bone marrow donation, and more generally for further and/or improved methods for obtaining MSC-derived cells and cell products useful in among others regenerative therapy.

SUMMARY

In an aspect of the present disclosure, there is provided a method for obtaining mesenchymal stem cell (MSC)-derived cells of chondro-osteoblastic lineage from MSC, the method comprising:

(a) culturing MSC recovered from a biological sample of a subject in a culture medium comprising fibroblast growth factor-2 (FGF-2), transforming growth factor beta (TGFβ) and heparin or a derivative or analogue thereof at a concentration of at least 0.01 IU/ml, thereby obtaining mesenchymal stem cell (MSC)-derived cells;

(b) passaging the MSC-derived cells for a first time and further culturing the MSC-derived cells in a medium as defined in (a); and

(c) passaging the MSC-derived cells for a second time and further culturing the MSC-derived cells in a medium as defined in (a), thereby obtaining the MSC-derived cells of chondro-osteoblastic lineage,

wherein the MSC-derived cells are cultured in step (b) for a period of x days, wherein day x is the last day on which at least 20% of the MSC-derived cells are proliferating.

In another aspect of the present disclosure, there is provided a method for obtaining MSC-derived cells of chondro-osteoblastic lineage from MSC, the method comprising:

(a) culturing MSC recovered from a biological sample of a subject in a culture medium comprising FGF-2, TGFβ and heparin or a derivative or analogue thereof at a concentration of at least 0.01 IU/ml, thereby obtaining MSC-derived cells;

(b) passaging the MSC-derived cells for a first time and further culturing the MSC-derived cells in a medium as defined in (a); and

(c) passaging the MSC-derived cells for a second time and further culturing the MSC-derived cells in a medium as defined in (a), thereby obtaining the MSC-derived cells of chondro-osteoblastic lineage,

wherein the MSC-derived cells are plated for the further culturing in step (b) at a density of $3x10^2$ to $1x10^3$ cells/cm^2, preferably at a density of $3x10^2$ to $8x10^2$ cells/cm^2.

In a further aspect of the present disclosure, there is provided a method for obtaining MSC-derived cells of chondro-osteoblastic lineage from MSC, the method comprising:

(a) culturing MSC recovered from a biological sample of a subject in a culture medium comprising FGF-2, TGFβ and heparin or a derivative or analogue thereof at a concentration of at least 0.01 IU/ml, thereby obtaining MSC-derived cells;

(b) passaging the MSC-derived cells for a first time and further culturing the MSC-derived cells in a medium as defined in (a);

(c) passaging the MSC-derived cells for a second time and further culturing the MSC-derived cells in a medium as defined in (a), thereby obtaining the MSC-derived cells of chondro-osteoblastic lineage;

(d) resuspending the MSC-derived cells of chondro-osteoblastic lineage in a cryopreservation medium suitable for administration to a subject; and

(e) cryopreserving the MSC-derived cells of chondro-osteoblastic lineage.

In another aspect of the present disclosure, there is provided a population of MSC-derived cells of chondro-osteoblastic lineage obtainable or obtained by *in vitro* or *ex vivo* expansion of MSC, whereby at least 90% of the MSC-derived cells of chondro-osteoblastic lineage in suspension have a diameter equal to or less than 25 µm ($D_{90} \leq 25$ µm) and wherein at most 1 % of the MSC-derived cells of chondro-osteoblastic lineage in suspension have a diameter of more than 35 µm.

In a further aspect of the present disclosure, there is provided a pharmaceutical formulation comprising the population of MSC-derived cells of chondro-osteoblastic lineage disclosed herein.

In another aspect of the present disclosure, there is provided a population of MSC-derived cells of chondro-osteoblastic lineage, or the pharmaceutical formulation disclosed herein, for use as a medicament, preferably for use in the treatment of a subject in need of transplantation of cells of chondro-osteoblastic lineage.

In a further aspect of the present disclosure, there is provided a use of a population of MSC-derived cells of chondro-osteoblastic lineage, or the pharmaceutical formulation disclosed herein, in the manufacture of a medicament for treating a subject in need of transplantation of cells of chondro-osteoblastic lineage.

As corroborated by the experimental section, which illustrates certain representative examples of the present disclosure, the inventors realized that the production volume of mesenchymal stem cells (MSC)-derived cells of chondro-osteoblastic lineage can be considerably improved when said cells are obtained by a manufacturing method comprising a tertiary culture (and hence addition of an intermediate cell passage “P2”), and controlling one or more settings of

the culturing duration of secondary culture, the culturing duration of tertiary culture, addition of a cryopreservation step at the end of tertiary culture, or the plating density.

Hence, in an example, the present disclosure provides a method for obtaining, such as differentiating and/or expanding, MSC-derived cells of chondro-osteoblastic lineage from MSC, the method comprising:

(a) culturing MSC recovered from a biological sample of a subject in a culture medium comprising fibroblast growth factor-2 (FGF-2), transforming growth factor beta (TGFβ) and heparin or a derivative or analogue thereof at a concentration of at least 0.01 IU/ml, thereby obtaining mesenchymal stem cell (MSC)-derived cells;

(b) passaging the MSC-derived cells for a first time and further culturing the MSC-derived cells in a medium as defined in (a); and

(c) passaging the MSC-derived cells for a second time and further culturing the MSC-derived cells in a medium as defined in (a), thereby obtaining the MSC-derived cells of chondro-osteoblastic lineage,

wherein the MSC-derived cells are cultured in step (b) for a period of x days, wherein day x is the last day on which at least 20% of the MSC-derived cells are proliferating (e.g. in S phase, G2 phase or M phase of the cell cycle).

In a further example, the present disclosure provides a method for obtaining MSC-derived cells of chondro-osteoblastic lineage from MSC, the method comprising:

(a) culturing MSC recovered from a biological sample of a subject in a culture medium comprising FGF-2, TGFβ and heparin or a derivative or analogue thereof at a concentration of at least 0.01 IU/ml, thereby obtaining MSC-derived cells;

(b) passaging the MSC-derived cells for a first time and further culturing the MSC-derived cells in a medium as defined in (a); and

(c) passaging the MSC-derived cells for a second time and further culturing the MSC-derived cells in a medium as defined in (a), thereby obtaining the MSC-derived cells of chondro-osteoblastic lineage,

wherein the MSC-derived cells are plated for the further culturing in step (b) at a density of $3x10^2$ to $1x10^3$ cells/cm^2, preferably at a density of $3x10^2$ to $8x10^2$ cells/cm^2.

In a further example, the present disclosure provides a method for obtaining MSC-derived cells of chondro-osteoblastic lineage from MSC, the method comprising:

(a) culturing MSC recovered from a biological sample of a subject in a culture medium comprising FGF-2, TGFβ and heparin or a derivative or analogue thereof at a concentration of at least 0.01 IU/ml, thereby obtaining MSC-derived cells;

(b) passaging the MSC-derived cells for a first time and further culturing the MSC-derived cells in a medium as defined in (a);

(c) passaging the MSC-derived cells for a second time and further culturing the MSC-derived cells in a medium as defined in (a), thereby obtaining the MSC-derived cells of chondro-osteoblastic lineage;

(d) resuspending the MSC-derived cells of chondro-osteoblastic lineage in a cryopreservation medium suitable for administration to a subject; and

(e) cryopreserving the MSC-derived cells of chondro-osteoblastic lineage.

In a further example, the present disclosure relates to a population of MSC-derived cells of chondro-osteoblastic lineage obtainable or obtained by the methods as defined herein.

In a further example, the present disclosure provides a population of MSC-derived cells of chondro-osteoblastic lineage obtainable or obtained by *in vitro* or *ex vivo* expansion of MSC, whereby at least 90% of the MSC-derived cells of chondro-osteoblastic lineage in suspension have a diameter equal to or less than 25 µm ($D_{90} \leq 25$ µm) and wherein at most 1 % of the MSC-derived cells of chondro-osteoblastic lineage in suspension have a diameter of more than 35 µm.

In a further example, the present disclosure provides a pharmaceutical formulation comprising the population of MSC-derived cells of chondro-osteoblastic lineage as defined herein.

In another example, the present disclosure provides the population of MSC-derived cells of chondro-osteoblastic lineage as defined herein, or the pharmaceutical formulation as defined herein, for use as a medicament, preferably for use in the treatment of a subject in need of transplantation of cells of chondro-osteoblastic lineage.

The inventors found that the present methods can provide for one or more advantages as discussed below. The present methods allow to substantially increase culture yields and productivity of MSC-derived cells of chondro-osteoblastic lineage obtained from one bone marrow sample. As a result, one donation of bone marrow is sufficient to cover satisfying production volumes. Furthermore, the present methods increase the availability of MSC-derived cells of chondro-osteoblastic lineage for clinical use. Furthermore, the cryopreservation of the MSC-derived cells of chondro-osteoblastic lineage obtained by the present methods leads to a cell product which is directly administrable to a subject in need of transplantation of cells of chondro-osteoblastic lineage. The cryopreservation of the MSC-derived cells of chondro-osteoblastic lineage obtained by the present methods allows direct distribution and delivery of the cell product upon request, and hence immediately treatment of a subject in need of transplantation of cells of chondro-osteoblastic lineage. Further, cryopreservation of the MSC-derived cells of chondro-osteoblastic lineage obtained by the present methods allows to conduct all release tests of the cell product and obtain the results before administration of the cell product. In addition, the MSC-derived cells of chondro-osteoblastic lineage obtained by the methods as defined herein advantageously have both osteoinductive and osteogenic potential.

These and further preferred examples of the present disclosure are described in the following sections and in the appended claims. The subject-matter of the appended claims is hereby specifically incorporated in this specification.

DESCRIPTION OF THE DRAWINGS

FIG. 1 represents graphs illustrating CD73 (FIG. 1A) and CD44 (FIG. 1B) expression levels analysed by flow cytometry in A: MSC; B: cell product A; C: cell product B; D: cell product C – fresh (N=12, 6, 22, 15 (CD73) and N= 22, 8, 22, 18 (CD44) for MSC, cell product A, B and C respectively).

FIG. 2 represents graphs illustrating the cell size of comparative cell products according to the prior art and cell products illustrating the invention: A: MSC; B: cell product A; C: cell product B; D: cell product C – fresh; E: cell product C – cryo CS10; F: cell product C – cryo CS10 diluted HSA 1:1; G: cell product C – cryo CS10 5%HSA; H: cell product C – cryo HTS 10%HSA 10%DMSO.

FIG. 3 represents a graph illustrating the cell density according to the culture duration. The cells were counted: FIG. 3A: manually (Trypan Blue method with Bürker chamber) or FIG. 3B: by cytometry (BD Trucount^{TM}).

CLAIMS

1. A method for obtaining mesenchymal stem cell (MSC)-derived cells of chondro-osteoblastic lineage from MSC, the method comprising:

   (a) culturing MSC recovered from a biological sample of a subject in a culture medium comprising fibroblast growth factor-2 (FGF-2), transforming growth factor beta (TGFβ) and heparin or a derivative or analogue thereof at a concentration of at least 0.01 IU/ml, thereby obtaining mesenchymal stem cell (MSC)-derived cells;

   (b) passaging the MSC-derived cells for a first time and further culturing the MSC-derived cells in a medium as defined in (a); and

   (c) passaging the MSC-derived cells for a second time and further culturing the MSC-derived cells in a medium as defined in (a), thereby obtaining the MSC-derived cells of chondro-osteoblastic lineage,

   wherein the MSC-derived cells are cultured in step (b) for a period of x days, wherein day x is the last day on which at least 20% of the MSC-derived cells are proliferating.

2. The method according to claim 1, wherein the MSC-derived cells are cultured in step (b) for a period of from about 8 days to about 12 days, preferably for a period of about 10 days.

3. The method according to claim 1 or 2, wherein the MSC-derived cells are cultured in step (a) for a period of from about 13 days to about 15 days, preferably for a period of about 14 days.

4. The method according to any one of claims 1 to 3, wherein the MSC-derived cells are cultured in step (c) for a period of from about 10 days to about 14 days, preferably for a period of about 12 days.

5. The method according to any one of claims 1 to 4, wherein the proliferating MSC-derived cells are in S phase, G2 phase or M phase of the cell cycle.

6. A method for obtaining MSC-derived cells of chondro-osteoblastic lineage from MSC, the method comprising:

   (a) culturing MSC recovered from a biological sample of a subject in a culture medium comprising FGF-2, TGFβ and heparin or a derivative or analogue thereof at a concentration of at least 0.01 IU/ml, thereby obtaining MSC-derived cells;

   (b) passaging the MSC-derived cells for a first time and further culturing the MSC-derived cells in a medium as defined in (a); and

(c) passaging the MSC-derived cells for a second time and further culturing the MSC-derived cells in a medium as defined in (a), thereby obtaining the MSC-derived cells of chondro-osteoblastic lineage,

wherein the MSC-derived cells are plated for the further culturing in step (b) at a density of $3x10^2$ to $1x10^3$ cells/cm^2, preferably at a density of $3x10^2$ to $8x10^2$ cells/cm^2.

7. The method according to claim 6, wherein the MSC-derived cells are plated for the further culturing in step (c) at a density of $3x10^2$ to $1x10^3$ cells/cm^2, preferably at a density of $3x10^2$ to $8x10^2$ cells/cm^2.

8. A method for obtaining MSC-derived cells of chondro-osteoblastic lineage from MSC, the method comprising:

(a) culturing MSC recovered from a biological sample of a subject in a culture medium comprising FGF-2, TGFβ and heparin or a derivative or analogue thereof at a concentration of at least 0.01 IU/ml, thereby obtaining MSC-derived cells;

(b) passaging the MSC-derived cells for a first time and further culturing the MSC-derived cells in a medium as defined in (a);

(c) passaging the MSC-derived cells for a second time and further culturing the MSC-derived cells in a medium as defined in (a), thereby obtaining the MSC-derived cells of chondro-osteoblastic lineage;

(d) resuspending the MSC-derived cells of chondro-osteoblastic lineage in a cryopreservation medium suitable for administration to a subject; and

(e) cryopreserving the MSC-derived cells of chondro-osteoblastic lineage.

9. The method according to claim 8, wherein the MSC-derived cells of chondro-osteoblastic lineage are resuspended in the cryopreservation medium in step (d) at a concentration of between about $1x10^7$/ml and about $1x10^8$/ml, preferably at a concentration of between about $2x10^7$/ml and about $4x10^7$/ml.

10. The method according to claim 8 or 9, wherein the cryopreservation medium comprises dimethylsulfoxide (DMSO), human serum albumin (HSA), adenosine, polypeptide, benzopyran, or a combination thereof.

11. The method according to any one of claims 1 to 10, wherein TGFβ is selected from the group consisting of TGFβ1, TGFβ2, TGFβ3, and mixtures thereof; preferably wherein TGFβ is TGFβ1.

12. The method according to any one of claims 1 to 11, wherein:

    - the concentration of heparin or derivative or analogue thereof is about 0.1 IU/ml; and/or
    - heparin or heparin derivative or analogue thereof is selected from the group consisting of unfractionated heparin (UFH); low molecular weight heparin (LMWH), such as enoxaparin, dalteparin, nadroparin, tinzaparin, certoparin, reviparin, ardeparin, parnaparin, bemiparin, or mixtures thereof; a heparinoid, such as heparan sulfate, dermatan sulfate, chondroitin sulfate, acharan sulfate, keratan sulfate, or mixtures thereof, such as danaparoid; a heparin salt; a heparinoid salt; a heparin fragment; a heparinoid fragment; and mixtures thereof.

13. The method according to any one of claims 1 to 12, wherein the MSC or MSC-derived cells are additionally contacted with, such as wherein the medium additionally comprises one or more of, plasma, serum or a substitute thereof.

14. The method according to any one of claims 1 to 13, wherein the subject is a human subject.

15. A population of MSC-derived cells of chondro-osteoblastic lineage obtainable or obtained by *in vitro* or *ex vivo* expansion of MSC, whereby at least 90% of the MSC-derived cells of chondro-osteoblastic lineage in suspension have a diameter equal to or less than 25 µm ($D_{90} \leq$ 25 µm) and wherein at most 1 % of the MSC-derived cells of chondro-osteoblastic lineage in suspension have a diameter of more than 35 µm.

16. The population of MSC-derived cells of chondro-osteoblastic lineage according to claim 15, wherein the MSC-derived cells of chondro-osteoblastic lineage are obtainable or obtained by the methods as defined in any one of claims 1 to 14.

17. A pharmaceutical formulation comprising the population of MSC-derived cells of chondro-osteoblastic lineage as defined in claim 15 or 16.

18. The pharmaceutical formulation according to claim 17, wherein the pharmaceutical formulation further comprises a component with osteo-conductive properties, such as tricalcium phosphate, hydroxyapatite, combination of hydroxyapatite/tricalcium phosphate particles, poly-lactic acid, poly-lactic glycolic acid, hyaluronic acid or a derivative thereof, chitosan, poly-L-lysine, gelatine, collagen, osteonectin, fibrinogen, osteocalcin, or a combination thereof.

19. The population of MSC-derived cells of chondro-osteoblastic lineage according to claim 15 or 16, or the pharmaceutical formulation according to claim 17 or 18, for use as a medicament,

preferably for use in the treatment of a subject in need of transplantation of cells of chondro-osteoblastic lineage.

20. The population or the pharmaceutical formulation for use according to claim 19, wherein:

- the MSC-derived cells of chondro-osteoblastic lineage are present at a concentration between about $1x10^7$/ml and about $1x10^8$/ml, preferably between about $2x10^7$/ml and about $4x10^7$ cells/ml; and/or
- the population of MSC-derived cells of chondro-osteoblastic lineage or the pharmaceutical formulation is suitable for percutaneous, intra-osseous, intra-articular, intervertebral or intravascular administration;
- the population of MSC-derived cells of chondro-osteoblastic lineage or the pharmaceutical formulation is suitable for administration at a site of a bone defect.

21. Use of a population of MSC-derived cells of chondro-osteoblastic lineage according to claim 15 or 16, or the pharmaceutical formulation according to claim 17 or 18 in the manufacture of a medicament for treating a subject in need of transplantation of cells of chondro-osteoblastic lineage.

22. The use according to claim 21, wherein:

- the MSC-derived cells of chondro-osteoblastic lineage are present at a concentration between about $1x10^7$/ml and about $1x10^8$/ml, preferably between about $2x10^7$/ml and about $4x10^7$ cells/ml; and/or
- the population of MSC-derived cells of chondro-osteoblastic lineage or the pharmaceutical formulation is suitable for percutaneous, intra-osseous, intra-articular, intervertebral or intravascular administration;
- the population of MSC-derived cells of chondro-osteoblastic lineage or the pharmaceutical formulation is suitable for administration at a site of a bone defect.