM STEMPRO[®] MSC SFM base.

Example 6

Example 6 represents illustrative protocols for quality assurance and construct finalization involving cryopreservation which entails preparation the construct for shipment following defined good manufacturing processes (GMP) for cell therapy applications and include:

i. Obtaining an appropriate volume of SYNTH-A-FREEZE[®] cryopreservation medium (Thermo Fisher Scientific) and storing the medium at 2°C to 8°C until use;

ii. Preparing, harvesting and determining cell density using COUNTESS[®] Automated Cell Counter prior to centrifugation a desired quantity of cells where typical cell densities for cryopreservation with SYNTH-A-FREEZE[®] medium are 5×10^5 to 3×10^6 ;

iii. Re-suspending the cell pellet in the pre-determined volume of 2°C to 8°C of SYNTH-A-FREEZE[®] medium;

iv. Immediately dispensing aliquots of the obtained suspension into cryovials according to the manufacturer's specifications;

v. Placing the cryovials into an appropriate cryosystem, such as a MR. FROSTYTM system available from Thermo Fisher Scientific Inc. that maintains freezer temperatures at -80°C;

vi. Transferring the vials to a liquid nitrogen long-term vapor-phase storage at -200°C to -125°C.

I claim:

1. A composition, comprising:
 - (i) a multi-dimensional support selected from the group consisting of scaffolding, collagen, matrix, particle, and fiber; and
 - (ii) living LGR-expressing epithelial stem cells;wherein said composition is a minimally polarized, micro-aggregate, multi-cellular composition.
2. The composition of claim 1, further comprising soluble growth factors.
3. The composition of claim 1, further comprising enhancing factors or analytes.
4. The composition according to any one of claims 1 to 3, further comprising LGR-specific binding elements selected from a ligand family, R-spondin, an epidermal-derived growth factor (EDGF), a platelet-derived growth factor (PDGF), a Wnt protein, a vascular endothelial growth factor (VEGF), and an antimicrobial peptide.
5. The composition according to any one of claims 1 to 4, wherein the LGR-expressing epithelial stem cells are selected from LGR4-expressing cells, LGR5-expressing cells or LGR6-expressing cells.
6. A method of producing a minimally polarized micro-aggregate multi-cellular composition, the method comprising:
 - a) extracting at least one minimally polarized functional unit containing LGR-expressing cells from a mammalian tissue specimen;
 - b) processing of hypodermis and subdermal fat cellular components of the mammalian tissue specimen to provide a mesenchymal-derived cellular population;

- c) adding at least a portion of the mesenchymal-derived cellular population to the at least one minimally polarized functional unit to create at least one epithelial stem cell functional singularity unit;
- d) enriching the at least one epithelial stem cell functional singularity unit;
- 5 and
- e) adding the at least one enriched epithelial stem cell functional singularity unit to a delivery substrate.

10 7. The method of claim 6, wherein the delivery substrate is selected from scaffolding, collagen, matrix, particle, cells and fiber.

15 8. The method of claim 6, wherein the LGR-expressing stem cells are selected from LGR4-expressing cells, LGR5-expressing cells and LGR6-expressing cells.

9. The method of claim 6, further comprising supplementing the composition with one or more supplements selected from a growth factor, an analyte and an LGR-specific binding element.

20 10. The method of claim 9, wherein the one or more supplements is selected from a ligand family, R-spondin, an epidermal-derived growth factor (EDGF), a platelet-derived growth factor (PDGF), a Wnt protein, a vascular endothelial growth factor (VEGF), and an antimicrobial peptide.

25 11. The method of claim 6, further comprising adding cornification medium to the composition.

30 12. The method of claim 6, further comprising cryopreserving the composition.

13. The method of claim 6, further comprising adding enhancing factors or analytes to the composition.

14. A composition produced by the method of claim 6.

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15. A composition as defined in any one of claims 1 to 5 for use in therapy.

16. A composition as defined in any one of claims 1 to 5 for use:

10 i) in the treatment of wounds, wherein the composition accelerates healing of the wounds; and/or

ii) in the treatment of tissue, wherein the composition repairs or restores tissue; and/or

iii) as a therapeutic construct for treating any one of a tissue region, wound, void, defect tissue, or blood; and/or

15 iv) as a therapeutic construct in a treatment to alter surrounding adjacent tissues.

17. The composition of claim 1, wherein the living LGR-expressing epithelial stem cells are cutaneous epithelial stem cells.