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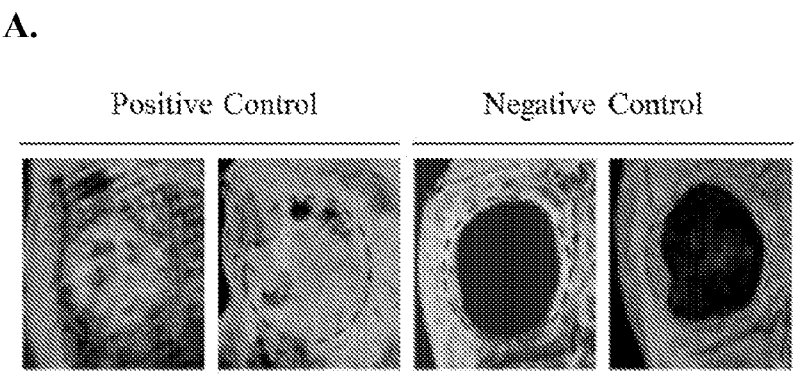
(54) Title: SOMATIC STEM CELLS FOR TREATING BONE DEFECTS

FIG. 1



(57) Abstract: A method of treating a bone defect in a subject, comprising administering to a subject in need thereof at a bone defect site an effective amount of isolated somatic stem cells, wherein the somatic stem cells are about 2 to 8.0 μm in size and are Lgr5+ or CD349+.

SOMATIC STEM CELLS FOR TREATING BONE DEFECTS

CROSS REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Provisional Application No. 62/081,880, filed on November 19, 2014, the entire content of which is hereby incorporated by reference herein.

BACKGROUND

Stem cells are pluripotent or totipotent cells that can differentiate *in vivo* or *in vitro* into many or all cell lineages. Due to their pluripotency, embryonic stem (ES) cells hold great promise for treating various diseases. Yet, ethical considerations have hampered the use of human ES cells. Stem cells of a non-embryonic origin would circumvent this obstacle. These adult stem cells have the same capability for differentiation as do ES cells.

Multipotent adult progenitor cells from bone marrow have been isolated that can differentiate into ectoderm, mesoderm and endoderm. Other types of cells, including marrow-isolated adult multi-lineage inducible cells and single cell clones derived from bone marrow also have the same multi-potential ability for differentiation. Such multipotent somatic cells are difficult to obtain, culture, and expand.

SUMMARY

Described herein is a method of treating a bone defect in a subject. The method includes administering to a subject in need thereof at a bone defect site an effective amount of isolated somatic stem cells. The somatic stem cells are about 2 to 8.0 μm in size and are Lgr5+ or CD349+.

The isolated somatic stem cells can be obtained by the following procedure: incubating a sample from a donor subject with EDTA or heparin in a container until the sample is separated into an upper layer and a lower layer; collecting the upper layer; and isolating from the upper layer a population of somatic stem cells that are about 2 to 8.0 μm in size and are Lgr5+ or CD349+.

The details of one or more embodiments are set forth in the accompanying drawing and the description below. Other features, objects, and advantages of the embodiments will be apparent from the description and drawing, and from the claims.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 is a set of images that demonstrate repair of a cranial defect using SB cells. (A): positive and negative controls. (B): SB cells.

DETAILED DESCRIPTION

It was unexpectedly discovered that small adult stem cells, i.e., SB cells, can be isolated from samples from subjects. SB cells are pluripotent or totipotent stem cells that can differentiate into cell types associated with the three embryonic germ layers, namely, ectoderm, endoderm, and mesoderm. See US2012/0034194.

SB cells isolated from a biological sample (e.g., a bone marrow sample) are about 2 to 6.0 μm in size, CD133-, CD34-, CD90-, CD66e-, CD31-, Lin1-, CD61-, Oct4+, Nanog+, and Sox2-. Among the SB cell population, there is a unique subpopulation of cells that are CD9- and Lgr5+ ("Lgr5+ SB cells"). There is another subpopulation of SB cells that are CD9+ and CD349+ ("CD349+ SB cells").

SB cells can be isolated from a sample using the following procedure. The sample is incubated with EDTA or heparin in a container (e.g., in an EDTA tube) until the sample separates into an upper layer and a lower layer. The incubation can be performed for 6 to 48 hours at 4°C. The upper layer produced by the above incubating step contains SB cells (e.g., Lgr5+ SB cells and CD349+ SB cells), which can be isolated using methods based on cell size (e.g., centrifuging and filtering) or those based on cell surface markers (e.g., flow cytometry, antibodies, and magnetic sorting).

To enrich SB cells, Lin+ cells and CD61+ cells can be removed from the cell population in the upper layer. Alternatively, Lin- cells and CD61- cells can be selected from the cell population. Lin+ and CD61+ cells can be removed or selected using methods known in the art, e.g., EasySep Biotin Selection Kit and EasySep PE Selection Kit.

To further enrich SB cells, granulocyte-colony stimulating factor (GCSF) or fucoidan can be administered to a subject before a sample is obtained from the subject. For example, the subject can be injected with 5μg/kg/per day of GCSF for 1

to 5 days prior to obtaining the sample. Data described below show that GCSF can mobilize SB cells. GCSF-mobilized SB cells are slightly larger in size, i.e., about 4 to 8 μm.

SB cells can be isolated from a sample such as a blood, bone marrow, skeletal muscle, or adipose tissue sample. In an embodiment where the sample is a skeletal muscle or adipose tissue sample, prior to the incubating step, the tissue sample can be first digested with a collagenase to release individual cells from the extracellular matrix. The sample can be obtained from a human subject.

Isolated SB cells, Lgr5+ SB cells, or CD349+ SB cells can be further propagated in a non-differentiating medium for more than 10, 20, 50, or 100 population doublings without indications of spontaneous differentiation, senescence, morphological changes, increased growth rate, or changes in ability to differentiate. These stem cells can be stored by standard methods before use.

The term “stem cell” refers to a cell that is totipotent or pluripotent, i.e., capable of differentiating into a number of final, differentiated cell types. Totipotent stem cells typically have the capacity to develop into any cell type. Totipotent stem cells can be embryonic or non-embryonic in origin. Pluripotent cells are typically cells capable of differentiating into several different, final differentiated cell types. Unipotent stem cells can produce only one cell type, but have the property of self-renewal which distinguishes them from non-stem cells. These stem cells can originate from various tissue or organ systems, including blood, nerve, muscle, skin, gut, bone, kidney, liver, pancreas, thymus, and the like.

The stem cells disclosed herein are substantially pure. The term "substantially pure", when used in reference to stem cells or cells derived there from (e.g., differentiated cells), means that the specified cells constitute the majority of cells in the preparation (i.e., more than 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95%). Generally, a substantially purified population of cells constitutes at least about 70% of the cells in a preparation, usually about 80% of the cells in a preparation, and particularly at least about 90% of the cells in a preparation (e.g., 95%, 97%, 99% or 100%).

The terms "proliferation" and "expansion," as used interchangeably herein with reference to cells, refer to an increase in the number of cells of the same type by division. The term "differentiation" refers to a developmental process whereby cells become specialized for a particular function, for example, where cells acquire one or

more morphological characteristics and/or functions different from that of the initial cell type. The term "differentiation" includes both lineage commitment and terminal differentiation processes. Differentiation may be assessed, for example, by monitoring the presence or absence of lineage markers, using immunohistochemistry or other procedures known to a worker skilled in the art. Differentiated progeny cells derived from progenitor cells may be, but are not necessarily, related to the same germ layer or tissue as the source tissue of the stem cells. For example, neural progenitor cells and muscle progenitor cells can differentiate into hematopoietic cell lineages.

The terms "lineage commitment" and "specification," as used interchangeably herein, refer to the process a stem cell undergoes in which the stem cell gives rise to a progenitor cell committed to forming a particular limited range of differentiated cell types. Committed progenitor cells are often capable of self-renewal or cell division.

The term "terminal differentiation" refers to the final differentiation of a cell into a mature, fully differentiated cell. For example, neural progenitor cells and muscle progenitor cells can differentiate into hematopoietic cell lineages, terminal differentiation of which leads to mature blood cells of a specific cell type. Usually, terminal differentiation is associated with withdrawal from the cell cycle and cessation of proliferation. The term "progenitor cell," as used herein, refers to a cell that is committed to a particular cell lineage, which gives rise to cells of this lineage by a series of cell divisions. An example of a progenitor cell would be a myoblast, which is capable of differentiation to only one type of cell, but is itself not fully mature or fully differentiated.

Lgr5+ or CD349+ SB cells can be used to treat or repair a bone defect in a patient. To treat a bone defect in a patient, Lgr5+ or CD349+ SB cells alone can be administered to the subject at the defect site. The cells can also be administered together with a bone graft (e.g., an autograft or allograft) or a bone graft substitute (e.g., demineralized bone matrix, collagen-based matrix, hydroxyapatite, calcium phosphate, and calcium sulfate).

Lgr5+ or CD349+ SB cells can also be first implanted in a scaffold or matrix. The scaffold or matrix can then be implanted at the defect site. Stem cell scaffolds composed of one or more materials (e.g., collagen, agarose, alginate, hyaluronan, chitosan, PLGA, and PEG) are known in the art.

A "bone defect" refers to a lack or deficiency of bone tissue (i.e., the mineralized matrix of a bone) in an area in a bone. A bone defect can result from various causes, such as trauma, cancer, or a congenital condition.

Both heterologous and autologous Lgr5+ or CD349+ SB cells can be used to treat a patient. If heterologous cells are used, HLA-matching should be conducted to avoid or minimize host reactions. Autologous cells can be enriched and purified from a subject and stored for later use. The cells may be cultured in the presence of host or graft T cells *ex vivo* and re-introduced into the host. This may have the advantage of the host recognizing the cells as self and better providing reduction in T cell activity.

Genetically engineered histocompatible universal donor Lgr5+ or CD349+ SB cells can also be prepared using methods known in the art. More specifically, the stem cells described herein can be genetically engineered to not express on their surface class II MHC molecules. The cells can also be engineered to not express substantially all cell surface class I and class II MHC molecules. As used herein, the term "not express" means either that an insufficient amount is expressed on the surface of the cell to elicit a response or that the protein that is expressed is deficient and therefore does not elicit a response.

"Treating" refers to administration of a composition (e.g., a cell composition) to a subject, who is suffering from or is at risk for developing that disorder, with the purpose to cure, alleviate, relieve, remedy, delay the onset of, prevent, or ameliorate the disorder, the symptom of the disorder, the disease state secondary to the disorder, or the predisposition toward the damage/disorder. An "effective amount" refers to an amount of the composition that is capable of producing a medically desirable result in a treated subject. The treatment method can be performed alone or in conjunction with other drugs or therapies.

The specific example below is to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. Without further elaboration, it is believed that one skilled in the art can, based on the description herein, utilize the present disclosure to its fullest extent. All publications cited herein are hereby incorporated by reference in their entirety.

EXAMPLE

A bone marrow sample was drawn from a human subject and placed in an anti-clotting EDTA tube. After incubating the tube for 6 to 48 hours at 4°C, the

sample separated into two layers. The top layer contained a somatic stem cell population (SB cells), which was further analyzed by C6 accuri flow cytometry, immunocytochemistry, and RT-PCR. The bottom layer contained red and white blood cells, which are not smaller than 6.0 μm.

Using sizing beads to run the flow cytometry, the size of the SB cells was determined. The size of the SB cells was between 2 and 6 microns. SB cells were either Lgr5+ or CD349+. Lgr5 was expressed by 32% of the cell population in gate P2.

We found that SB cells can be mobilized by injection of GCSF. The same human subject was injection with 5μg/kg/per day of GCSF for 5 days. A peripheral blood sample was collected about 3.5 hours after the last injection. SB cells were isolated from the blood sample as described above and analyzed by flow cytometry. As compared to SB cells isolated from the subject prior to the GCSF injection, the cell size increased to 4~8 micron and the percentage of Lgr5+ cells also increased.

Normal human blood (purchased from AllCell) was placed in an anti-clotting EDTA tube, to which HetaStarch (purchased from StemCell) was added. The blood sample separated into two layers. CD61+ platelets and Lin+ cells, which include red and white blood cells, were removed from the top layer using the EasySep Biotin Selection Kit and EasySep PE Selection Kit, respectively, following the manufacturer's instructions. After Lin+ and CD61+ cells were removed, a purified population of Lgr5+ or CD349+ SB cells was obtained.

1 million of the above purified SB cells together with collagen sponges were engrafted into a SCID mouse at a cranial defect site created by surgical removal of a section of the bone from the skull. The mouse was analyzed by microcomputed tomography images 3 or 5 months after engraftment of the SB cells in the defected site. As shown in Fig. 1, SB cells were able to form bone structures to repair the defect site. A mouse treated with human bone marrow cells that overexpress a human bone morphogenetic protein 7 (hBMP7) was used as a positive control. A mouse treated with collagen sponges and PBS only was used as a negative control.

OTHER EMBODIMENTS

All of the features disclosed in this specification may be combined in any combination. Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose. Thus, unless

expressly stated otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.

From the above description, one skilled in the art can easily ascertain the essential characteristics of the described embodiments, and without departing from the spirit and scope thereof, can make various changes and modifications of the embodiments to adapt it to various usages and conditions. Thus, other embodiments are also within the claims.

CLAIMS

1. A method of treating a bone defect in a subject, comprising administering to a subject in need thereof at a bone defect site an effective amount of isolated somatic stem cells, wherein the somatic stem cells are 2 to 8.0 μm in size and are Lgr5+ or CD349+.

2. The method of claim 1, wherein the somatic stem cells are CD133-, CD34-, CD90-, CD66e-, CD31-, Lin1-, CD61-, Oct4+, Nanog+, and Sox2-.

3. The method of claim 1 or 2, wherein the somatic stem cells are Lgr5+.

4. The method of claim 1 or 2, wherein the somatic stem cells are obtained by the following procedure:

incubating a sample from a donor subject with EDTA or heparin in a container until the sample is separated into an upper layer and a lower layer,

collecting the upper layer, and

isolating from the upper layer a population of somatic stem cells that are 2 to 8.0 μm in size and are Lgr5+ or CD349+.

5. The method of claim 4, wherein the sample is a blood or bone marrow sample.

6. The method of claim 5, wherein, prior to obtaining the sample from the donor subject, the donor subject is administered with a granulocyte-colony stimulating factor or fucoidan.

7. The method of claim 1 or 2, wherein the somatic stem cells are autologous or heterologous to the subject.

8. The method of claim 1 or 2, further comprising, prior to the administering step:

incubating a sample from a donor subject with EDTA or heparin in a container until the sample is separated into an upper layer and a lower layer,

collecting the upper layer, and

isolating from the upper layer a population of somatic stem cells that are about 2 to 8.0 μm in size and are Lgr5+ or CD349+.

9. The method of claim 8, wherein the sample is a blood or bone marrow sample.

10. The method of claim 9, further comprising removing Lin+ cells and CD61+ cells from the upper layer.

11. The method of claim 9 or 10, wherein, before the sample is obtained from the donor subject, the donor subject is administered with a granulocyte-colony stimulating factor or fucoidan.

12. The method of claim 11, wherein the donor subject is the subject with a bone defect or another subject.

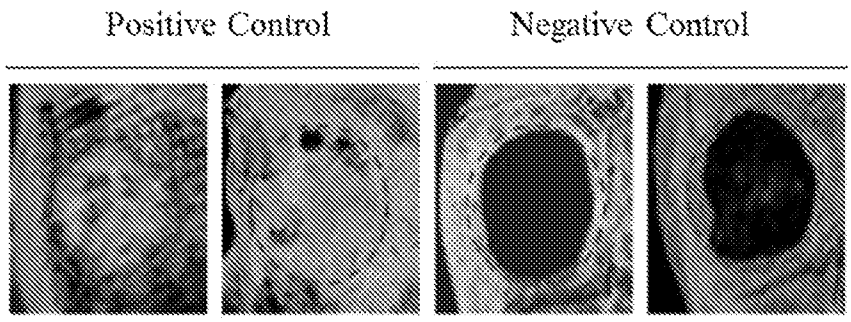
13. The method of any of claims 1-12, further comprising administering a bone graft or bone graft substitute to the bone defect site.

14. The method of any of claims 1-13, wherein the somatic stem cells are implanted in a scaffold.

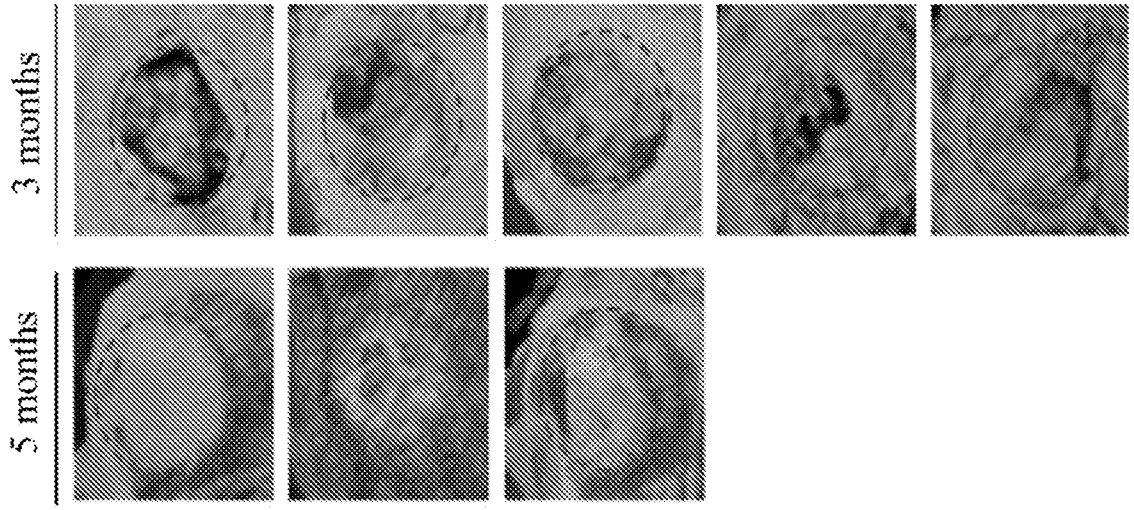
15. Isolated somatic stem cells for use in treating a bone defect in a subject, wherein the somatic stem cells are 2 to 8.0 μm in size and are Lgr5+ or CD349+.

FIG. 1

A.



B.



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/061257

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 35/28 (2016.01)
CPC - A61K 35/28 (2015.12)
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) - A61K 35/14, 35/28, 38/19 ; C12N 5/074, 5/0775 (2016.01) (2016.01)
CPC - A61K 35/14, 35/28, 38/193 ; C12N 5/0607, 5/0663 (2015.12)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/85.1, 93.7 ; 435/366, 325
CPC - A61K 35/14, 35/28, 38/193 ; C12N 5/0607, 5/0663 (2015.12) (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Orbit, Google Patents, Google Scholar.
Search terms used: LGR5+, CD349+, bone, osteo+, repair, defect, injury, fracture, regeneration, differentiation, mobilization, administration, granulocyte, G-CSF, fucoidan, stem cells

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2014/0161774 A1 (STEMBIOS TECHNOLOGIES INC) 12 June 2014 (12.06.2014) entire document	1-5, 7-10, 15 --- 6
Y	US 2014/0219952 A1 (CAMERON) 07 August 2014 (07.08.2014) entire document	6
A	US 2011/0305673 A1 (SPEES) 15 December 2011 (15.12.2011) entire document	1-10, 15
A	US 2007/0190023 A1 (BATTISTA et al) 16 August 2007 (16.08.2007) entire document	1-10, 15

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* 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	Date of mailing of the international search report
13 January 2016	01 FEB 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/061257

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 11-14
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

(19)中华人民共和国国家知识产权局

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A61P 19/08(2006.01)

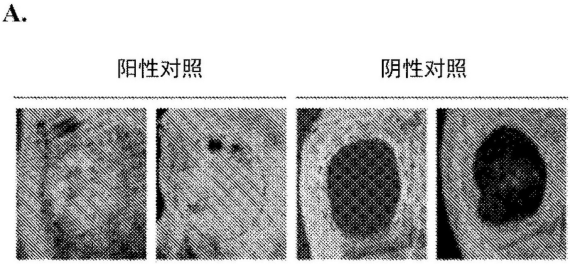
权利要求书1页 说明书4页 附图1页

(54)发明名称

用于治疗骨骼缺损的体干细胞

(57)摘要

一种治疗个体内的骨骼缺损的方法，其包含施用有效量的分离的体干细胞至需要其的个体内的骨骼缺损处，其中所述体干细胞大小为大约2至8.0μm且为Lgr5+或CD349+。



CN 106573018 A

1.一种治疗个体内的骨骼缺损的方法,其包含施用有效量的分离的体干细胞至需要其的个体内的骨骼缺损处,其中所述体干细胞大小为2至8.0μm且为Lgr5+或CD349+。

2.如权利要求1所述的方法,其中所述体干细胞为CD133-、CD34-、CD90-、CD66e-、CD31-、Lin1-、CD61-、Oct4+、Nanog+,以及Sox2-。

3.如权利要求1或2所述的方法,其中所述体干细胞为Lgr5+。

4.如权利要求1或2所述的方法,其中所述体干细胞通过下列的程序来获得:

于容器中以EDTA或肝素培养源自供体个体的样本直到所述样本分离成上层和下层,

收集所述上层,以及

从所述上层分离体干细胞群,其大小为2至8.0μm且为Lgr5+或CD349+。

5.如权利要求4所述的方法,其中所述样本为血液或骨髓样本。

6.如权利要求5所述的方法,其中,在从所述供体个体获得所述样本之前,所述供体个体被施用粒细胞集落刺激因子或褐藻糖胶(fucoidan)。

7.如权利要求1或2所述的方法,其中所述体干细胞对所述个体为自体的或异源性的。

8.如权利要求1或2所述的方法,在所述施用步骤之前,所述方法进一步包含:

于容器中以EDTA或肝素来培养源自供体个体的样本直到所述样本分离成上层和下层,

收集所述上层,以及

从所述上层分离体干细胞群,其大小为大约2至8.0μm且为Lgr5+或CD349+。

9.如权利要求8所述的方法,其中所述样本为血液或骨髓样本。

10.如权利要求9所述的方法,其进一步包含从所述上层移除Lin+细胞及CD61+细胞。

11.如权利要求9或10所述的方法,其中,在从所述供体个体获得所述样本之前,所述供体个体被施用颗粒球群落刺激因子或褐藻糖胶。

12.如权利要求11所述的方法,其中所述供体个体为具有骨骼缺损的供体或是另一个个体。

13.如权利要求1-12中任一项所述的方法,其进一步包含施用骨骼移植物或骨骼移植物代用品至所述骨骼缺损处。

14.如权利要求1-13中任一项所述的方法,其中所述体干细胞植入于支架内。

15.一种分离的体干细胞,其用于治疗个体内的骨骼缺损,其中所述体干细胞大小为2至8.0μm且为Lgr5+或CD349+。

用于治疗骨骼缺损的体干细胞

[0001] 相关申请案的交叉引述

[0002] 本申请案主张2014年11月19日提交的美国专利临时申请案第62/081,880的优先权,其整体内容并入本文以作为参考数据。

发明领域

[0003] 本发明有关于用于治疗骨骼缺损的体干细胞。

[0004] 发明背景

[0005] 干细胞为多潜能的或全能的干细胞,其能于活体内或活体外分化成许多的或者全部的细胞谱系。由于其多能性,胚胎干(ES)细胞保有治疗各种各样的疾病的巨大潜力。然而,道德的考虑已妨碍了人类ES细胞的用途。非胚胎起源的干细胞能规避此障碍。这些成体干细胞具有如同ES细胞一样的分化能力。

[0006] 已分离源自骨髓的多潜能的成体祖细胞,其能分化成外胚层、中胚层以及内胚层。其他类型的细胞,包括骨髓分离的成体多谱系可诱导的细胞以及衍生自骨髓的单细胞克隆,也具有相同多潜能的分化能力。这些多潜能的体细胞不易获得、培养,及扩增。

发明概要

[0007] 本文所描述的是一种治疗个体内的骨骼缺损的方法。该方法包括施用有效量的分离的体干细胞至需要其的个体内的骨骼缺损处。该体干细胞大小为大约2至8.0μm且为Lgr5+或CD349+。

[0008] 该分离的体干细胞可以通过下列的程序来获得:于容器中以EDTA或肝素来培养源自供体个体的样本直到该样本分离成上层和下层;收集该上层;以及从该上层单离体干细胞群,其大小为大约2至8.0μm且为Lgr5+或CD349+。

[0009] 以下的附图及说明中列举一个或更多个实施方案的细节。从本说明及图示,以及从权利要求将更明了该实施方案的其他特征、目的及优点。

[0010] 附图简述

[0011] 图1为一组影像,其展现出利用SB细胞进行的颅缺损的修复。(A):阳性和阴性对照。(B):SB细胞。

[0012] 较佳实施例的详细说明

[0013] 意外地发现小成体干细胞(small adult stem cells),也即SB细胞,可以从来自个体的样本分离。SB细胞为多潜能的或全能的干细胞,其能分化成与三种胚胎胚层,即外胚层、内胚层及中胚层,有关联的细胞类型。参见,US2012/0034194。

[0014] 从生物样本(例如,骨髓样本)分离的SB细胞大小为大约2至6.0μm,CD133-、CD34-、CD90-、CD66e-、CD31-、Lin1-、CD61-、Oct4+、Nanog+,以及Sox2-。在SB细胞群中,有一种独特的细胞亚群,其为CD9-及Lgr5+(“Lgr5+SB细胞”)。有另一种SB细胞亚群,其为CD9+及CD349+(“CD349+SB细胞”)。

[0015] SB细胞可以使用下列的程序从样本分离。该样本用EDTA或肝素于容器中(例如,于

EDTA管中)培养,直到该样本分离成上层和下层。培养可以在4℃下执行历时6至48小时。由以上的培养步骤生产的上层含有SB细胞(例如,Lgr5+SB细胞及CD349+SB细胞),其分离可以使用根据细胞大小的方法(例如,离心和过滤),或者根据细胞表面标志的那些方法(例如,流式细胞术、抗体,以及磁分选)。

[0016] 为了富集(enrich)SB细胞,可以从上层中的该细胞群移除Lin+细胞及CD61+细胞。备选地,可以从该细胞群选择Lin-细胞及CD61-细胞。Lin+及CD61+细胞可以使用本领域已知的方法予以移除或选择,例如,EasySep Biotin Selection Kit及EasySep PE Selection Kit。

[0017] 为了进一步富集SB细胞,在从该个体获得样本之前,可以施用粒细胞集落刺激因子(GCSF)或褐藻糖胶(fucoidan)至该个体。举例而言,在获得样本以前,该个体可以注射以5μg/kg/每天的GCSF历时1至5天。以下所描述的数据显示GCSF可以动员SB细胞。GCSF-动员的SB细胞的大小稍微大一些,也即,大约4至8μm。

[0018] SB细胞可以从样本分离,例如血液、骨髓、骨骼肌,或者脂肪的组织样本。在样本为一种骨骼肌或者脂肪组织样本的一个实施方案中,在培养步骤以前,组织样本首先可以用胶原蛋白酶来消化以从细胞外的基质释放个别的细胞。该样本可以获得自人类个体。

[0019] 分离的SB细胞,Lgr5+SB细胞,或者CD349+SB细胞可以于非分化培养基中进一步增殖超过10、20、50,或者100群倍增,而没有显示自发性的分化、衰老、形态变异、增高的生长速率,或者分化能力改变的情况。这些干细胞在使用前可以用标准的方法予以储存。

[0020] 术语“干细胞”系是指一种细胞,其为全能或多潜能的,电即能够分化成一些最终的、分化细胞类型。全能干细胞典型地具有发展成任一种细胞类型的能力。全能的干细胞的起源可以为胚胎或者非胚胎。多潜能的细胞典型为细胞,其能够分化成数种的不同的、最终分化的细胞类型。单潜能干细胞仅可以生产一种细胞类型,但是其具有能与非干细胞区分的自我更新的性质。这些干细胞可以发源自各种各样的组织或者器官系统,包括血液、神经、肌肉、皮肤、肠、骨骼、肾脏、肝脏、胰脏、胸腺,以及类似物。

[0021] 本文中所公开的干细胞为实质纯的。术语"实质纯的",当参照干细胞或者干细胞衍生的细胞(例如,分化的细胞)来使用时,意指该特定的细胞组成制备物中多数的细胞(也即,超过20%、30%、40%、50%、60%、70%、80%、90%,或者95%)。一般说来,一种实质纯化的细胞群组成制备物中至少大约70%的细胞,通常为制备物中大约80%的细胞,以及特别地为制备物中至少大约90%的细胞(例如,95%、97%、99%或者100%)。

[0022] 当在本文中可交换地使用时,有关于细胞的术语"增生(proliferation)"和"扩增(expansion)",意指相同类型的细胞数目通过分裂而增加。术语"分化"是指一种发展过程,通过此发展过程细胞变成特化成一种特定的功能,举例而言,细胞获得与最初的细胞类型相异的一种或多种形态特征及/或功能。术语"分化"包括谱系确定(lineage commitment)及终末分化(terminal differentiation)过程二者。分化的评估可以,举例而言,通过使用免疫组织化学法或者本领域技术人员已知的其他程序,来监测存在或者缺少谱系标志。衍生自祖细胞的分化的后代细胞可以,但是非必需,相关于干细胞的来源组织相同的胚层或者组织。举例而言,神经祖细胞及肌肉祖细胞可以分化成造成血细胞谱系。

[0023] 当在本文中可交换地使用时,术语"谱系确定(lineage commitment)"及"规格(specification)",涉及干细胞经历的过程,其中所述干细胞产生祖细胞确定会形成一种

特定的限定范围的分化细胞类型。确定的祖细胞通常能够自我更新或者细胞分裂。

[0024] 术语"终末分化"是指一种细胞成为成熟、完全分化的细胞的最终的分化。举例而言，神经祖细胞及肌肉的祖细胞可以分化成造血细胞谱系，其终末分化导致一种特定的细胞类型的成熟的血液细胞。通常，终末分化与退出细胞周期及增生中断有关联。当使用于本文中，术语"祖细胞"是指一种细胞，其确定会成一种特定的细胞谱系，其通过一系列的细胞分裂来产生此谱系的细胞。祖细胞的实例会是一种成肌细胞(myoblast)，其仅仅能够分化成一种细胞类型，但是其自身不是完全成熟或者完全分化的。

[0025] Lgr5+或者CD349+SB细胞可以用来治疗或修复病人体内的骨骼缺损。为了治疗病人体内的骨骼缺损，Lgr5+或者CD349+SB细胞可以单独地施用至该个体缺损处。该细胞也可以连同骨骼移植物(例如，自体移植物或同种异体移植物)或骨骼移植物代用品(例如，去矿质骨骼基质、基于胶原蛋白的基质、羟磷石灰、磷酸钙，以及硫酸钙)一起施用。

[0026] Lgr5+或者CD349+SB细胞也可以先植入于支架或基质内。该支架或基质继而植入缺损处。组成干细胞支架的一种或多种材料(例如，胶原蛋白、琼脂糖、藻酸盐、透明质酸(hyaluronan)、壳聚糖、PLGA，以及PEG)为本领域已知的。

[0027] "骨骼缺损"是指于骨骼中的一个区域缺少或者缺乏骨骼组织(也即，矿化的骨骼基质)。骨骼缺损可以起因于各种各样的原因，例如创伤、癌症，或者先天性疾病。

[0028] 异源性及自体的Lgr5+或者CD349+SB细胞二者均可以用来治疗病人。如果使用异源性细胞，应该要进行HLA-配型(HLA-matching)以避免宿主反应或者使宿主反应减到最少。自体的细胞可以从个体富集及纯化以及储存以备后用。该细胞可以在活体外(ex vivo)宿主或者移植物T细胞存在下予以培养，以及再导入至宿主内。此可能有的优点为宿主将细胞识别为自我的以及对T细胞活性提供更佳缩减。

[0029] 遗传工程改造的组织相容性万能供血者(histocompatible universal donor)Lgr5+或者CD349+SB细胞，也可以使用本领域已知的方法来制备。更特别地，本文所描述的干细胞可以予以遗传工程改造成不表达其表面II型MHC分子。该细胞也可以予以工程改造成基本上不表达全部的细胞表面I型和II型MHC分子。当使用于本文中时，术语"不表达"意指不足以引起反应的量表达在细胞表面上，或者表达的蛋白质是有缺陷的并且且因而不会引起反应。

[0030] "治疗(Treating)"是指施用一种组合物(例如，一种细胞组合物))至个体，该个体患有该病症或者处于发展该病症的风险，目的为了治愈、缓和、缓解、医治(remedy)、延迟发作、预防，或者减轻该病症，病症的症状，病症继发的疾病状态，或者损伤/病症的素因。"有效量"是指组合物的量能够在治疗的个体内产生医学上所需的结果。治疗的方法可以单独地执行或者结合其他的药物或者疗法来执行。

[0031] 以下特定的实施例要解读为仅仅作例证的，以及无论以任何方式不限制本公开的其余部分。在没有进一步详细阐述的情况下，据信技术人员根据本文的说明、能够最大程度地使用本公开。本文中列举的全部出版物以其整体并入以作为参考数据。

实施例

[0032] 从人类个体取出骨髓样本以及放置于抗凝血的EDTA管中。在4℃下培养历时6至48小时之后，该样本分离成两层。顶层含有体干细胞群(SB细胞)，其经由C6accuri流式细胞

术、免疫细胞化学法，以及RT-PCR予以进一步的分析。底层含有红及白血液细胞，其不小于6.0μm。

[0033] 使用分级小珠来进行流式细胞术，决定SB细胞的大小。SB细胞的大小系介于2和6微米之间。SB细胞不是Lgr5+就是CD349+。P2闸(gate)中的细胞群的32％表达Lgr5。

[0034] 我们发现SB细胞可以经由注射GCSF予以动员。相同的人类个体注射以5μg/kg/每天的GCSF历时5天。最后的注射大约3.5小时之后，收集周边的血液样本。SB细胞系如以上所述从血液样本分离以及经由流式细胞术来分析。当与GCSF注射以前从该个体分离的SB细胞比较，细胞的大小增加至4～8微米以及Lgr5+细胞的百分比也增加。

[0035] 将正常的人类血液(购自于AllCell)放置于抗凝血的EDTA管中，添加HetaStarch(购自于StemCell)。血液样本分离成两层。CD61+血小板和Lin+细胞，其包括红及白血液细胞，分别依照制造商的说明来使用EasySep Biotin Selection Kit和EasySep PE Selection Kit而从顶层移开。之后，移动Lin+和CD61+细胞，获得一种经纯化的Lgr5+或者CD349+SB细胞群。

[0036] 一百万个以上经纯化的SB细胞连同胶原蛋白海绵一起植入至SCID小鼠于颅缺损处，该处通过从颅骨移除一块骨骼所产生。在SB细胞植入于缺损处3或5个月之后，通过微型计算机断层摄影影像来分析小鼠。如同图1中所显示的，SB细胞能够形成骨骼结构以修复缺损处。一种以人类骨髓细胞来治疗的小鼠，该人类骨髓细胞过度表达一种人类骨成形性蛋白质7(hBMP7)，用作阳性对照。用只有胶原蛋白海绵的及PBS来治疗的小鼠用作阴性对照。

[0037] 其他实施方案

[0038] 本说明书所公开的全部特征可以以任何组合进行组合。本说明书所公开的各个特征可以用提供相同的、均等的，或者相似的目的的替代的特征来代替。因而，除非另有明确陈述，否则所公开的各个特征仅仅为一种通用系列的均等物或者相似的特征的实例。

[0039] 从以上的说明，本领域技术人员可以容易地确定所描述实施方案的必须的特征，以及在没有背离其精神和范畴的情况下，可以做出实施方案各种各样的改变和修饰以适应各种各样的用法和条件。因而，其他的实施方案也落在权利要求的范围内。

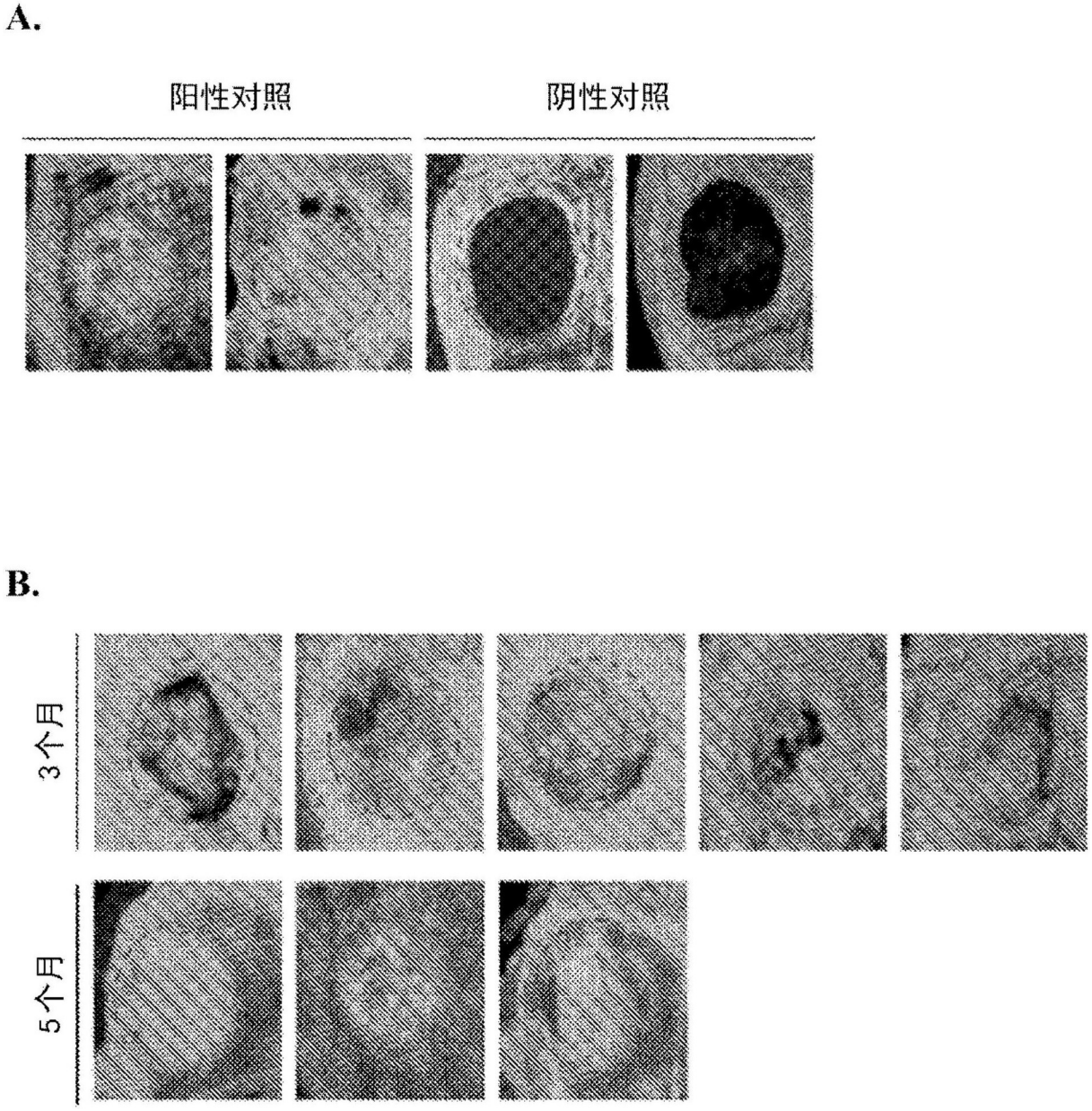


图1