

Claims

1. A method for producing a population of cardiac stromal cells from pluripotent stem cells, the method comprising the steps of:
 - i. Inducing epithelial-mesenchymal transition of epicardial cells obtained by differentiation of pluripotent stem cells, wherein the epicardial cells express Wilms tumor antigen (WT-1), wherein by inducing epithelial-mesenchymal transition the epicardial cells are differentiated into cardiac stromal cells, wherein inducing epithelial-mesenchymal transition comprises a1) culturing said epicardial cells under suitable conditions in the presence of a first extracellular matrix protein in a serum-free basal medium; followed by a2) culturing the cells of step (i) a1) under suitable conditions in the presence of a second extracellular matrix protein in a serum-free basal medium; wherein at least about 80 % of the cells of the obtained population of cardiac stromal cells express CD90, CD73, and CD44; and
 - ii. Amplifying the number of said cardiac stromal cells by culturing said population of cardiac stromal cells of step (i) in the presence of at least one third extracellular matrix protein in a serum-free basal medium, wherein at least 80 % of the cells of the cardiac stromal cell population maintain the expression of CD90, CD73, and CD44.
2. The method of claim 1, wherein step (ii) stably amplifies the population of cardiac stromal cells as determined by the maintained expression of at least 80% of CD90, CD73, and CD44, preferably at least 81%, more preferably at least 82%, even more preferably at least 83 %, even more preferably at least 84%, even more preferably at least 85%, even more preferably at least 86%, even more preferably at least 87%, even more preferably at least 88%, even more preferably at least 89%, and most preferably at least 90%, as determined by flow cytometry.
3. The method of claim 1 or 2, wherein the method comprises the steps of:
 - i. Inducing epithelial-mesenchymal transition of epicardial cells obtained by differentiation of pluripotent stem cells, wherein the epicardial cells express Wilms tumor antigen (WT-1), wherein by inducing epithelial-mesenchymal transition the epicardial cells are differentiated into cardiac stromal cells, wherein inducing epithelial-mesenchymal transition comprises
 - a1) culturing said epicardial cells under suitable conditions in the presence of an first extracellular matrix protein in a serum-free basal medium comprising effective amounts of (a) FGF2, (b) vascular endothelial growth factor (VEGF),

(c) glutamine and (d) a GSK-3 inhibitor, wherein said amounts result in the expression of CD90 in at least 50% of the cells obtained by step (i) a1), the expression of CD73 in at most 50% of the cells obtained by step (i) a1), and the expression of CD44 in at most 30% of the cells obtained by step (i) a1); followed by

a2) culturing the cells of step (i) a1) under suitable conditions in the presence of a second extracellular matrix protein in a serum-free basal medium comprising effective amounts of (a) FGF2, (b) VEGF, and (c) glutamine; wherein said amounts result in the expression of CD90, CD73, and CD44 in at least 80 % of the obtained population cardiac stromal cells; and

ii. Amplifying the number of said cardiac stromal cells by culturing said population of cardiac stromal cells of step (i) in the presence of at least one third extracellular matrix protein in a serum-free basal medium comprising effective amounts of (a) FGF2, (b) VEGF, and (c) glutamine, wherein said amounts result in the maintained expression of CD90, CD73, and CD44 in at least 80 % of said cardiac stromal cell population obtained by step (ii).

4. The method any of the preceding claims, wherein the serum-free basal medium in step (i) a1) comprises a final concentration of 10-200 ng/ml FGF2, preferably 15-100 ng/ml, more preferably 20-80 ng/ml, even more preferably 30-70 ng/ml, most preferably 40-60 ng/ml, and most preferably about 50 ng/ml;

wherein serum-free basal medium in step (i) a1) comprises a final concentration of 5-100 ng/ml VEGF, preferably 7-50 ng/ml, more preferably 10-40 ng/ml, even more preferably 15-35 ng/ml, most preferably 20-30 ng/ml, and most preferably about 25 ng/ml VEGF;

wherein serum-free basal medium in step (i) a1) comprises a final concentration of 0.2-20 mM glutamine, preferably, 0.5-10 mM glutamine, more preferably 0.75-5 mM glutamine, more preferably 1-3 mM glutamine, more preferably 1.5-2.5 mM glutamine, even more preferably about 2 mM glutamine, and most preferably wherein the glutamine is present as a L-alanine-L-glutamine dipeptide; and/or

wherein the basal medium in step (i) a1) comprises a final concentration of 0.1-10 μ M CHIR99021, preferably 0.2-9 μ M, more preferably 0.3-8 μ M, even more preferably 0.4-7 μ M, still more preferably 0.5-6 μ M, more preferably 0.6-5 μ M, more preferably 0.7-4 μ M, more preferably 0.8-3 μ M, most preferably 0.9-2 μ M, and even most preferably about 1 μ M CHIR99021.

5. The method of any of the preceding claims, wherein the serum-free basal medium in step (i) a2) comprises a final concentration of 10-200 ng/ml FGF2, preferably 15-100 ng/ml, more preferably 20-80 ng/ml, even more preferably 30-70 ng/ml, most preferably 40-60 ng/ml, and most preferably about 50 ng/ml;

wherein the serum-free basal medium in step (i) a2) comprises a final concentration of 5-100 ng/ml VEGF, preferably 7-50 ng/ml, more preferably 10-40 ng/ml, even more preferably 15-35 ng/ml, most preferably 20-30 ng/ml, and most preferably about 25 ng/ml VEGF; and/or

wherein the serum-free basal medium in step (i) a2) comprises a final concentration of 0.2-20 mM glutamine, preferably, 0.5-10 mM glutamine, more preferably 0.75-5 mM glutamine, more preferably 1-3 mM glutamine, more preferably 1.5-2.5 mM glutamine, even more preferably about 2 mM glutamine, and most preferably wherein the glutamine is present as a L-alanine-L-glutamine dipeptide.

6. The method of any of the preceding claims, wherein the serum-free basal medium in step (ii) comprises a final concentration of 10-200 ng/ml FGF2, preferably 15-100 ng/ml, more preferably 20-80 ng/ml, even more preferably 30-70 ng/ml, most preferably 40-60 ng/ml, and most preferably about 50 ng/ml;

wherein the serum-free basal medium in step (ii) comprises a final concentration of 5-100 ng/ml VEGF, preferably 7-50 ng/ml, more preferably 10-40 ng/ml, even more preferably 15-35 ng/ml, most preferably 20-30 ng/ml, and most preferably about 25 ng/ml VEGF; and/or

wherein the serum-free basal medium in step (ii) comprises a final concentration of 0.2-20 mM glutamine, preferably, 0.5-10 mM glutamine, more preferably 0.75-5 mM glutamine, more preferably 1-3 mM glutamine, more preferably 1.5-2.5 mM glutamine, even more preferably about 2 mM glutamine, and most preferably wherein the glutamine is present as a L-alanine-L-glutamine dipeptide.

7. The method of any of the preceding claims, wherein the first extracellular matrix protein comprises laminin, vitronectin, collagen, in particular gelatine, fibronectin, elastin, preferably wherein the first extracellular matrix protein comprises laminin, more preferably wherein the extracellular matrix protein is laminin, most preferably wherein laminin is laminin-521;

wherein the second extracellular matrix protein is different from the first extracellular matrix protein and comprises vitronectin, laminin, collagen, in particular gelatine, fibronectin, and elastin, preferably wherein the second extracellular matrix protein is vitronectin; and/or

wherein the at least one third extracellular matrix protein is selected from vitronectin, laminin, collagen, in particular gelatine, fibronectin, and elastin, preferably wherein the extracellular matrix protein is vitronectin.

8. The method of any of the preceding claims, wherein the epicardial cells express Wilms tumor antigen 1 (WT-1) as determined by fluorescent microscopy;

wherein the epicardial cells express Wilms tumor antigen 1 (WT-1) RNA at least 2-fold more than TBP (TATA-binding protein), preferably 3-fold, more preferably 4-fold, even more preferably at least 5-fold, most preferably at least 6-fold; and at most 15-fold, as determined by qRT-PCR; and/or

wherein the epicardial cells are obtained as described in Schlick (2018), Doctoral Thesis, November 2018, University of Göttingen, Witty et al. Nat Biotechnology 32, 1026–1035 (2014), or any other suitable method for providing epicardial cells.

9. The method of any of the preceding claims, wherein the method comprises the steps of:

i*. Inducing mesodermal differentiation by culturing said pluripotent stem cells under suitable conditions on laminin coated substrate in a serum-free basal medium comprising effective amounts of (a) bone morphogenetic protein 4 (BMP4), (b) Activin A (ActA), (c) a GSK-3 inhibitor, (d) basic fibroblast growth factor (FGF2), (e) glutamine, and (f) a serum-free supplement comprising albumin, transferrin, ethanol amine, selenium or a bioavailable salt thereof, L-carnitine, fatty acid supplement, and triodo-L-thyronine (T3), wherein said amounts result in the expression of CD90 in at least 90% of cells obtained by step (i*), the expression of CD73 in at most 10% of cells obtained by step (i*), and the expression of CD44 in at most 10% of the cells obtained by step (i*), as determined by flow cytometry;

i**. Inducing epicardial differentiation by culturing the cells of step (i*) under suitable conditions on laminin coated substrates in a serum-free basal medium comprising effective amounts of (a) BMP4, (b) retinoic acid (RA), (c) a GSK-3 inhibitor, (d) insulin, (e) glutamine and (f) the serum-free supplement as in (i); whereby the obtained cells express Wilms tumor antigen 1 (WT-1), as determined by fluorescent microscopy

i. Inducing epithelial-mesenchymal transition by a1) culturing the cells of step (i**) under suitable conditions in the presence of an first extracellular matrix protein in a serum-free basal medium comprising effective amounts of (a) FGF2, (b) vascular endothelial growth factor (VEGF), (c) glutamine and (d) a GSK-3 inhibitor, wherein said amounts result in the expression of

CD90 in at least 50% of the cells obtained by step (i) a1), the expression of CD73 in at most 50% of the cells obtained by step (i) a1), and the expression of CD44 in at most 30% of the cells obtained by step (i) a1); followed by

a2) culturing the cells of step (i) a1) under suitable conditions in the presence of a second extracellular matrix protein in a serum-free basal medium comprising effective amounts of (a) FGF2, (b) VEGF, and (c) glutamine; wherein said amounts result in the expression of CD90, CD73, and CD44 in at least 80 % of the obtained population of cardiac stromal cells; and

ii. Amplifying the number of said cardiac stromal cells by culturing said population of cardiac stromal cells of step (i) in the presence of at least one third extracellular matrix protein in a serum-free basal medium comprising effective amounts of (a) FGF2, (b) VEGF, and (c) glutamine, wherein said amounts result in the maintained expression of CD90, CD73, and CD44 in at least 80 % of said cardiac stromal cell population.

10. An isolated population of cardiac stromal cells, wherein the cardiac stromal cells have been obtained by differentiation of pluripotent stem cells and wherein at least about 80 % of the cells of the population of cardiac stromal cells express CD90, CD73, and CD44.
11. An engineered organ tissue comprising a population of cardiac stromal cells as defined in claim 10.
12. Use of the population of cardiac stromal cells (cStC) obtained by the method according to any of claims 1-9 and 16, or as defined in claim 10, or use of the engineered organ tissue as defined in claim 11 in an *in vitro* model for drug screening, preferably *in vitro* model for drug efficacy screening or in an *in vitro* model for drug toxicity screening.
13. Use of the population of cardiac stromal cells (cStC) obtained by the method according to any of claims 1-9 and 16, or as defined in claim 10 in an *in vitro* production of an engineered organ tissue, preferably of a human engineered organ tissue, more preferably wherein the engineered human organ tissue is engineered human myocardium or engineered human connective tissue.
14. The population of cardiac stromal cells (cStC) obtained by the method according to any of claim 1-9 and 16, or as defined in claim 10 for use in organ repair, preferably heart repair or soft tissue repair.
15. A serum-free cell culture medium suitable for amplification of cardiac stromal cells comprising (a) a serum-free basal medium, (b) 10-200 ng/ml FGF2, (c) 5-100 ng/ml VEGF, (d) 0.2-20 mM glutamine, and (e) an eukaryotic cell culture medium supplement

comprising 6.6-165 µg/ml ascorbic acid, 2-50 µg/ml insulin, 1.1-27.5 µg/ml transferrin, 1660-41500 µg/ml albumin, and 11-145 nM selenium.