Derivation and self-renewal of Isl1+ cells and uses thereof

Technical field

The present invention pertains inter alia to methods for deriving multipotent $Isl1^+$ cells (i.e. methods for inducing a cell to enter a multipotent $Isl1^+$ lineage), methods for differentiating $Isl1^+$ cells to cardiac cells, cells obtainable by such methods, kits and compositions for carrying out the methods in accordance with the invention, and also medical applications of said cells.

Background Art

The turnover of cardiomyocytes is low in the adult heart but can increase during ischemia through upregulation of Islet-1 positive (Isl1+) or C-Kit positive (C-Kit+) multipotent cardiac progenitor cells (Genead *et al.*, *PlosOne* 7, e36804 (2012)). The upregulation of endogenous progenitor cells is not sufficient to replace the damaged musculature after substantial ischemic damage, resulting in heart failure. An attractive approach to prevent the development of heart failure would be to provide progenitor cells capable of repairing tissue damage in the heart. These progenitor cells should be capable of giving rise to cardiomyocytes, smooth muscle cells, nerve cells and endothelium. Isl1+ cells have this capacity and form 2/3 of the developing heart including the sino-atrial node (SA), part of the atrial-ventricular node (AV), right atrium, right ventricle, proximal aorta, trunk of the pulmonary arteries and proximal parts of the coronary arteries (Lam *et al.*, *Pediatr. Cardiol.* 30, 690 (2009)).

However, one considerable limitation in using these progenitor cells for regenerative medicine pertains to the difficulty of expanding well-characterized clonal progenitor cell populations, from either adult human tissue or from fetal/embryonic stem cell tissues. In an earlier study, Laugwitz and coworkers showed that it was possible to expand postnatal Isl1+ cells from transgenic mice if they were co-cultured with cardiac mesenchymal feeder cells (Laugwitz *et al.*, *Nature* 433, 647 (2005)).

Therefore there is great need in the art for methods that efficiently enable the formation of cardiovascular progenitors and maintaining and expanding these cardiovascular progenitors in a multipotent state to enable the generation of a diverse set of heart lineages. A desired method would enable the production of, and subsequent unlimited expansion of progenitors capable of entering different cardiac lineages. Such a method is highly desirable as it will circumvent many of the issues relating to tissue rejection commonly associated with transplantation therapies, and overall provide alternative regenerative medicine strategies and improve treatment outcomes for patients suffering from a variety of cardiac and cardiovascular disorders.

Summary of the Invention

It is hence an object of the present invention to overcome the above-identified problems and satisfy the existing needs within the art, i. e. to provide for facile and efficient derivation of multipotent $Isl1^+$ cells using clearly defined and highly controllable methods. Furthermore, the present invention enables not only efficient derivation of multipotent $Isl1^+$ cells but also substantially indefinite self-renewal and proliferation (i.e. clonal expansion) of said multipotent $Isl1^+$ cells, as well as cellular differentiation, e.g. into cardiac tissue. The cells obtainable using the methods in accordance with the present invention are highly suitable for various clinical applications and exhibit strong homing ability to tissues and sites of interest, for instance cardiac tissue after differentiation into cardiomyocytes. Specifically, $Isl1^+$ cells that have been differentiated into cardiac tissue, using the derivation and differentiation methods according to the present invention, home strongly to infarcted (ischemic) regions of the heart, where they become elongated and arrange in parallel with the surrounding cardiomyocytes.

Thus, the present invention pertains to, in a first aspect, methods for deriving multipotent $Isl1^+$ cells (i.e. methods for inducing a cell to enter a multipotent $Isl1^+$ lineage). Such methods may comprise the step of culturing a mesenchymal cell (also known as a mesenchymal stem cell (MSC)), e.g. a cell from a mesenchymal cell fraction, in the presence of at least one laminin comprising an α5 chain (the α5 chain

is represented by SEQ ID No 3), and in a medium comprising at least one agent which activates the Wnt canonical pathway, and to continue culturing the cell culture for a sufficient period of time to derive the $Isl1^+$ cell (and/or a sufficient population of $Isl1^+$ cells).

In a further aspect, the instant invention relates to methods for self-renewal of multipotent $Isl1^+$ cells, said methods may comprise the steps of (i) culturing cells obtainable through the methods as per the above-described aspect in the presence of at least one laminin comprising an α5 chain, and in a medium comprising at least one agent which activates the Wnt canonical pathway; and, (ii) maintaining and/or expanding the cells obtainable from step (i) in cell culture. In a third aspect, the present invention pertains to a method for differentiating $Isl1^+$ cells into cardiac cells (e.g. cardiomyocytes, smooth muscle cells, and/or cardiac endothelial cells). In a further aspect, the present invention relates to cells obtainable by the methods in accordance with the invention, notably multipotent $Isl1^+$ cells and $Isl1^+$ cells differentiated into cardiac cells (normally expressing e.g. Troponin T and other markers for heart cells) (both the multipotent $Isl1^+$ cells and the $Isl1^+$ cells differentiated into cardiomyocytes may optionally be cryopreserved).

In additional aspects, the instant invention pertains to compositions and kits (e.g. kits of parts) for carrying out the methods of the present invention. The compositions according to the invention may comprise at least one laminin comprising an α5 chain and at least one agent which activates the Wnt canonical pathway, and the kit (i.e. a kit of parts), similarly, may comprise at least one laminin comprising an α5 chain and a cell culture medium comprising at least one agent which activates the Wnt canonical pathway. In a further aspect, the present invention relates to kits and/or compositions for differentiating cells (notably $Isl1^+$ cells) into cardiomyocytes. Such kits may comprise at least one laminin selected from the group comprising laminin-111, laminin-211, laminin-221, and any combination thereof. The components of the kit (e.g. the laminin and the Wnt-activating agent) may advantageously be provided in separate containers (for instance vials, ampoules, tubes, or the like), in order to enable combining the components when carrying out the methods as per the present invention. The laminins of the kits and/or compositions may be provided either in a suitable medium, lyophilized, or coated on dishes/plastic suitable for cell culture.

In other aspects, the present invention pertains to the cells as per the present invention for for use in medicine. More specifically, the cells may be used in the treatment and/or prophylaxis of heart insufficiency, heart failure, myocardial infarction, and/or congenital heart disease due to cardiac defects affecting parts derived from the second heart field (for instance right atrium, right ventricle, outflow tracts (aorta, pulmonary arteries) and ventricular septum). Further aspects as per the instant invention relates to pharmaceutical compositions comprising cells as per the invention, together with at least one pharmaceutically acceptable excipient, for use in medicine, for instance for use in the treatment and/or prophylaxis of heart insufficiency, heart failure, myocardial infarction, and/or congenital heart disease due to cardiac defects affecting parts derived from the second heart field (for instance right atrium, right ventricle, outflow tracts (aorta, pulmonary arteries) and ventricular septum).

A further aspect in accordance with the present invention pertains to methods of treatment for improving, alleviating or preventing heart insufficiency, heart failure, myocardial infarction, and/or congenital heart disease due to cardiac defects affecting parts derived from the second heart field (for instance right atrium, right ventricle, outflow tracts (aorta, pulmonary arteries) and ventricular septum) in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of the cells according to the instant invention.

In yet another aspect, the present invention pertains to the use of a combination of at least one laminin comprising an α5 chain and at least one agent which activates the Wnt canonical pathway, for deriving a multipotent $Isl1^+$ cell. In a further aspect, the present invention also relates to the use of laminin-111, laminin-211, laminin-221, and/or any combination thereof for differentiating cells (preferably $Isl1^+$ cells in accordance with the present invention) into cardiac cells, for instance cardiomyocytes.

The present invention thus provides methods for deriving multipotent $Isl1^+$ cell, for self-renewing such cells, and for differentiating such cells into cardiac cells, with considerably improved efficiency, less variability, under defined clinically acceptable conditions, and with enhanced control compared to the methods of the current art.

Furthermore, the compositions and kits as per the present invention facilitate carrying out the methods as per the present invention, enabling facile culturing and handling, for both clinical and experimental purposes.

Brief Description of the Drawings

Figure 1. Derivation of Isl1+ cells from embryonic rat cardiac mesenchymal stem cells or from rat bone marrow. (a) In the initial adherent stem cell fraction, there was only a small number of Isl1+ cells (<1%) (in the heart tissue these were mixed with cardiomyocytes expressing cardiac α-actinin). During the first week of culture in a Wnt-medium on laminin 511, laminin 521, or a combination of the two, the mesenchymal stem cells switched into cells expressing Isl1. Along with the Isl1+ cells there were more differentiated cells expressing cardiac α-actinin. During subsequent passages, the relative proportion of cells expressing cardiac α-actinin was reduced and after two weeks in culture (5 passages), 73±18% of the cells expressed Isl1 and there was no differentiated cells left. (b) Within two weeks of culture, the initial fraction had expanded 16 times. (c) Western blot analysis confirmed the activation of the Wnt/β-catenin pathway in the cultured Isl1+ cells, demonstrating increased levels of both phosphorylated Lrp6 on Serine 1490 and active (dephosphorylated) β-catenin (ABC) compared to the initial adherent cell fraction. In panel a, bars represent 50 μm.

Figure 2. Derivation of Isl1+ cells from bone marrow, cord blood, and fetal/embryonic heart mesenchymal stem cells (MSCs). (A) Schematic representation of the experimental procedure used to generate and expand multipotent Isl1+ cells from a mesenchymal stem cell fraction. (B) Before initiation of the Wnt and laminin protocol, less than 5% of the mesenchymal stem cell fraction expressed Isl1 (when using fetal/embryonic heart tissue these cells were mixed with cells expressing cardiac α-actinin (α-act)). (C) During the first week in culture on the at least one α5-containing laminin and in the Wnt-medium, the mesenchymal stem cell fraction switched into cells expressing Isl1. (D) After two weeks of culture, 92±2 % of the cells were Isl1 positive and more than 40% of the cells were still proliferating expressing Ki67. (E)

The cultured Isl1+ cells could be differentiated into cells expressing cardiac α-actinin, von Willebrand factor (vWF) and smooth muscle actin (SMA). (F) After three weeks of culture, the Isl1+ cell population had expanded more than 1000-fold. In order to verify the protocol, the initial mesenchymal stem cell fraction was cultured under different conditions: Wnt-medium+at least one α5-containing laminin, Wnt-medium+plastic, Medium without Wnt3a+at least one α5-containing laminin and Medium without Wnt3a+plastic. (G) The qRT-PCR-analysis demonstrates that the combination of Wnt-medium and at least one laminin comprising an α5 chain (e.g. either laminin 511 (a composition comprising SEQ ID No. 3, SEQ ID No. 4, and SEQ ID No. 6) or laminin 521 (a composition comprising SEQ ID No. 3, SEQ ID No. 5, and SEQ ID No. 6) or a combination of the two) is necessary for derivation and expansion of undifferentiated Isl1+ cells. In panel B to E, nuclear staining with DAPI is shown in magenta. Cells costaining for Isl1 and magenta are shown in white. Bars represent 50 µm. In panel F, error bars represent mean±SD. In figure: ** p <0.01; *** p <0.001.

Figure 3. Flow cytometry analysis of the adherent fraction from a human fetal/embryonic heart and bone marrow aspirate from healthy human donors. The cells expressed the mesenchymal stem cell markers CD105, CD90, CD73 and CD44, while being negative for the hematopoietic lineage markers CD14, CD19, CD34, CD45 and the endothelial marker CD31.

Figure 4. Heat maps representative of the gene-expression profiles of cultured Isl1+ cells in comparison to *in vivo* present cells in the Isl1-positive and -negative regions of a fetal heart. The origin of the cells is in (A) rat and (B) human. Average linkage and log2 transformation of signals are presented. The heatmap color scale range from red (high expression) via black (average expression) to green (low expression).

Figure 5. Heat map representative of the gene-expression profile and flow cytometry analysis defining different progenitor populations of the cultured human Isl1+ cells. (A) Total RNA was isolated from Isl1-positive (Islet1+ve, red cells) and -negative (Islet-1-ve) regions from a human fetal heart (9.5 weeks) and through laser capture microdissection. In figure: OFT: outflow tract; LA: left atrium; LV: left ventricle: RA: right atrium; RV: right ventricle. Nuclei are stained blue by DAPI. Bar represent 50

μm. (B) Heat map: The cultured human Isl1+ cells (obtained from human fetal heart) had a higher expression of Isl1 than the Isl1-positive region of the human embryonic heart, and the expression of differentiation markers was much lower. The expression of pluripotency markers of the cultured Isl1+ cells increased during the culturing process (from 2 to 3 weeks). In figure, average linkage and log2 transformation of signals are presented. The heat map color scale ranges from red (high expression) via yellow to blue (low expression). (C) In flow cytometry analysis of human fetal heart, human cord blood MSCs, and human bone marrow MSCs, the CD34+ and CD45+ cell populations were excluded (and this may be preferably under certain circumstances). All human Isl1+ cells co-express KDR, 59.5% SSEA-1 and 4.3% c-kit. All SSEA-1+ cells co-express KDR, and they constitute 2.7% of the c-kit+ cell population.

Figure 6. Detection by immunohistochemistry of the implanted rat Isl1+ cells (obtained either from embryonic or fetal heart or from rat bone marrow) labelled with luciferase and β-gal expressing transposons. (A) Hematoxylin and eosin staining demonstrating the site of injection of labelled Isl1+ cells into the left ventricular wall. 24 hours after injection, X-gal staining identified the labelled $Isl1^+$ cells (blue cells) both at the site of injection (insert in figure A) and in the outflow tract region close to the PA and Ao (insert in figure B). After two weeks, few Isl1+ cells could be detected at the site of injection (C). These cells had changed their appearance and were elongated and organized in parallel to the surrounding cardiomyocytes. (D, E) When labelled Isl1+ cells were injected into the peri-ischemic region of the left ventricle, the majority of the cells stayed in the infarction area (insert in figure D) and few cells were found in the outflow tract region 24 hours post injection (insert in figure E). (F) After two weeks, labelled Isl1+ cells were still found in the infarct area and again the implanted cells were elongated and interspersed between the surrounding cardiomyocytes (insert in figure F). In figures, bars represent 1000 μm and in insert 100 μm. Abbreviations; Ao: aorta; PA: pulmonary artery; RA: right atrium; LV: left ventricle.

Figure 7. Study of *in vivo* survival and migration of rat Isl1+ cells (obtained either from bone marrow or from embryonic heart) labelled with luciferase and β-gal expressing transposons. (A) Labelled Isl1+ cells were injected into the left ventricular

wall of a normal rat heart, where the In Vitro Imaging System (IVIS system) detected a strong signal a few hours after injection. (B) After 24 hours, the strongest signal was detected in a region corresponding to the outflow tract, where it stayed during the detection period of one week (C). Labeled Isl1+ cells were in the next step injected into the left ventricular wall of a normal heart. Within 24 hours, the strongest signal was detected in the outflow tract region (D). After induction of myocardial infarction, the strongest signal was again detected in the ischemic region where it increased from 24 hours (E) to one-week post infarction (F). (G) Injection of labelled Isl1+cells into a tail vein, 8 hours after induction of myocardial infarction. The Isl1+cells seemed to home into the infarction region where the signal was strongest. Signaling can also be seen in the lungs, which is probably indicative of some of the cells being maintained in the capillary network of the lungs.

Figure 8. Laminin comprising α5 chain crucial for derivation of Isl1+ cells. Laminin 511, laminin 521, and a combination of the two, induce mesenchymal stem cells (obtainable either from bone marrow of healthy human donors, from human cord blood, from the human amniotic sac, or from human fetal or embryonic heart tissue) to enter a multipotent $Isl1^+$ lineage, whereas other laminins are incapable of promoting derivation of $Isl1^+$ cells. Other laminins comprising an α5 chain (for instance laminins 522 and 523) are naturally also within the scope of the present invention.

Figure 9. Laminins 111 (a composition comprising SEQ ID No. 1, SEQ ID No. 2, and SEQ ID No. 6), 211 (a composition comprising SEQ ID No. 2, SEQ ID No. 2, and SEQ ID No. 6), 221 (a composition comprising SEQ ID No. 2, SEQ ID No. 5, and SEQ ID No. 6), and a combination of the two promotes cellular differentiation. Laminin 111, laminin 211, laminin 221, or any combination thereof, promote differentiation of Isl1+ cells into cardiac cells (e.g. cardiomyocytes, smooth muscle cells, and/or endothelial cells).

Detailed Description of the Invention

The present invention pertains inter alia to methods for deriving multipotent Isl1$^+$ cells (i.e. methods for inducing a cell to enter a multipotent Isl1$^+$ lineage), comprising the step of culturing a mesenchymal stem cell (MSC) (e.g. a cell from a mesenchymal cell fraction) in the presence of at least one laminin comprising an α5 chain, and in a medium comprising at least one agent which activates the Wnt canonical pathway. The Isl1$^+$ cell obtained using said method may subsequently be cultured in the presence of at least one laminin selected from the group comprising laminin 111, laminin 221, and laminin 211, or any combination thereof, in order to further differentiate the cell population. Also, the present invention pertains to methods for self-renewal and/or proliferation of Isl1$^+$ cells (optionally obtainable via the derivation methods as per the present invention but the self-renewal and/or maintenance procedure may also be applied to Isl1+ cells obtained via other methods or from other sources), comprising the steps of (i) culturing an Isl1$^+$ cell in the presence of at least one laminin comprising an α5 chain, and in a medium comprising at least one agent which activates the Wnt canonical pathway; and, (ii) maintaining and/or expanding the cells obtainable from step (i), in cell culture. The Isl1$^+$ cell obtained in step (ii) of the self-renewal (proliferation) method may subsequently be cultured in the presence of at least one laminin selected from the group comprising laminin 111, laminin 211, laminin 221, and a combination thereof, in order to further differentiate the cell population (into cardiac cells, specifically cardiomyocytes).

The present invention additionally relates to cells obtainable by the methods in accordance with the present invention, compositions comprising at least one laminin comprising an α5 chain and at least one agent which activates the Wnt canonical pathway, as well as a kit (i.e. a kit of parts) comprising at least one laminin comprising an α5 chain and a cell culture medium containing at least one agent which activates the Wnt canonical pathway (e.g. in different vials), or, alternatively, a differentiation kit comprising at least one laminin selected from the group comprising laminin 111, laminin 211, laminin 221, and a combination thereof. Furthermore, the present invention also pertains to cells obtainable by the methods of the instant invention for use in medicine, for instance in cardiology-related indications, as well as pharmaceutical compositions comprising the cells and at least one pharmaceutically acceptable excipient, in order to enable efficient delivery of the

cells to the target site/tissue. Finally, the present invention moreover relates to the use of a combination of at least one laminin comprising an α5 chain and at least one agent which activates the Wnt canonical pathway, for deriving a multipotent $Isl1^+$ cell (i.e. for inducing a cell to enter a multipotent $Isl1^+$ lineage). Similarly, the present invention moreover relates to the use of a combination of (i) laminin 111, laminin 211, laminin 221, or a combination thereof, and optionally (ii) at least one agent which activates the Wnt canonical pathway, for differentiating a multipotent $Isl1^+$ cell into a cardiac cell. The agent which activates the Wnt canonical pathway may be included in the differentiation step although its presence is not absolutely necessary.

Where features, embodiments, or aspects of the present invention are described in terms of Markush groups, a person skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. The person skilled in the art will further recognize that the invention is also thereby described in terms of any combination of individual members or subgroups of members of Markush groups. Additionally, it should be noted that embodiments and features described in connection with one of the aspects and/or embodiments of the present invention also apply mutatis mutandis to all the other aspects and/or embodiments of the invention. For example, the at least one agent which activates the Wnt canonical pathway described in connection with the method for deriving the multipotent Isl1+ cells is to be understood to be relevant also for the kit of parts (interchangeably termed the kit). Furthermore, certain embodiments described in connection with certain aspects, for instance bromoindirubin-3'-oxime (BIO) as described in relation to the aspect pertaining to the method for deriving the multipotent Isl1+ cells, may naturally also be relevant in connection with other aspects and/or embodiment, in the case of BIO for instance in the aspects/embodiments pertaining to the compositions or the kit of parts, in accordance with the present invention.

In a first aspect, the present invention relates to a method for deriving multipotent $Isl1^+$ cells (i.e. a method for inducing a cell to enter a multipotent $Isl1^+$ lineage), comprising the steps of (i) culturing a mesenchymal cell (i.e. a cell from a mesenchymal cell fraction) in the presence of at least one laminin comprising an α5 chain, and in a medium comprising at least one agent which activates the Wnt

canonical pathway (and continuing to culture the cell for a sufficient period of time to derive the $Isl1^+$ cell and/or to derive sufficient populations of $Isl1^+$ cells).

It shall be understood that within the context of the present invention the term "culturing [...] in the presence of" may be interpreted as encompassing contacting the cell/cells with e.g. at least one laminin comprising an α5 chain and/or at least one agent which activates the Wnt canonical pathway. The time of culturing in the presence of for instance the at least one agent which activates the Wnt canonical pathway may naturally be of importance for the present invention, as is outlined in greater detail below. Normally, the laminins used within the context of the present invention are coated on the cell/tissue culture equipment, e.g. a cell culture dish or stent that may be implanted into the human or animal body for a therapeutic purpose, for instance to derive $Isl1^+$ cells and subsequently differentiate said cells to cardiomyocytes, using the method steps of the present invention.

In a further embodiment in accordance with the present invention, the mesenchymal (stem) cells (e.g. cells from a mesenchymal fraction) may be cardiac mesenchymal cells, embryonic stem cells, cord blood stem cells, and/or amniotic stem cells, or any combination of these sources of cells. Mesenchymal cells, also known as mesenchymal stem cells (MSCs) are multipotent stromal cells that can differentiate into a variety of cell types, including but not limited to osteoblasts, chondrocytes, and adipocytes. MSCs may be derived from the bone marrow, mesoderm, umbilical cord blood, Wharton's jelly, adult muscle, developing tooth buds, and/or amniotic fluid. Cells from the "mesenchymal fraction" shall within the context of the present invention be understood to relate to cells that may be derived from any of the above sources, e.g. bone marrow, mesoderm, umbilical cord blood, Wharton's jelly, adult muscle, developing tooth buds, and/or amniotic fluid. Bone marrow aspirate is a suitable source for cells of a mesenchymal cell fraction (i.e. mesenchymal cells). Bone marrow aspirate may be obtained through entering the crista iliaca with an aspiration needle, followed by extracting a certain volume of cell-containing aspirate, which may subsequently be sorted, cultured or frozen for future use. Alternatively, cord blood or amniotic cells may be extracted and isolated for the purposes of the present invention. Isolation of said cells may be carried out in connection with partus through conventional techniques.

The MSCs (i.e. the cells from a mesenchymal fraction) that are employed for treating a patient may be of allogeneic origin, i.e. it is normally not necessary to use autologous cells from the patient in need of treatment.

The inventors have unexpectedly realized that using at least one laminin comprising an α5 chain is highly advantageous and results in surprisingly enhanced derivation of the $Isl1^+$ cells (Figure 8). When using the culturing methods of the present invention Isl1 expression was, after two weeks in culture, stably detected in more than 90% of the mesenchymal cell population, and after 3 weeks of culturing the cell population had expanded more than 1000-fold. The at least one laminin comprising an α5 chain may be selected from the group comprising laminin 511, laminin 521, a combination of laminin 511 and laminin 521, any natural, recombinant, or synthetic protein, which has at least approximately 70% (preferably at least 80% and even more preferably at least 90%) sequence identity to the polypeptide sequence of laminin 511 or laminin 521, any natural, recombinant, or synthetic protein comprising the polypeptide sequence of the laminin α5 chain G-domain, any natural, recombinant, or synthetic protein comprising a polypeptide sequence, which has at least approximately 70% (preferably at least 80% and even more preferably at least 90%) sequence identity to the polypeptide sequence of the laminin α5 chain G-domain, and any cell culture growth substratum or cell culture medium additive comprising polypeptides from the polypeptide sequence of laminin 511 or laminin 521.

Further in accordance with the present invention, the at least one agent which activates the Wnt canonical pathway may be selected from the group comprising Wnt-1, Wnt-3a, Wnt-8, Wnt-8b, and any combination thereof. Additional agents activating the Wnt canonical pathway are naturally also within the scope of the present invention, irrespective of whether the activation of the Wnt canonical pathway is effected via direct activation of the pathway in question (for instance mediated by Wnt-3a) or via indirect stimulation of the Wnt canonical pathway by blockage of the non-canonical pathway (for instance via blocking glycogen synthase kinase 3 (GSK3) which causes an upregulation of beta-catenin) (i.e. using BIO). Also, various types of agents may be utilized within the scope of the instant invention, as is outlined in more detail below.

The inventors have realized that selecting an appropriate concentration interval of the at least one agent which activates the Wnt canonical pathway is important for the present invention, suitably a concentration of approximately 10-250 ng/ml, but preferably a concentration of approximately 50-150 ng/ml, or even more preferably approximately around 100 ng/ml. Naturally, the concentration interval depends on the activity and the potency of the Wnt-activating agent employed in the specific case. When utilizing BIO, either in combination with e.g. a Wnt-activating polypeptide (e.g. Wnt-3a) or alone, a suitable concentration range may be approximately 0.1-5 mM, preferably approximately 0.5-3 mM, or even more preferably approximately 2.5 mM. Furthermore, epidermal growth factor (EGF), at a concentration ranging from 1ng/ml to 100 ng/ml (preferably around 10 ng/ml) may be included in the culture medium, in order to enhance cell expansion. Additionally, when DMEM/F12 medium is used for the cell culturing methods, the medium may be supplemented with B27.

A second step of the method in accordance with the present invention (i.e. step (ii)) may further comprise expanding the $Isl1^+$ cell population, in order to increase the cell numbers. Additionally, the second step may furthermore comprise self-renewal and/or proliferation of the $Isl1^+$ cells (i.e. maintaining the $Isl1^+$ cells in culture basically indefinitely, to enable further usage or experimentation).

In order to further differentiate the cells obtainable by the methods of the present invention, a further differentiation step (iii) may be included, wherein $Isl1^+$ cells (either obtained via the derivation methods of the present invention or $Isl1^+$ cells obtainable from any other source) is cultured in the presence of at least one laminin selected from the group comprising laminin 111, laminin 211, laminin 221, and any combination of thereof, and any natural, recombinant, or synthetic protein comprising a polypeptide sequence, which has at least approximately 70% sequence identity to the polypeptide sequence(s) of either laminin 111, laminin 221, or laminin 211. When culturing the cells in the presence of the at least one laminin selected from the group comprising laminin 111, laminin 211, laminin 221, and a combination thereof, and any natural, recombinant, or synthetic protein comprising a polypeptide sequence, which has at least approximately 70% sequence identity to the polypeptide sequence(s) of either laminin 111, laminin 221 or laminin 211, the Wnt-activating agent may be excluded from the culture medium, in order to further enhance

differentiation to the desired cell type, in this case cardiac (heart) cells (and possibly tissues) (Figure 9). Laminin 111 and other α1-containing laminins (such as laminin 121) may in some instances be utilized also to derive Isl1$^+$ cells from mesenchymal stem cells as per the present invention (in addition to their use for differentiating Isl1$^+$ cells into cardiac cells), although derivation using α5-containing laminins is generally significantly more efficient and reliable. α1-containing laminins may be present in a 3-dimensional structure (such as a 3-dimensional matrix and/or gel, e.g. Geltrex^{TM}), in order to efficiently induce Isl1$^+$ expression (when using the same protocol as employed for the α5-containing laminins).

The present invention relates, at least partially, to methods for the production and expansion of Isl1$^+$ progenitors, for example cardiovascular progenitors, while maintaining their multipotency and capacity for multi-lineage differentiation. In a further aspect, the present invention pertains to a method for self-renewal of a multipotent Isl1$^+$ cell, comprising the steps of (i) culturing a cell, obtainable through the derivation methods as per the present invention, in the presence of at least one laminin comprising an α5 chain, and in a medium comprising at least one agent which activates the Wnt canonical pathway; and (ii) maintaining and/or expanding the cells obtainable from step (i) in cell culture, essentially indefinitely.

As abovementioned, in one aspect, the present invention provides a method to induce a cell to enter the Isl1$^+$ lineage. More specifically, the present invention is based on the realization that activation of Wnt signalling in the presence of at least one laminin comprising an α5 chain triggers the entry of cells into islet 1+ lineage and can be used to pre-specify cells to become Isl1$^+$ progenitors. Thus, the present invention pertains to methods to induce cells, for example uncommitted progenitors, into Isl1$^+$ progenitors, for example Isl1$^+$ cardiovascular progenitors. Furthermore, the instant invention provides methods for expanding Isl1$^+$ progenitors by triggering their renewal.

The cells in accordance with the present invention may be stem cells, including, but not limited to, embryonic stem cells, embryoid body (EB) cells, adult stem cells and fetal or postnatal stem cells, optionally obtained from from tissue, for instance embryonic, fetal, postnatal or adult tissue. The tissue may for instance be cardiac

tissue. More specifically, the cells in accordance with the present invention may be cells from a mesenchymal fraction (i.e. mesenchymal cells, also known as mesenchymal stem cells), for instance derived from bone marrow, mesoderm, umbilical cord blood, Wharton's jelly, adult muscle, developing tooth buds, and/or amniotic fluid.

The tissue may comprise, but is not limited to, fibroblasts, cardiac fibroblasts, circulating endothelial progenitors, pancreas, liver, adipose tissue, bone marrow, kidney, bladder, palate, umbilical cord, amniotic fluid, dermal tissue, skin, muscle, spleen, placenta, bone, neural tissue or epithelial tissue.

The cells may further be obtained from a subject with a disease or disorder, for example from a subject with an acquired or congenital cardiac or cardiovascular disorder, disease or dysfunction, for example a cardiac defect. The cell may be of mammalian origin, for instance human, and it may also be a genetically modified cell. Additionally, the mesenchymal stem cells may also be obtained from a young healthy donor (preferably below 35 years of age), for instance through aspirating cells (e.g. around 50 ml) from the bone marrow.

The present invention also pertains to methods to expand $Isl1^+$ progenitors (i.e., progenitors that are already $Isl1^+$) by activating or enhancing the Wnt/β-catenin pathway. In particular, the present invention is based on the discovery that activating, increasing, or enhancing Wnt signalling when cells are cultured in the presence of at least one laminin comprising an α5 chain triggers renewal of $Isl1^+$ progenitors and can be used to expand $Isl1^+$ progenitors while maintaining their capacity for multi-linage differentiation. Accordingly, the present invention relates to methods for expanding $Isl1^+$ progenitors, for example $Isl1^+$ cardiovascular progenitors. The activation, increasing, or enhancing of the Wnt canonical pathway is effectuated by at least one agent activating the Wnt canonical pathway. In this context, agents activating the Wnt canonical pathway are any agents which activate the Wnt canonical pathway. Preferably, such agent(s) activate the Wnt canonical pathway in a selective manner. In some embodiments, the agents that activate the Wnt canonical pathway are directly applied to the $Isl1^+$ progenitor, for example, agents that activate the Wnt canonical pathway are applied to the culture media.

In another aspect, the present invention pertains to cells obtainable by the methods of the instant invention, i.e. $Isl1^+$ cells, optionally further differentiated into e.g. cardiac cells, for instance cardiomyocytes (cells differentiated using the methods of the present invention normally display at least a 100-fold increase in Troponin T expression, and frequently a 150-fold increase in expression). The cells as per the present invention may optionally be cryopreserved, in order to enable storage, facilitated handling, and subsequent use and experimentation.

In yet another aspect, the present invention relates to a composition comprising at least one laminin comprising an α5 chain and at least one agent which activates the Wnt canonical pathway. The at least one laminin comprising an α5 chain may be selected from the group comprising laminin 51, laminin 521, a combination of laminin 511 and laminin 521, any natural, recombinant, or synthetic protein, which has at least approximately 70% sequence identity to the polypeptide sequence of laminin 511 or laminin 521, any natural, recombinant, or synthetic protein comprising the polypeptide sequence of the laminin α5 chain G-domain, any natural, recombinant, or synthetic protein comprising a polypeptide sequence, which has at least approximately 70% sequence identity to the polypeptide sequence of the laminin α5 chain G-domain, and any cell culture growth substratum or cell culture medium additive comprising polypeptides from the polypeptide sequence of laminin 511 or laminin 521.

Further in accordance with the present invention, the at least one agent which activates the Wnt canonical pathway, and which is comprised in the composition, may be selected from the group comprising Wnt-1, Wnt-3a, Wnt-8, Wnt-8b, and any combination thereof. Additional agents activating the Wnt canonical pathway are naturally also within the scope of the present invention, irrespective of whether the activation of the Wnt canonical pathway is effectuated via direct activation of the pathway in question (for instance mediated by Wnt-3a) or via indirect stimulation of the Wnt canonical pathway. The composition may be present in a number of different forms, for instance is selected from the group comprising a liquid, a solid, a matrix, a dispersion, a suspension, an emulsion, a microemulsion, a gel, or a solution, and any combination thereof. The composition in accordance with the present invention may for instance be a combination of a liquid (preferably comprising the at least one

agent which activates the Wnt canonical pathway) and a substantially solid and/or a gel and/or a matrix (preferably comprising the laminin comprising an α5 chain).

In a further aspect, the instant invention relates to a kit (i.e. a kit of parts) comprising at least one laminin comprising an α5 chain and a cell culture medium comprising at least one agent which activates the Wnt canonical pathway. The components of the kit (i.e. the laminin and the Wnt-activating agent) may advantageously be provided in separate containers (for instance vials, ampoules, tubes, or the like), in order to enable combining the components when carrying out the methods as per the present invention. The kit may for instance comprise a cell culture medium comprising the at least one agent which activates the Wnt canonical pathway (at a suitable concentration), and the at least one laminin comprising an α5 chain, coated e.g. on a surface suitable for cell culture (such as a cell culture plate, a well of a multi-well plate, a petri dish, a cell culture flask, etc.). Furthermore, the kit may also comprise additional components for differentiating the $Isl1^+$ cells to cardiac cells (e.g. cardiomyocytes), namely at least one laminin selected from the group comprising laminin 111, laminin 211, laminin 221, and any combination thereof. A suitable derivation and differentiation kit as per the present invention would thus comprise a cell culture medium comprising the at least one agent which activates the Wnt canonical pathway, at least one laminin comprising an α5 chain, a cell culture differentiation medium that may be devoid of the at least one agent which activates the Wnt canonical pathway, and at least one laminin selected from the group comprising laminin 111, laminin 211, laminin 221, and any combination thereof. In one embodiment, the laminin comprising an α5 chain may be coated on a 1^{st} set of cell culture equipment, whereas the at least one laminin selected from the group comprising laminin 111, laminin 211, laminin 221, and any combination thereof, are coated on a 2^{nd} set of cell culture equipment, and where a cell culture medium comprising the at least one agent which activates the Wnt canonical pathway is provided in one container whereas cell culture differentiation medium devoid of the Wnt-activating agent(s) is provided in another container.

Additionally, stents or other medical devices for implantation into a human or animal body may also be coated with suitable combinations of either the laminin comprising an α5 chain or the at least one laminin selected from the group comprising laminin

111, laminin 211, laminin 221, and any combination thereof, to enable derivation and/or cardiomyocyte differentiation inside the human or animal body. The stent may comprise gold, cobalt-chromium, tanatalum, nitinol, silicone, polyethylene, and/or polyurethane or any combination thereof. The stents in question may e.g. be a coronary stent, a blood vessel bypass stent, and/or a trachea stent. The stent may be a drug-eluting stent, wherein the drug to be eluted may for instance be a Wnt-activating agent, an agent for vascularisation, an immunomodulating agent, or any other suitable agent(s) to optimize the engraftment and stent function. The stents may be implanted with or without cells, e.g. $Isl1^+$ cells or $Isl1^+$ cells that have been further differentiated into cardiac cells (e.g. cardiomyocytes or cardiac endothelial or smooth muscle cells).

The at least one laminin comprising an α5 chain may be selected from the group comprising laminin 511, laminin 521, a combination of laminin 511 and laminin 521, any natural, recombinant, or synthetic protein, which has at least approximately 70 % sequence identity to the polypeptide sequence of laminin 511 or laminin 521, any natural, recombinant, or synthetic protein comprising the polypeptide sequence of the laminin α5 chain G-domain, any natural, recombinant, or synthetic protein comprising a polypeptide sequence, which has at least approximately 90 % sequence identity to the polypeptide sequence of the laminin α5 chain G-domain, and any cell culture growth substratum or cell culture medium additive comprising polypeptides from the polypeptide sequence of laminin 511 or laminin 521. Further in accordance with the present invention, the at least one agent which activates the Wnt canonical pathway, and which is comprised in the composition, may be selected from the group comprising Wnt-1, Wnt-3a, Wnt-8, Wnt-8b, and any combination thereof.

In another aspect, the present invention pertains to the cells as per the present invention for use in for use in medicine. More specifically, the cells may be used in the treatment and/or prophylaxis of heart insufficiency, heart failure, myocardial infarction, and/or congenital heart disease due to cardiac defects affecting parts derived from the second heart field (for instance right atrium, right ventricle, outflow tracts (aorta, pulmonary arteries) and ventricular septum). Further, the instant invention also relates to pharmaceutical compositions comprising cells as per the

invention, together with at least one pharmaceutically acceptable excipient, for use in medicine, for instance for use in the treatment and/or prophylaxis of heart insufficiency, heart failure, myocardial infarction, and/or congenital heart disease due to cardiac defects affecting parts derived from the second heart field (for instance right atrium, right ventricle, outflow tracts (aorta, pulmonary arteries) and ventricular septum). Both the $Isl1^+$ cells, derived through the derivation methods of the present invention (i.e. through culturing a mesenchymal cell in the presence of at least one laminin comprising an α5 chain, and in a medium comprising at least one agent which activates the Wnt canonical pathway), and the cells that have been further differentiated using the differentiation methods of the present invention (i.e. through culturing in the presence of at least one of laminin 111, laminin 211, laminin 221, or any combination thereof) may be included in a pharmaceutical composition for the treatment or prophylaxis of various illnesses and ailments. The pharmaceutical composition may comprise at least 100,000 ($1*10^5$) cells per ml, preferably at least 500,000 ($5*10^5$) per ml, in a pharmaceutically acceptable carrier, normally an aqueous solution comprising around 9% NaCl.

Furthermore, preferably at least 100,000 cells may be used per kg of body weight, preferably at least 500,000 per kg of body weight, or even more preferably at least 1,000,000 MSCs per kg of body weight.

A further aspect in accordance with the present invention pertains to methods of treatment for improving, alleviating or preventing heart insufficiency, heart failure, myocardial infarction, and/or congenital heart disease due to cardiac defects affecting parts derived from the second heart field (for instance right atrium, right ventricle, outflow tracts (aorta, pulmonary arteries) and ventricular septum) in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of the cells according to the instant invention. The subject is preferably a mammal, for instance a human being. The cells may be administered via intravascular administration, intravenous administration, intraventricular administration, epicardial administration, intramuscular administration, intraportal administration, intrathecal administration, and/or subcutaneous administration, or via any other suitable administration method that delivers the cells to the target site/tissue. Specifically, in the context of myocardial

infarction, the cells may advantageously be administered to a patient via endovascular injection/infusion or via percutaneous administration in the occluded vessel (before or after re-opening), using a cathether. Additionally, for the treatment of sinus node dysfunction, $Isl1^+$ cells (optionally differentiated into cardiomyocytes, in this case pacemaker cells) may be administered to a patient via percutaneous administration using a catheter introduced into the right atrium, preferably through the sinus coronarius.

Alternatively, the cells of the present invention (either $Isl1^+$ cells or $Isl1^+$ cells further differentiated into cardiomyocytes) may be administered to the patient via the introduction of a suitable stent (e.g. a coronary stent that is introduced in connection with the treatment procedure).

Furthermore, *in utero* administration of cells may be applied to treat fetal cardiac defects.

In a further aspect, the present invention pertains to the use of a combination of at least one laminin comprising an α5 chain and at least one agent which activates the Wnt canonical pathway, for deriving a multipotent $Isl1^+$ cell (i.e. for inducing a cell to enter a multipotent $Isl1^+$ lineage). The at least one laminin comprising an α5 chain may be selected from the group comprising laminin 511, laminin 521, a combination of laminin 511 and laminin 521, any natural, recombinant, or synthetic protein, which has at least approximately 70% sequence identity to the polypeptide sequence of laminin 511 or laminin 521, any natural, recombinant, or synthetic protein comprising the polypeptide sequence of the laminin α5 chain G-domain, any natural, recombinant, or synthetic protein comprising a polypeptide sequence, which has at least approximately 70% sequence identity to the polypeptide sequence of the laminin α5 chain G-domain, and any cell culture growth substratum or cell culture medium additive comprising polypeptides from the polypeptide sequence of laminin 511 or laminin 521. Further in accordance with the present invention, the at least one agent which activates the Wnt canonical pathway, and which is comprised in the composition, may be selected from the group comprising Wnt-1, Wnt-3a, Wnt-8, Wnt-8b, and any combination thereof.

In another aspect, methods are provided that use the Isl1$^+$ progenitors generated and expanded by the methods described herein. In one embodiment, the Isl1$^+$ progenitors generated and expanded by the methods described herein are used for the production of a pharmaceutical composition, for example a composition for regenerative medicine. Additionally, the Isl1+ cells that have been differentiated into cardiomyocytes may also be used in pharmaceutical compositions, for instance for the treatment of various cardiology-related illnesses and ailments. The compositions may be for use in transplantation into subjects in need of cardiac transplantation, for example but not limited to subjects with congenital and/or acquired heart disease and/or subjects with vascular diseases and/or cardiovascular diseases.

The subjects may have or be at risk of heart disease and/or vascular disease and/or cardiovascular disease, and the Isl1$^+$ progenitors generated and expanded by the methods of the present invention may be autologous and/or allogenic (and the cells may optionally be genetically modified).

The Isl1$^+$ progenitors and the further differentiated cardiac cells generated and expanded by the methods of the present invention may be used in assays and experiments, for instance for screening agents, for example agents for the development of therapeutic interventions of diseases, including, but not limited to, therapeutics for congenital and adult heart failure. Alternatively, the cells of the present invention may be employed in assays for screening agents that are toxic to the cell (e.g. cardiotoxicity tests).

Thus, the present invention provides methods for; (i) triggering or inducing cells, typically mesenchymal stem cells, to enter the Isl1$^+$ lineage pathway by activating the Wnt canonical pathway (i.e. a method for deriving an Isl1$^+$ cell) and culturing in the presence of laminin(s) having certain characteristics, (ii) expanding Isl1$^+$ cells, for example any Isl1$^+$ progenitor of the Isl1 $^+$ cardiovascular progenitor hierarchy, by continued renewal of the cells so obtained, and (iii) differentiation of said Isl1$^+$ cells into e.g. cardiac cells, such as cardiomyocytes, smooth muscle cells, and/or endothelial cells.

The at least one agent that activate, increase or enhance the Wnt canonical pathway, herein alternatively termed "agent that activate the Wnt canonical pathway" or "activating agents" shall be understood to comprise agents that activate the Wnt canonical pathway directly or indirectly.

For convenience and clarity, certain terms employed herein collected below. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

The term "laminin" pertains to a family of heterotrimeric glycoproteins composed of α, β, and γ chains that exist, respectively, as five, three, and three genetically distinct types forming 15 different combinations in human tissues. They are named according to the chain composition; for example, laminin-511 (LN-511) consists of α5, β1, and γ1 chains. Earlier, laminins were named according to the order of their discovery. Thus, laminin-111 was named laminin-1; laminin-511 was named laminin-10; laminin-521 was named laminin-11.

Laminins are the main component of basement membranes and are in contact with various cells *in vivo* and different laminins show various spatio-temoral expression patterns in developing organisms as well tissue-specific location and functions. Thus, laminin-211 and laminin-221 are primarily present in basement membranes around muscle cells and motor neuron synapses, laminin-332 is specific for subepithelial basement membranes, and laminin-511 is practically ubiquitous. The first discovered laminin, laminin-111, which is also known as laminin-1 or just laminin, is restricted to the early embryo and is a very rear isoform in adult human tissues. Laminin-111 has been studied most extensively, because it is possible to isolate it from the mouse Engelbreth-Holm-Swarm (EHS) sarcoma, which is easy to induce. Other laminins are hard to isolate from tissues due to extensive cross-linking and they should preferably be expressed as recombinant proteins in mammalian cells.

The cellular effects of laminins are largely mediated via ligand binding to cell membrane receptors such as integrins, dystroglycan, and Lutheran glycoprotein. They can induce direct outside-in signaling in the cells, which has been shown to

alter transcription levels of genes and even influence chromatin remodeling of the gene promoters. Laminins are very different in their ligand specificity. Thus, laminins containing an α5 chain can avidly bind integrin α6β1, integrin α3β1 and Lutheran glycoprotein, while laminin-111 (or laminin) has highest affinity to integrin α7X2β1 and dystroglycan. Laminin β and γ chains can modulate laminin-integrin binding, but the cellular interaction is mediated primarily via binding of the α chains to the receptors. Therefore, α5-containing laminins should be regarded as different from, e.g. α2-containing laminins.

Thus, the term "laminin comprising an α5 chain" used herein refers to any laminin polypeptide comprising an α chain of type five (5), e.g. laminin-511, 521, 522, 523, etc. Additional laminins comprising an α5 chain are naturally also within the scope of the present invention. As used herein, an α5 chain refers to any polypeptide chain that has at least 70% sequence identity to the laminin α5 chain, e.g. of SEQ ID No. 1, preferably a sequence identity of at least 80%, and even more preferably a sequence identify of at least 90%. More generally, all polypeptides and/or nucleotides disclosed in the present application encompass polypeptide and/or nucleotide sequences that have at least 70% sequence identity to the polypeptide and/or nucleotide in question, preferably a sequence identity of at least 80%, and even more preferably a sequence identify of at least 90%.

The term "Isl1$^+$ cell" refers to cells that may under normal circumstances express the following markers (in addition to Isl1); mesodermal markers; Tbx6 and Brachyury T; C-Kit; activation markers of early cardiogenesis: GATA4, Mef2c, Nkx2.5; multipotency markers, such as Sox2, SSEA1, Nanog, Tra-1, KDR, or a combination of these; transcription factors involved in the transcriptional network of the second heart field: Tbx1, Hand2, FoxH1; other relevant factors linked to the Isl1 expression like Fgf8 and 10.

The term "cardiac cell" or "heart cell" or "cardiomyocyte" shall be understood as a cell that may under normal circumstances express markers such as: myosin heavy chains (MyH), Troponin T (TnT) and/or Troponin I (TnI) and the proteins are normally organized in contractile elements.

The term "progenitor cells" or “progenitors” is to be understood to refer to cells that have a cellular phenotype that is more primitive (i.e. is at an earlier stage along a developmental pathway or progression than is a fully differentiated cell) relative to a cell which it can give rise to by differentiation. Progenitor cells also frequently have significant or very high proliferative potential, and they can give rise to multiple distinct differentiated cell types or to a single differentiated cell type, depending on the developmental pathway and on the surrounding environment in which the cells develop and differentiate. A cell can begin as progenitor cell and proceed toward a differentiated phenotype, but then revert and re-express the progenitor cell phenotype. Consequently, a progenitor cell can be derived from a non-stem cell.

The term "stem cell" is to be understood to refer to an undifferentiated cell which is capable of proliferation and to give rise to more progenitor cells having the ability to generate a large number of mother cells which may in turn give rise to differentiated, or differentiable daughter cells. The daughter cells themselves can be induced to proliferate and produce progeny that subsequently differentiate into one or more mature cell types, while at the same time retaining one or more cells with parental developmental potential. The phrase "stem cell" refers to a subset of progenitors that have the capacity or potential, under particular circumstances, to differentiate to a more specialized or differentiated phenotype, and which retains the capacity, under certain circumstances, to proliferate without substantially differentiating. The term “stem cell” may generally refer to a naturally occurring mother cell whose descendants (progeny) specialise, often in different directions, through differentiation, e.g., by acquiring completely individual characters, as occurs in progressive diversification of embryonic cells and/or tissues. Cellular differentiation is a complex process typically occurring through many cell divisions and a differentiated cell may be derived from a multipotent cell which is itself derived from a multipotent cell, etc. While each of these multipotent cells may be considered to be stem cells, the range of cell types each can give rise to may vary substantially. Some differentiated cells also have the capacity to give rise to cells of greater developmental potential and such capacity may be be induced artificially upon treatment with various factors, or may be natural. Stem cells are also in many instances "multipotent" because they can produce progeny of more than one distinct

cell type, but this is not required for their "stem-ness." Self-renewal is the other typical part of the stem cell definition, and it is important for the purposes of this invention. Theoretically, self-renewal can occur by either of two major mechanisms, i.e. symmetrically or asymmetrically. Asymmetric division refers to one daughter retaining the stem state and the other daughter expressing some distinct other specific function and phenotype. Symmetric division refers to the process when some of the stem cells in a population divide symmetrically into two stems, thus maintaining some stem cells in the population as a whole, while other cells in the population give rise to differentiated progeny only. It is formally possible that cells that begin as stem cells proceed toward a differentiated phenotype, but then revert and re-express the stem cell phenotype, a process which is often referred to as e.g. "dedifferentiation" or "reprogramming" or "retrodifferentiation".

The term "differentiation" in the present context is to be understood to mean the formation of cells expressing markers or characteristics known to be associated with cells that are more specialized and closer to becoming terminally differentiated cells, which are incapable of further division or differentiation. Progressive differentiation or progressive commitment refers to the pathway along which cells progress from a less committed cell, to a cell that is increasingly committed to a particular cell type, and eventually to a cell that is terminally differentiated. Cell which are more specialized (for instance have begun to progress along a path of progressive differentiation) but not yet terminally differentiated are referred to as partially differentiated. Differentiation is a development process whereby cells acquire a specialized phenotype (for instance acquire one or more characteristics, features, or functions distinct from other cell types). In certain cases, the differentiated phenotype may refer to a cell phenotype that is at the mature endpoint in some developmental pathway (a so called terminally differentiated cell). In many (but not all) tissues, the process of differentiation is connected with exit from the cell cycle and in these instances, the terminally differentiated cells lose or greatly restrict their capacity to proliferate. For the purposes of the present invention, the term "differentiation" or "differentiated" is to be understood to refer to cells that are more specialized in their fate and/or function than at a previous point in their development, consequently including both cells that are terminally differentiated and cells that (although not

terminally differentiated) are more specialized than at a previous point in their development.

In the context of cell origins and development (i.e. cell ontogeny), "differentiated", or "differentiating" is a relative term meaning a "differentiated cell" is a cell that has progressed further down a developmental pathway than the cell it is being compared with. Stem cells can thus differentiate to precursor cells restricted to a certain lineage (such as for instance a mesodermal stem cell), which can in turn differentiate into other types of precursor cells further down the pathway in question (such as a cardiomyocyte precursor), and then to a terminally differentiated cell (which may play a characteristic role in a certain tissue type, and may or may not (depending on the circumstances) retain the capacity to proliferate further).

The term "embryonic stem cell" is to be understood to refer to the pluripotent stem cells of the inner cell mass of the embryonic blastocyst, and such cells can also be obtained from the inner cell mass of blastocysts derived from somatic cell nuclear transfer. The distinguishing features of an embryonic stem cell define an embryonic stem cell phenotype. Consequently, a cell has the phenotype of an embryonic stem cell if it possesses one or more of the unique characteristics/features of an embryonic stem cell, such that the cell is distinguishable from other cells. Exemplary distinguishing embryonic stem cell characteristics/features include but are not limited to proliferative capacity, gene expression profile, karyotype, differentiation capacity, responsiveness to particular culture conditions, as well as possible additional characteristics/features.

The term "adult stem cell" or "ASC" is used to refer to any multipotent stem cell derived from non-embryonic tissue, including fetal, juvenile, and adult tissue. Stem cells have been isolated from a wide variety of adult tissues including blood, bone marrow, brain, olfactory epithelium, skin, pancreas, skeletal muscle, and cardiac muscle. Examples of adult stem cells include but are not limited to mesenchymal stem cells, hematopoietic stem cells, neural stem cells, neural crest stem cells, and pancreatic stem cells. As indicated above, stem cells have been found resident in

basically every tissue type and within the context of the present invention, therefore, stem cell populations can be isolated from virtually any animal tissue.

The terms "proliferating" and "proliferation" is to be understood to refer to an increase in the number of cells in a population (growth) by means of cell division. Cell proliferation is generally understood to be an implication of coordinated activation of multiple signal transduction pathways (in response to the environment surrounding a cell), including growth factors and other mitogens. Cell proliferation may also be promoted by release from negative or blocking actions of intra- and/or extracellular signals and mechanisms.

The terms "renewal" or "self-renewal" or "proliferation" are used interchangeably herein, and are to be understood to refer to the ability of stem cells to renew themselves by dividing into the same non-specialized cell type over long periods, for instance many months to years. Proliferation may in some cases refer to expansion of cells by the repeated division of a single cell into two identical daughter cells.

The term “embryonic” may within the context of the present invention be used to refer to fetal material (i.e. cells or tissues), i.e. cells/tissues obtainable from a developing foetus. By definition, an embryo enters the foetal stage (i.e. becomes a foetus) at the time of organogenesis (i.e. when organs start to form) and the tissues and cells used in certain examples in accordance with the present invention are obtained from foetal material, i.e. from gestational week 6-10. Normally, the definition in humans is that the embryonic period stretches from fertilization to the eight week after fertilization, whereas the period starting from the eight week and lasting till partus is defined as the fetal period. Essentially, within the context of the present invention, when pre-natal cells and/or tissues are utilized they are normally of a fetal character, but sometimes also of an embryonic character.

The terms "mesenchymal cell" or "mesenchyme" or “cells from a mesenchymal fraction” or “mesenchymal stem cells” or “MSCs” are used interchangeably herein and may for the purposes of the present invention refer to in some instances the fusiform or stellate cells that are found between the ectoderm and endoderm of young embryos, or cells that are found in adult tissue such as bone marrow,

mesoderm, umbilical cord blood, Wharton's jelly, adult muscle, developing tooth buds, and/or amniotic fluid. Most mesenchymal cells are derived from established mesodermal layers, but they may also develop from neural crest or neural tube ectoderm in the cephalic region. A "mesenchymal stem cell" or a "mesenchymal cell" is to be understood to refer to a cell from any suitable adult tissue such as bone marrow, mesoderm, umbilical cord blood, Wharton's jelly, adult muscle, developing tooth buds, and/or amniotic fluid, or from fetal or embryonic connective tissue. Furthermore, induced pluripotent stem cells (i.e. iPS cells), which may be derived from e.g. a human fibroblast, a human hepatocyte, and/or a human blood cell, may be utilized in the context of the present invention to derive $Isl1^+$ cells. Further, mesenchymal progenitor or mesodermal progenitors may refer to progenitor cells of mesodermal origin. The mesoderm is the middle embryonic germ layer, lying between the ectoderm and the endoderm, from which connective tissue, muscle, bone, and the urogenital and circulatory systems develop. A cell from the "mesenchymal fraction" shall within the context of the present invention be understood to relate to any type of mesenchymal cell (i.e. a mesenchymal stem cell (MSC)) that may be derived from e.g. any of the following sources: bone marrow, mesoderm, umbilical cord blood, Wharton's jelly, adult muscle, developing tooth buds, and/or amniotic fluid. The MSCs as per the present invention may for instance be obtained by aspiration from the bone marrow, or by isolation from umbilical cord blood, or extracted from the mesoderm of either a foetus and/or an embryo.

The term "lineages" as used herein is to be understood to pertain to a cell with a common ancestry or cells with a common developmental fate. A cell that has entered an "islet 1+ lineage" is to be understood to refer to the cell as being an $Isl1^+$ progenitor and expressing $Isl1^+$, and which may differentiate along the Isl1+ progenitor lineage restricted pathways. Such pathways may be one or more developmental lineage pathways, for instance an endothelial lineage, a cardiac lineage or a smooth muscle lineage. For example, a cell that has entered the Isl1+ lineage is a cell which is has the capacity of differentiating into three major cell types in the heart (i.e. cardiac, smooth muscle, and endothelial cells).

A "marker" is to be understood to describe the characteristics/features and/or phenotype of a cell and markers can often be used for selection/identification of cells comprising characteristics of interests. Markers will vary with specific cells. Markers are characteristics, whether morphological, functional or biochemical (enzymatic) characteristics of the cell of a particular cell type, or molecules expressed by the cell type. Markers are preferably proteins and more preferably possess at least one epitope for antibodies or other binding molecules that may be available. However, a marker may be any molecule found in or on a cell including but not limited to proteins (peptides and polypeptides), polysaccharides, nucleic acids, lipids, and steroids. Some examples of morphological characteristics/features or traits include for instance shape, size, and nuclear-to-cytoplasmic ratio. Examples of functional characteristics or traits comprise for instance the capacity to migrate under certain conditions, the ability to adhere to certain substrates, and the capacity to differentiate along particular lineages.

The terms "subject" and "individual" and “patient” may be used interchangeably herein and are to be understood to refer to an animal, for instance a human being, from whom cells can be obtained and/or to whom treatment, including prophylaxis or preventative treatment (for instance using the cells as per the present invention) is provided. Advantageously, the subject of the treatments as described in the context of the present invention is a mammal, preferably a human, or other mammals, preferably domesticated or production mammals.

Cardiovascular diseases, conditions or disorders are to be understood to comprise medical conditions related to the cardiovascular (heart) or circulatory system (blood vessels). A response to myocardial injury follows a clearly defined path in which some cells die while others enter a state of hibernation (wherein they are not yet dead but are dysfunctional). This is followed by infiltration of inflammatory cells and deposition of collagen as part of a scarring process. This happens in parallel with in-growth of new blood vessels and a certain extent of continued cell death. The term cardiovascular diseases is intended to comprise all disorders and diseases characterized by insufficient, undesired or abnormal cardiac function, for instance congenital heart disease and any condition which leads to congestive heart failure in a subject, particularly a human subject, ischemic heart disease, hypertensive heart

disease and pulmonary hypertensive heart disease, and valvular disease. Insufficient or abnormal cardiac function can an implication of disease, injury, trauma, and/or aging, comprising for instance diseases and/or disorders of the pericardium, heart valves (for instance stenosed valves, incompetent valves, rheumatic heart disease, aortic regurgitation, mitral valve prolapse), myocardium (myocardial infarction, coronary artery disease, heart failure, angina, ischemic heart disease) blood vessels (for instance arteriosclerosis or aneurysm) or veins (for instance varicose veins, haemorrhoids). The term "ischemia" is to be understood to refer to any localized tissue ischemia due to reduction of the inflow of blood, and the term "myocardial ischemia" is to be understood to comprise circulatory disturbances caused by coronary atherosclerosis and/or inadequate oxygen supply to the myocardium. An acute myocardial infarction may represent an irreversible ischemic insult to myocardial tissue. This insult may result in an occlusive (for instance thrombotic or embolic) event in the coronary circulation, producing a milieu in which the myocardial metabolic demands are exceeding the supply of oxygen to the myocardial tissue.

The term "agent" refers to any chemical, entity or moiety, comprising for instance (without any limitation) synthetic and naturally-occurring non-proteinaceous and proteinaceous entities. An agent may be a nucleic acid, nucleic acid analogues, peptides, proteins, antibodies, aptamers, oligomers of amino acids, nucleic acids, or carbohydrates comprising for instance oligonucleotides, ribozymes, DNAzymes, glycoproteins, proteins, siRNAs, lipoproteins, aptamers, and any modifications and combinations thereof. Agents are to also be understood to possibly include small molecules having certain chemical moieties, comprising for instance chemical moieties such as unsubstituted or substituted alkyl, aromatic, or heterocyclyl moieties including macrolides, leptomycins, and related natural products or analogues thereof. As used herein, the term "Wnt activating agent" refers any agent that activates the Wnt canonical pathway, or inhibits or suppresses the activity of inhibitors of Wnt canonical pathway. The activation is preferably selective activation, which means that the Wnt3 pathway is activated to the substantial exclusion of the effects (i.e. activation of inhibition) on non-Wnt3 pathways.

The term "therapeutically effective amount" is to be understood to refer to an amount thatresults in an improvement, allevation, or remediation of the disease, disorder, or symptoms of the disease or condition.

The terms "administering," "introducing" and "transplanting" are used interchangeably for the purposes of the present invention, for instance in the context of the placement of $Isl1^+$ progenitors (for example $Isl1^+$ cells or Troponin T-expressing cardiomyocytes differentiated using the methods of the present invention) into a subject. A suitable method or route is one which leads to at least partial localization of the cardiovascular stem cells at a desired site. The cells may be administered (delivered) by any appropriate route which results in delivery to a desired location/tissue/site in the subject where at least a portion of the cells or components of the cells remain viable (the period of viability of the cells after administration may be as short as a few hours to a few days, to as long as several years).

The modes of administration suitable for the purposes of the present invention comprise for instance (without limitation) intravenous, intramuscular, intraarterial, intrathecal, intraventricular, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, sub capsular, subarachnoid, intraspinal, intracerebro spinal, and intrasternal injection and infusion.

The phrase "pharmaceutically acceptable excipient" as used herein is to be understood to relate to a pharmaceutically acceptable material, composition or vehicle, for instance a solid or liquid filler, a diluent, an excipient, a carrier, a solvent or a encapsulating material, involved in suspending, maintaining the activity of or carrying or transporting the subject agents from one organ, or portion of the body, to another organ, or portion of the body.

The term "derivative" or "variant" as used herein is to be understood to refer to for instance a peptide, chemical or nucleic acid that differs from the naturally occurring polypeptide or nucleic acid by at least one amino acid or nucleic acid, for instance deletions, additions, substitutions or side-chain modifications, but at the same time retains one or more specific functions and/or activities of the naturally occurring

molecule. Amino acid substitutions may comprise alterations in which an amino acid is replaced with a different naturally occurring or a non-conventional amino acid residue, and these substitutions may be conservative or non-conservative.The present invention pertains inter alia to use of agents that activate the Wnt canonical pathway. Examples of Wnt activating agents may include for example polypeptides, peptides, nucleic acids nucleic acid analogues, phage, , peptidomimetics, antibodies, small or large organic molecules, ribozymes or inorganic molecules or any combination of thereof (optionally naturally occurring).The agents that activate the Wnt canonical pathway may include for example antibodies (polyclonal or monoclonal), neutralizing antibodies, antigen-binding antibody fragments, peptides, proteins, peptidomimetics, aptamers, oligonucleotides, hormones, small molecules, nucleic acids, nucleic acid analogues, carbohydrates or variants thereof that function to inactivate or activate one or more, as the case may be, nucleic acid and/or protein participant in a Wnt pathway as described herein or as known in the art. It shall be understood that the above described exemplifying embodiments can be modified without departing from the scope of the invention, inter alia with respect to the described constituents, materials, and process parameters applied. The invention will now be further exemplified with the enclosed examples, which naturally also can be modified without departing from the scope of the invention.

Experimental Section

In order to develop a feeder free culturing system where the origin and expansion of the Isl1+ cells could be followed, the inventors synthesized a plasmid construct comprising parts of the Isl1-promotor region linked to the fluorescent marker green fluorescent protein (GFP). The plasmid was introduced into the cells by means of electroporation. Mesenchymal cells (i) derived from rat embryonic hearts, (ii) obtained from rat bone marrow, (iii) obtained from the bone marrow of young healthy donors, (iv) obtained from human cord blood, or (v) isolated from human amniotic sources, were labeled with the Isl1 gene construct concomitant to the addition of Wnt-containing medium (100 ng/ml Wnt-3a and 2,5 mM BIO). The feeder cells used in previous studies were in this protocol replaced with dishes pre-coated with various

laminins comprising an α5 chain (notably laminins 511, laminin 521, and a combination thereof). In culture the cells grew to confluency and were passaged every other day. At each passage, cells were analysed immunohistochemically for the expression of Isl1. With each successive passage the ratio of Isl1+ cells increased, which also corresponded to the Isl1 expression registered directly on the plate through activation of the Isl1 gene construct. In the initial fraction less than 1% of the cells expressed Isl1, which increased to 73±18% after two weeks in culture. Within two weeks, the Isl1+ cell population had expanded 16 times from the initiation of the Wnt+laminin-protocol and 35% of the cells were still proliferating expressing Ki67 at the end of the culturing process.

Pure populations of Isl1+ cells have not previously been derived from human fetal or embryonic hearts. To study the ability of the adherent fraction of human fetal or embryonic hearts (gestational weeks 5 to 10) to give rise to Isl1+ cells, these cells, and cells aspirated from human bone marrow or isolated from cord blood, were labeled with the Isl1+ gene construct after the cells had grown to confluence and before being seeded on plates pre-coated with α5-containing laminin(s) in accordance with the present invention. Additionally, 100 ng/ml Wnt-3a was again utilized to activate the Wnt canonical pathway, but this time BIO was excluded and the concentration of fetal bovine serum (FBS) was increased to 10%, cells proliferated in a similar manner to rat $Isl1^+$ cells. These cells demonstrated a similar growth pattern as the rat cells and MSCs from other human tissues (i.e. mesenchymal cells obtainable from bone marrow, amniotic or cord blood cells), and after two weeks the cells had expanded almost 70 times from the initiation of the protocol, generating 92±2 % pure populations of Isl1+ cells. At the end of the culturing process, more than 40% of the cells were still proliferating expressing Ki67.

The initial adherent fraction of the fetal/embryonic hearts, that give rise to both human and rat Isl1+ cells, have a similar appearance as mesenchymal stem cells. These cells can be characterized as regards to their expression of surface antigens. We therefore prepared adherent cells from human embryonic hearts (gestational week 6), which were expanded for five weeks followed by FACS analysis using the mesenchymal panel. The adherent fraction expressed the mesenchymal stem cell markers CD105, CD90, CD73 and CD44, while being negative for the hematopoietic

lineage markers CD14, CD19, CD34, CD45 and endothelial marker CD31 (Figure 3). Similar expression profiles were detected using mesenchymal cells obtainable from bone marrow or cord blood cells. In order to ensure that the cells did not change their phenotype during expansion, samples of cells from the different sources were removed and stimulated according to the Wnt/laminin-protocol. This yielded similar amounts and ratio of Isl1+ cells. These findings lead us to conclude that Isl1+ cells can be generated from various mesenchymal cell sources by stimulation of the Wnt canonical pathway when culturing on laminin(s) comprising an α5 chain.

The gene-expression profile of cultured rat and human Isl1+ cells were studied and compared to the Isl1+ cells present *in vivo* in the embryonic hearts (Figure 4). These studies were performed in order to confirm that the cultured Isl1+ cells were similar to the Isl1+ cells that give rise to the second heart field (SHF). In order to identify a core group of transcription factors that most probably are activated in Isl1+ cells, we identified relevant transcription factors shown to be important for regulating the SHF development in mice. These studies have identified a set of transcription factors that are intimately involved in the transcriptional network of the SHF. These include members of the NK class of homeodomain proteins, the zinc-finger transcription factor GATA4, the MADS domain transcription factor Mef2c, T-box and Forkhead transcription factors as well as Hand class of basic-loop-helix (Hand) factors. Downstream to the Isl1 transcription factor, the Isl1-GATA4-Mef2c pathway seems to be playing a pivotal role for activation of the cascade that induces derivation of cardiomyocytes. The Isl1 and GATA4 factors seem to interact to induce activation of the Mef2c transcription factor, followed by upregulation of the transcription factors Nkx2.5 and Hand2. Other key-regulators of the SHF, are the Forkhead transcription factor FoxH1, the Tbx1 transcription factor, which is dependent on Forkhead factors for its activation and finally Fgf8, which in turn is regulated by Tbx1. Furthermore, blockage of Fgf-signaling prevents expansion of Isl1+ cells in the SHF, indicating an upstream effect on the Isl1-pathway. The cultured rat and human Isl1+ cells express the mesodermal markers TBX6 and Brachyury T, together with the above-mentioned key transcription factors involved in the transcriptional network of the SHF, i.e. Isl1, Mef2c, Nkx2.5, FoxH1, Tbx1, Fgf8 and Fgf10. Furthermore, they also express the early cardiomyocyte marker mesoderm posterior 1 (MESP1), while being negative

for the more mature cardiomyocyte markers troponin I (TnI), troponin T (TnT) and myosin heavy chains (Myh) 6 and 7.

The cultured Isl1+ cells demonstrate a similar gene-expression profile as the Isl1+ cells isolated from the embryonic hearts, except that these cells have an even higher expression of the late cardiomyocyte markers Myh, TnT and TnI. In the cultured human Isl1+ cells, the Isl1-GATA4-MEF2c pathway is more upregulated than in both the cultured rat Isl1+ cells and in the *in vivo* present Isl1+ cells. This correlates well with the upregulation of bone morphogenic peptides (BMPs), which are important for stimulation of early cardiogenesis and concomitant upregulation of the early cardiomyocyte markers Nkx2.5 and MESP1.

Other important findings are that both the cultured and the *in vivo* present Isl1+ cells express the pluripotency markers Tra-1, Sox2, Nanog and stage-specific embryonic antigen-1 (SSEA-1) while at the same time expressing nestin (NES), paired box 3 (PAX3), known to be expressed in neural crest cells and to a smaller extent the smooth muscle markers myocardin (MYOCD) and Myh11.

According to micro-array data the cultured human and rat Isl1+ cells are multipotent stem cells with a genetic signature (Isl1+, KDR+, Nkx2.5+, Mef2c+) that has shown to be effective in differentiating into the three cardiovascular lineages with emphasis on cardiomyocytes. The cultured Isl1+ cells also express the stem cell markers C-Kit, the epicardial marker Tbx18, together with the multipotent surface marker SSEA1, which make the relationship between these selection markers used to identify different populations of cardiac progenitors a bit confusing. Perhaps these markers identify different states of activation of Isl1+ cells, which at the same time demonstrates the plasticity of these cells.

In prior studies we have shown that Isl1+ cells are during the embryonic and post-natal period localized to the outflow tract (OFT). Even in the adult heart, resident Isl1+ cells can be found in this region and respond to ischemia by migrating from the OFT into the ischemic regions. This could imply that Isl1+ cells respond to myocardial ischemia by actively homing into this region. In order to follow the homing capacity of Isl1+ cells *in vivo* and at the same time confirm this migration immunohistochemically, the cells were labelled with luciferase and β-gal expressing

transposons by means of electroporation. Cells expressing luciferase can be followed *in vivo* with bioluminescent imaging and β-gal expressing cells can be stained for immunohistochemistry. By utilising the Sleeping-Beauty-100X (SB-100X) transposon system the transgenes were stably integrated into the genome. Labeled rat Isl1+ cells, were injected into the left ventricle wall in immunoincompetent rats and the migration of the Isl1+cells was detected by an In Vivo Imaging System (IVIS®Spectrum CT) (PerkinElmer Inc., USA). Within 24 hours, the majority of the Isl1+ cells have started to migrate towards the outflow tract and to a lesser extent there were cells left at the site of injection (Figures 5A-C and 6A-C). In contrast, if the Isl1+ cells were injected into the peri-ischemic region induced by ligation of the left anterior descending artery (LAD), the majority of the cells did not migrate to the OFT. Instead they were found in the infarct where they at 2 weeks had become elongated and arranged in parallel to the surrounding cardiomyocytes. In order to further characterize the homing ability of Isl1+ cells, they were injected into the left ventricle of normal hearts and their migration followed with IVIS. Within 24 hours the Isl1+ cells were detected in the OFT, but after induction of myocardial infarction through LAD ligation, the cells migrated into the ischemic region. To further study the homing characteristics of Isl1+ cells, a myocardial infarction was induced followed by intravenous administration of labeled Isl1+cells through a tail vein. Again the cells migrated to the myocardial infarct. These results imply that the Isl1+ cells have the ability to home, especially to ischemic regions of the heart but also to areas where the Isl1+ cells are resident in the embryonic and adult heart. The explanation for this migratory response might be that during myocardial ischemia, the surrounding cardiomyocytes up-regulate CXCR4, which is the receptor for stromal cell derived factor-1 (SDF-1). Mesenchymal stem cells are known to express SDF-1 and during ischemia these cells home from the circulation into the infarcted region stimulated through the SDF-1-CXCR4 axis. Moreover, engrafted mesenchymal stem cells secrete SDF-1, which were shown to cause homing of the cardiac progenitors into the infarct. According to our micro-array data, both cultured and especially *in vivo* present Isl1+ cells express CXCR4, which probably mediates the homing of Isl1+ cells into the ischemic region. The SDF-1-CXCR4 axis might also be important for homing of Isl1+ cells into the OFT of a normal heart, since *in vivo* present Isl1+ cells have a high expression of this receptor.

In this study we have shown that it is possible to derive pure populations of rat and human Isl1+ cells from mesenchymal cells from the bone marrow of healthy young donors, from mesenchymal cells obtained from cord blood, from amniotic sac sources, and from embryonic/fetal cardiac mesenchymal cells in a culturing system where feeder cells have been replaced by laminins comprising an α5 chain (notably laminins 511, laminin 521, and a combination thereof), and where we use a culturing medium that stimulates the Wnt canonical pathway. Both the human and rat Isl1+ cells have a similar gene-expression profile as their respective *in vivo* present cells, but they are more immature. The cultured Isl1+ cells have a genetic signature that is favorable for differentiation into the three cardiovascular lineages and they actively home into ischemic myocardium. In this study we have also devised a method for differentiating Isl1+ cells into cardiac cells (cardiomyocytes, smooth muscle cells, and endothelial cells), by including either laminin 111, 211, or 221, or any combination thereof, in the culture medium. These characteristics are beneficial for the use of Isl1+ cells to repair damaged hearts after myocardial infarction. Another field is the congenital heart diseases, where defects affecting the aortic arch, proximal pulmonary arteries and the outflow tract account for almost 30% of all congenital cardiac defects. These defects might be due to alterations in SHF migration, differentiation or proliferation and perhaps in the future, *ex vivo* cultured Isl1+ cells can be used for *in utero* treatment of these cardiac defects. This reflects the magnitude of importance of our findings, which hopefully will add a new dimension to regenerative cardiology.

Animals and ethics

The animal care committée of the Karolinska University Hospital approved all experimental animal procedures. Pregnant Sprague-Dawley (B&K Universal AB, Sollentuna, Sweden), gestational day 14 and Rowett nude rats (RNU, genotype rn/rnu) (Charles River Deutchland Inc., Germany) weighing 250-300g were used in this study. To collect bone marrow tissue and cord blood individual permission was obtained using a standard informed consent procedure and prior approval by the regional ethical committee. To collect human embryonic tissue (gestational weeks 5 to 10), individual permission was obtained using a standard informed consent

procedure and prior approval by the regional ethical committee. The investigation conforms to the principles outlined in the Declaration of Helsinki.

Cloning of the Isl1-Promoter Construct and transfection of the mesenchymal cells

A 3kb genomic human DNA fragment upstream of an *Isl1* start codon (-3 to -3170 from ATG) was amplified by PCR with a high-fidelity Phusion DNA polymerase (Thermo Scientific). Forward primer 5´- aaa gag ctc GGT GTA ACA GCC ACA TTT -3´ and reverse primer 5´- gga gaa ttc CTG TAA GAG GGA GTA ATG TC -3´. The PCR product was cloned into the MCS of the vector pEGFP-1 (Clontech Laboratories Inc, USA) between the restriction enzyme cleavage sites SacI and EcoRI. The Isl1-plasmid was introduced into the rat and human cells using the Neon™ Electroporation system (Invitrogen, US). Pig pancreatic Isl1+ cells were used as positive controls, while Human Aortic Endothelial Cells (HAEC) were used as negative controls for the Isl1-gene construct.

Derivation and expansion of Isl1+ cells from embryonic rat hearts

For each experiment 10 pregnant sprague-dawley rats (gestational day, GD, 13 to14) were used. The rats were eutanized in a CO_2 chamber after which the whole embryos (approximately 10 embryos/rat) were removed through a low midline abdominal incision. From each embryo the heart was removed under microscope, minced into small pieces and rinced repetitively with Hank´s buffered salt solution (HBSS) (GibcoBRL, NY, USA). The heart pieces were then predigested over night in 4°C in 0,5mg/ml Trypsin-solution in HBSS. The next step was to prepare the mesenchymal fraction, which is a modification of the protocol developed by Laugwitz and coworkers (Laugwitz *et al.*, 2005). The predigested heart pieces were treated with collagenase type II (Worthington Biochemical Corp, Lakewood, NJ) 240U/ml in HBSS, 2-3ml per round in the incubator at 37°C, for 10 to 15 minutes under gentle stirring. The supernatant was then centrifuged at 330-350g for 8 minutes, resuspended in ice cooled HBSS. This procedure was repeated until the heart pieces were dissociated. The pooled cells were again washed, centrifuged at 330-350g twice and resuspended in Dulbecco´s Modified Eagle´s Medium (DMEM) 4.5/M199 (4:1) (GibcoBRL) containing 10 % horse serum (GibcoBRL) and 5% fetal bovine serum (FBS) (PAA Laboratories Inc., USA) with MycoZap (Lonza, Switzerland). The mesenchymal (adherent) fraction was separated from the

cardiomyocytes and endothelial cells by two rounds of culture on plastic wells for 1 hour in incubator at 37°C, 5 % CO_2 in 3 % O_2. In order to follow the derivation of Isl1+ cells from the adherent fraction, these cells were labeled with the Isl1-gene construct containing the promotor for the Isl1-gene linked to the green fluorescent protein (GFP) gene (described above). This gene construct enables us to follow the derivation of Isl1+ cells directly in the culture dish. To stimulate the transformation of the mesenchymal cells into Isl1+ cells, the adherent fraction was detached using TrypLE^{TM}Express (GibcoBRL) and recultured on plates coated with a thin layer of laminin 511, 521 or a combination of these, cultured in medium DMEM high glucose (GibcoBRL), MycoZap (Lonza), Hepes (25mM) (GibcoBRL), glutamin (2mM) (Fisher Scientific) and 15% FBS (PAA) in incubator at 37°C, 5% CO_2 in 3% O_2. At confluence (48 h), the cells were detached using TrypLE^{TM}Express (GibcoBRL) and recultured on plates coated with Laminin 511, 521 or a combination of these, in Wnt-medium containing DMEM/F12 supplemented with 1ml B27 (GibcoBRL), 2% FBS (PAA), MycoZap (Lonza), epidermal growth factor (EGF, 10ng/ml) (R&Dsystems, Minneapolis, USA), 2.5 mM BIO (Sigma-Aldrich, USA), Wnt 3a (100 ng/ml) (R&Dsystems). At confluence, the cells were passaged and recultured on wells coated with laminin 511, 521 or a combination of these, in the same medium. At each passage, cells were harvested for analyses described below. After two weeks treatment with the Wnt medium, the culturing process was finished and cells harvested for further analyses or injections.

Furthermore, we have also freezed and thawed adherent cells from different time points. Freezing of the cultured adherent cells was performed by detaching the cells and resuspending them after centrifugation in cooled Recovery^{TM}- cell culture freezing medium (GibcoBRL). The cells were transferred to storage vials and frozen gradually (−1 °C per minute) down to−70 °C and then transferred to liquid nitrogen (−180 °C). When the frozen cells were recultured, they were quickly thawed to 37 °C, washed, and recultured on plastic plates coated with Laminin 511, 521 or a combination of these, in the same Wnt-medium and culturing conditions as described above.

Derivation and expansion of human Isl1+ cells

Aspiration of bone marrow cells was carried out as previously described from young health human donors. Cord blood cells and amniotic cells were collected and isolated using standard conventional procedures. Abortion was performed according to a technique previously described by Westgren *et al.* (Acta Scand Obstet Gynecol 73, 601-604 1994)). The cardiac material was minced into small pieces and predigested over night as described for the rat embryonic hearts. The following procedures followed the same steps as for culturing of rat Isl1+ cells except that the preplating procedure was extended to 72 hours and performed in mesenchymal stem cell medium with DMEM low glucose (GibcoBRL), 10 % FBS (PAA) and MycoZap (Lonza). As an alternative, culture medium free from non-human components may be employed, wherein the FBS is replaced by lyzed platelets obtainable from human blood. In order to follow the derivation of Isl1+ cells, the mesenchymal cells were labeled using the same Isl1-gene construct as for derivation of rat Isl1+ cells. To stimulate transformation into Isl1+ cells, we used similar culturing conditions as for rat Isl1+ cells with laminin 511, 521 or a combination of these as coating matrix, but with a Wnt-medium where BIO was excluded and serum level increased to 15 % FBS (PAA) (or lyzed human platelets at a suitable concentration). After each passage cells were saved for further analyses and after 2 weeks the culturing process was finished and cells harvested for immunohistochemical staining and micro-array analyses. The mesenchymal cells from different time periods were also freezed and thawed as described for the rat Isl1+ cells.

Immunohistochemical analyses of the cultured Isl1+ cells

At each passage and at the end of the culturing process, cells were cytospinned and analyzed immunohistochemically for the presence of Isl1+ cells and also Ki67 to detect cell proliferation. The cytospinned cells were initially stored frozen, followed by fixation in 4 % formaldehyde, blocked with serum, and incubated with the primary antibodies; Isl1: goat anti-human Isl1 (R&D systems); Ki67: mouse anti-rat Ki67 (clone MIB-5, DakoCytomation, Denmark) and direct-conjugated mouse anti-human Ki67-FITC (clone MIB-1, DakoCytomation) respectively. To visualize the unconjugated primary antibodies, the sections were washed and incubated with the

fluorescence-labelled secondary antibodies; Isl1: Alexa Fluor 488 rabbit anti-goat (Molecular Probes, Invitrogen) and for Ki67: Alexa fluor 568 goat anti-mouse (Molecular Probes). Staining with control antibodies, as well as staining of negative and positive control tissues, was done to verify the specificity of all antibodies.

Expansion of the human mesenchymal cells for flow cytometry analysis

The human mesenchymal stem cells (MSCs) from different sources were prepared according to the same protocol as described above. Instead of changing to the Wnt-medium, the MSCs were maintained in DMEM high glucose (GibcoBRL), MycoZap (Lonza), Hepes (25mM) (GibcoBRL), glutamin (2mM) (Fisher Scientific) and 15% FBS (PAA) for five weeks at 37° C, humidified air containing 5% CO_2 and 3% O_2. Cells were passaged twice during this time, followed by flow cytometry. In order to test that the correct MSC was expanded, cells were transferred to wells coated with laminin 511, 521 or a combination of these, for continued culture for another two weeks in Wnt-medium, followed by immunohistochemical analysis of Isl1 expression.

Flow cytometry

Approximately $0{,}85x10^5$ cells per single staining were washed in PBS, centrifuged once at 300g for 5min and incubated at 4°C for 30min with appropriate amounts of antibody. The labelled cells were then washed in PBS as above. For characterization of the mesenchymal fraction we used the following panel containing fluorochrome-conjugated monoclonal antibodies (mAbs) against the human surface antigens: CD31 (WM59), CD34 (581), CD73 (AD2), CD44 (G44-26), CD14 (MoP9), CD19 (HIB19), CD105 (266) (all from BD Biosciences, San Jose, CA); HLA DR (Tu36), CD45 (HI30) (Invitrogen) and CD90 (eBio5E10) (eBioscience Inc., USA). Each antibody-clone was titrated to optimal staining concentration using primary human samples. Data acquisition was done on a CyFlow ML (Partec GmbH, Munster, Germany), followed by data analyses with the FloJo software (TreeStar Inc., USA).

Laser capture microdissection (LCM)

Cryosections (7 µm) of whole rat embryos (GD 13), sliced in a transverse plane from head to tail and cryosections (7 µm) of a human embryonic heart (gestational week 9.5) and bone marrow-derived MSCs were collected onto sterile glass slides as well as on a 1-µm-thick PEN-Membrane-coated glass slides (Carl Zeiss, Germany). Targeted areas including both Isl1 positive and negative, were selected for capture

using a 10x and 40x objectives. The negative regions were harvested from the developing left ventricle. Cryosectioning was performed using the 4-2-4-protocol, in which each slide was mounted with 3 sections. In using this protocol, two unstained membrane slides was preceeded and followed by four cryosectioned slides, which were subsequently stained towards Isl1, using the same primary and secondary antibodies as for the cultured cells. These protocols aimed to histoanatomically localize the Isl1 positive and negative areas as well as generating high quality RNA yield. The micro-dissection process was performed through identifying the cells of interest, which in the next step were delineated and cut out through laser dissection. Equivalent Isl1 positive and negative areas were laser captured and total RNA isolated using the PicoPure RNA isolation kit (PicoPure RNA isolation kit, Arcturus Engineering, Invitrogen) following the manufacturer's protocol. The quantity and quality of the RNA were determined using 2100 Agilent Bioanalyzer, RNA 6000 Pico LabChip (Agilent, Palo Alto, CA, USA). All RNA samples were stored under sterile conditions at −80 °C for future analysis. All safety handling measures to avoid RNA degradation were fulfilled.

Microarray analysis

Total RNA from cultured rat and human Isl1+ cells was isolated using the PicoPure RNA isolation kit (PicoPure RNA isolation kit) following the manufacturer's protocol as described in the previous section. Equivalent amounts (about 2 ng) of purified total RNA from micro-dissected tissues as well as from cultured cells were reversely transcribed and amplified using the Ovation Pico WTA system (NuGEN Technologies, CA, USA) following the manufacturer's instructions. Sense strand cDNA was generated using WT-Ovation Exon Module (NuGEN Technologies). cDNA was fragmented and labelled using the Encore Biotin Module (NuGEN). Labeled cDNA were hybridized to Affymetrix Rat and Human Gene ST 1.0 microarrays (Affymetrix Inc, CA, USA) respectively. GeneChips were washed, stained and scanned using the Fluidic Station 450 and GeneChip Scanner 3000 7G (Affymetrix).

Preprocessing of the microarray data was performed in the Affymetrix Expression Console (v. 1.1) (Affymetrix Inc.) using the following methods: Summarization: PLIER; Background Correction: PM-GCBG; Normalization: Global Median.

Generation of heatmaps

Heatmaps were created using Qlucore Omics Explorer 2.2. Hierarchical clustering of both samples and variables was done using the Euclidean metric and data where each variable was normalized to mean 0 and variance 1. Average linkage and log2 transformation of signals were used. The heatmap color scale range from red (high expression) via black (average expression) to green (low expression).

Anaesthesia and Postoperative Care

RNU rats (genotype rn/rnu, Charles River Deutschland Inc.) used for intramyocardial injection of labelled rat Isl1+ cells were anesthetized with a subcutaneous injection of Midazolam (Dormicum, 5 mg/kg) (Algol Pharma AB, Germany), Medetomidin hydrochloride (Domitor vet, 0.1 mg/kg) (Orion Corp., Espoo, Finland), Fentanyl (0.3 mg/kg) (B.Braun Medical AG, Seesatz, Switzerland) and subsequently endotracheally intubated to be able to perform the intramyocardial injections and induce myocardial infarctions described below. Positive-pressure ventilation was kept at a rate of 100 cycles per minute with a tidal volume of 4–5 ml with room air using a ventilator (7025 Rodent ventilator, UGO BASILE S.R.L, Italy).
The anesthesia was reversed by an intramuscular injection of Flumazenil (Lanexat, 0.1 mg/kg) (Hameln Pharma, Germany) and Tipamezol hydrochloride (Antisedan vet 5 mg/kg) (Orion Corp., Espoo, Finland). Postoperative analgesia was maintained by administrating Buprenorphin hydrochloride (Temgesic, 0.004 mg/kg/twice per day for 3 days) (Schering-Plough Corp., Belgium). Rats that showed signs of malfunction were excluded from the study.

Study of in vivo survival and migration of labeled Isl1+ cells

The RNU rats were divided into four groups depending on how the Isl1+ cells were injected. In each experiment the hearts were exposed through a left thoracotomy. In the myocardial infarction groups, the left anterior descending artery (LAD) was permanently ligated and infarction induction was confirmed by color change and dyskinesia of the antero-lateral wall of the left ventricle. In each experiment 1 million labeled Isl1+ cells (obtained either from cardiac tissue or from the bone marrow) were used except for the intravenous experiment where 4 million cells were injected.

Before injection, the Isl1+ cells were labelled with 2μg pT2/C-fluc (Addgene, US), 2μg pT2-β-gal and 1μg SB 100X using the Neon™ Electroporation system (Invitrogen). In order to detect the cells after implantation into the myocardium, D-Luciferin (300 mg/kg) was injected intraperitoneally, followed by 15 min incubation. Bioluminescence imaging was in the next step performed in the IVIS®Spectrum CT (Perkin Elmer Inc.) using 5 min exposure and high sensitivity settings. The rats were imaged in a ventral position to be able to detect the expression of the transplanted cells and quantify the total flux using the Living Image Software (Perkin Elmer Inc.). The corresponding CT image was performed on a Quantum FX μCT (Caliper, Perkin Elmer Inc.) with 17s scan time. The luminescent images were superimposed on the corresponding CT scan.

The rats were divided into the following groups: 1) injection into the left ventricular wall of a normal heart (n=4); 2) Injection into the peri-ischemic region of the left ventricle, directly after induction of a myocardial infarction (n=4). Cell survival and distribution were followed by IVIS (Perkin Elmer Inc.) and the hearts were harvested at 1 and 2 weeks for immunohistochemical analysis to confirm the IVIS data.; 3) Injection into the left ventricular wall of a normal myocardium, followed three days later by induction of a myocardial infarction through a re-thoracotomy and LAD ligation (n=3).; 4) Induction of a myocardial infarction followed by intravenous injection through the tail vein 8 hours after infarction induction (n=2). Since cell survival and distribution were found to correlate well with the IVIS signal, the animals in group 3 and 4 were only followed with IVIS for 1 week.

Detection of rat Isl1+ cells after intramyocardial injection

The hearts were harvested and freeze-sectioned into 7 μm thick sections. Hematoxylin and eosin stainings were used to get an overview of the different areas and subdomains of the hearts. From these sections, it was then possible to direct the X-gal staining to the regions of interest. The X-gal staning was done using a β-gal-staining kit (K1465-01) (Invitrogen) following the manufacturer's protocol.

Claims

1. A method for deriving a multipotent $Isl1^+$ cell, comprising the step of:

   (i) culturing a mesenchymal cell in the presence of at least one laminin comprising an α5 chain, and in a medium comprising at least one agent which activates the Wnt canonical pathway.

2. The method according to claim 1, wherein the mesenchymal cell is a cardiac mesenchymal cell, an embryonic or fetal stem cell, a cord blood stem cell, a bone marrow cell, and/or an amniotic stem cell.

3. The method according to any one of the preceding claims, wherein the at least one laminin comprising an α5 chain is selected from the group comprising laminin 511, laminin 521, and a combination of laminin 511 and laminin 521, and any synthetic, natural, or recombinant part or derivative or variant thereof.

4. The method according to any one of the preceding claims, wherein the at least one agent which activates the Wnt canonical pathway is selected from the group comprising Wnt-1, Wnt-3a, Wnt-8, Wnt-8b, and any combination thereof.

5. The method according to any one of the preceding claims, wherein the at least one agent which activates the Wnt canonical pathway is present at of concentration of 10-250 ng/ml.

6. The method according to any one of the preceding claims, wherein a second step (ii) further comprises expanding the $Isl1^+$ cell population.

7. The method according to any one of the preceding claims, further comprising a differentiation step, wherein the differentiation step comprises culturing the $Isl1^+$ cell in the presence of at least one laminin selected from the group comprising laminin 111, laminin 211, laminin 221, and any combination thereof.

8. A method for self-renewal of a multipotent Isl1$^+$ cell, comprising the steps of:

   (i) culturing an Isl1$^+$ cell in the presence of at least one laminin comprising an α5 chain, and in a medium comprising at least one agent which activates the Wnt canonical pathway; and,

   (ii) maintaining and/or expanding cells obtainable from step (i) in culture.

9. The method according to claim 8, further comprising a differentiation step, wherein the differentiation step comprises culturing the Isl1$^+$ cell in the presence of at least one laminin selected from the group comprising laminin 111, laminin 211, laminin 221, and any combination thereof.

10. Cell obtainable by the method of any one of the preceding claims.

11. A composition comprising at least one laminin comprising an α5 chain and at least one agent which activates the Wnt canonical pathway.

12. The composition according to claim 11, wherein the at least one laminin comprising an α5 chain is selected from the group comprising laminin 511, laminin 521, and a combination of laminin 511 and laminin 521, and any synthetic, natural, or recombinant part or derivative or variant thereof.

13. The composition according to any one of claims 11 to 12, wherein the composition is selected from the group comprising a liquid, a solid, a dispersion, a suspension, an emulsion, a microemulsion, a gel, or a solution, and any combination thereof.

14. A kit comprising at least one laminin comprising an α5 chain and a cell culture medium comprising at least one agent which activates the Wnt canonical pathway.

15. The kit according to claim 14, wherein the at least one laminin comprising an α5 chain is coated on a surface suitable for cell culture.

16. The kit according to any one of claims 16 to 17, further comprising at least one laminin selected from the group comprising laminin 111, laminin 211, laminin 221, and any combination thereof, said at least one laminin preferably

comprised in a separate container or on a separate surface suitable for cell culture.

17. The cell according to claim 10, for use in medicine.

18. The cell according to claim 10, for use in the treatment and/or prophylaxis of heart insufficiency, heart failure, myocardial infarction, and/or congenital heart disease due to cardiac defects affecting parts derived from the second heart field (for instance right atrium, right ventricle, outflow tracts (aorta, pulmonary arteries) and/or ventricular septum).

19. A pharmaceutical composition comprising the cell according claim 10 and at least one pharmaceutically acceptable excipient.

20. Use of a combination of at least one laminin comprising an α5 chain and at least one agent which activates the Wnt canonical pathway, for deriving a multipotent $Isl1^+$ cell.

21. Use of at least one laminin selected from the group comprising laminin 111, laminin 221, laminin 211, and any combination thereof, for differentiation of $Isl1^+$ cells to cardiac cells.