these events. Further, the inventors also found that the methods developed from the properties of the embryonic stem cells are also applicable to select or purify cardiomyocytes derived from fetuses or to select or purify cardiomyocytes derived from the adult stem cells. Based on these findings, the inventors have completed the present invention.

[0015] More specifically, in one embodiment, the present invention provides a method for selecting cardiomyocytes from a cell mixture containing cardiomyocytes and non-cardiomyocytes derived from embryonic stem cells, adult stem cells or fetuses, wherein said cell mixture is cultured in the culture medium under the following conditions: (i) a low-glucose-supplemented condition; and (ii) one or more conditions selected from the group consisting of a low calcium condition, a low-nutritional condition, a lactic acid-supplemented condition, an aspartic acid/glutamic acid-supplemented condition, and a pyruvic acid-supplemented condition. In this embodiment, the cell mixture can be prepared by inducing differentiation of embryonic stem cells, forming embryoid bodies comprising programmed cardiomyocytes (undifferentiated mesoblast cell), culturing the embryoid bodies in the culture medium under a low-serum-supplemented condition and/or a mildly-acidic pH condition.

[0016] In another embodiment, the prenset invention provides a method for selecting cardiomyocytes derived from embryonic stem cells, wherein the cardiomyocytes are selected by the following steps of: inducing differentiation of embryonic stem cells to form embryoid bodies comprising

undifferentiated mesoblast cell, the programmed cardiomyocytes) are referred to as a "non-cardiomyocyte".

[0026] The present inventors studied effects of the culture conditions such as a low-serum-supplemented condition, a low-glucose-supplemented condition, a low-nutritional condition, a low calcium condition, and a mildly-acidic pH condition on selection of the cardiomyocytes, in order to specifically discriminating the cardiomyocytes or cells which differentiate into the cardiomyocytes in the future from non-cardiomyocytes contained within the thus prepared embryoid bodies.

[0027] As a result, the present inventors found that when a cell mixture containing the undifferentiated mesoblast cell, the programmed cardiomyocytes and the cardiomyocytes is cultured under one or more conditions which are selected from the group consisting of a low-serum-supplemented condition, a low-glucose-supplemented condition, a low-nutritional condition, a low calcium condition, a mildly-acidic pH condition, alone or in any combination, the cardiomyocytes or cells which differentiate into the cardiomyocytes in the future are less sensitive to cytotoxic effect as compares with the non-cardiomyocytes under such condition.

[0028] On the basis of this finding, in the present invention, cells which are viable even under the above conditions were able to be selected as programmed cardiomyocytes and cardiomyocytes from a cell mixture containing the undifferentiated mesoblast cell, the programmed cardiomyocytes and the cardiomyocytes, by culturing the cell

mixture under the condition of a low-glucose-supplemented condition in combination with any one or any combinations of a low-serum-supplemented condition, a low-nutritional condition, a low calcium condition and a mildly-acidic pH condition.

[0029] The above described selection method is to select the cells of interst by culturing the cell mixture under the condition to which the cardiomyocytes are physiologically resistant, and therefore, is referred to as "physiological resistance-based selection method". The physiological resistance-based selection method of the present invention is characterized by culturing the cell mixture containing the cardiomyocytes in the culture medium under the condition selected from a low-glucose-supplemented condition, a low-serum-supplemented condition, a low-nutritional condition, a low calcium condition, or a mildly-acidic pH condition.

[0030] In the present invention, the term "a low-glucose-supplemented condition" is defined to be a condition under which the cell mixture is cultured in the culture medium with reduced level of sugars (i.e., group of substances including polysaccharides and monosaccharides (such as glucose, galactose, fructose, mannose), which are biochemically dissolve and converted in vivo or intracellularly to finally be catabolized by the glycolytic system). In the preferred embodiment, the term "a low-glucose-supplemented condition" means that the cell mixture is cultured in the culture medium in the absence of the sugars above or in the culture medium in which the level of sugars is limited to less than 1% as compared to the level of sugars in the culture medium used for

the differentiation induction. In the present invention, it is desirable to eliminate glucose, among others, as much as possible from the culture medium. For example, the commercially aveirable culture mediums which are generally used in the art (such as α-MEM, MEM [Hank's BSS], DMEM) contain 1 g/L of D-glucose (5.56 mM), RPMI 1640 contains 2.0 g/L of D-glucose (11.12 mM), and Ham's F-12 contains 1.82 g/L of D-glucose (10.12 mM), respectively. Therefore, the culture medium with the level of sugars being reduced to 1% means the culture medium containing 55.60-111.20 μM of sugars.

[0031] The present inventors found that, when the cell mixture is cultured in the culture medium under the limited level of sugars to less than 1% as compared to the level of sugars generally used in the art, the non-cardiomyocytes undergo cell death, while the undifferentiated mesoblast cell, the programmed cardiomyocytes and the cardiomyocytes can survive in the culture medium under such condition. In the present invention, a low-glucose-supplemented condition can be achieved by using, for example, RPMI culture medium (sugar-free), and DMEM culture medium (sugar-free) (both from GIBCO).

[0032] As used herein, the term "serum component" includes serum itself, components of biologically active agents containind in animal or human serum, and recombinantly or artificially produced components of the biologically active agent. In the present specification, the term "a low-serum-supplemented condition" refers to the condition where the level of the serum or the serum component, or the

ability to exclusively differentiate into the cardiomyocytes without a need to supplement of any additional substances to the culture medium. The "cardiomyocytes" refers to autonomously pulsating cells when the cells are viable and also cells expressing some markers such as Nkx2.5, GATA4, and Actinin, which can be detected after fixation.

[0043] For example, when the mouse embryonic stem cells are used as an origin of the cardiomyocytes, the cell population which can suvive under these culture conditions can be selected as a cell population consisting of the programmed cardiomyocytes or the cardiomyocytes from cells consisting of the embryoid bodies by the following steps: inducing differentiation of the mouse embryonic stem cells by eliminating LIF from the culture medium, then incubating for 4-7 days the mouse embryonic stem cells to generate embryoid bodies, and culturing the embryoid bodies under the physiological resistance-based selection method and/or the metabolism-based selection method to select the viable cell population as the programmed cardiomyocytes or the cardiomyocytes.

[0044] By way of example, after 5 days from the start of the differentiation induction, cells were selected for additional 24 hours under a low-glucose-supplemented condition and any one of or any combinations of four types of the conditions (i.e., a low-serum-supplemented condition, a low-nutritional condition, a low calcium condition, and a mildly-acidic pH condition). Though the selected cells were those with no pulsating ability, the cell population with no

CLAIMS

[1] A method for selecting cardiomyocytes from a cell mixture containing cardiomyocytes and non-cardiomyocytes derived from stem cells having similar characteristics to those of embryonic stem cells, wherein the stem cells exclude embryonic stem cells, adult stem cells, or fetuses, wherein said cell mixture is cultured in the culture medium under the following conditions:

(i) a low-glucose-supplemented condition; and

(ii) one or more conditions selected from the group consisting of a low calcium condition, a low-nutritional condition, a lactic acid-supplemented condition, an aspartic acid/glutamic acid-supplemented condition,and a pyruvic acid-supplemented condition.

[2] The method for selecting cardiomyocytes according to Claim 1, further comprising a step of eliminating dead cells adhered to the cardiomyocytes using collagenase.

[3] The method for selecting cardiomyocytes according to Claim 2, wherein the dead cells are eliminated based on the fact that the specific gravity of the dead cells is higher than that of the cardiomyocytes.

[4] The method for selecting cardiomyocytes according to any one of Claims 1-3, wherein the condition (ii) of the culture medium is a lactic acid-supplemented condition.

[5] The method for selecting cardiomyocytes according to any one of Claims 1-3, wherein the condition (ii) of the culture medium is an aspartic acid/glutamic acid-supplemented condition.

[6] The method for selecting cardiomyocytes according to any one of Claims 1-3, wherein the condition (ii) of the culture medium is a pyruvic acid-supplemented condition.

[7] The method for selecting cardiomyocytes according to Claim 1, wherein the cell mixture is prepared by the following steps of:

inducing differentiation of embryonic stem cells;

forming embryoid bodies comprising programmed cardiomyocytes (undifferentiated mesoblast cells) ;

preparing the cell mixture by culturing the embryoid bodies in the culture medium under a low-serum-supplemented condition and/or a mildly-acidic pH condition.

[8] A method for selecting cardiomyocytes derived from stem cells having similar characteristics to those of embryonic stem cells, wherein the stem cells exclude embryonic stem cells, wherein the cardiomyocytes are selected by the following steps of:

inducing differentiation of embryonic stem cells to form embryoid bodies comprising undifferentiated mesoblast cells;

then preparing a cell mixture comprising programmed cardiomyocytes by culturing the embryoid bodies in the culture medium under a low-serum-supplemented condition and a mildly-acidic pH condition; and

continuing the culture of the cell mixture in the same culture medium to obtain the cardiomyocytes.

[9] The method for selecting cardiomyocytes according to any one of Claims 1-6, wherein the low-glucose-supplemented condition is a sugar-free condition or a condition where sugar content is decreased less than 1% as compared to the sugar condition of the culture medium used for differentiation induction.

[10] The method for selecting cardiomyocytes according to any one of Claims 1-6, wherein the low calcium condition is a condition where the calcium concentration in the culture medium ranges between 0.3-1.3 mM.

[11] The method for selecting cardiomyocytes according to any one of Claims 1-6, wherein the low-nutritional condition is a condition where, when using a culture medium selected from the group consisting of RPMI culture medium, DMEM culture medium, MEM culture medium, F12 culture medium, and α-MEM culture medium as a culture medium, the contents of each nutritional components included in the culture medium are reduced to 10% or less as compared with the contents of each nutritional components originally included in these culture medium.

[12] The method for selecting cardiomyocytes according to any one of Claims 1-6, wherein the lactic acid-supplemented condition is a condition where 0.1-5 mM of lactic acid is supplemented to the culture medium.

[13] The method for selecting cardiomyocytes according to any one of Claims 1-6, wherein the aspartic acid/glutamic acid-supplemented condition is a condition where 20-100 mg/L of aspartic acid and 20-100 mg/L of glutamic acid are supplemented to the culture medium.

[14] The method for selecting cardiomyocytes according to any one of Claims 1-6, wherein the pyruvic acid-supplemented condition is a condition where 0.5-5 mM of pyruvic acid is supplemented to the culture medium.

[15] The method for selecting cardiomyocytes according to Claim 8, wherein the low-serum-supplemented condition is serum-free condition, or a condition where, when the concentration of serum or a serum components supplemented to the culture medium used in the process for obtaining undifferentiated mesoblast cells is considered as 100%, the concentration of serum or serum components is reduced to 0%-10%.

[16] The method for selecting cardiomyocytes according to Claim 8, wherein the mildly-acidic pH condition is a condition of pH6.5.

these events. Further, the inventors also found that the methods developed from the properties of the embryonic stem cells are also applicable to select or purify cardiomyocytes derived from fetuses or to select or purify cardiomyocytes derived from the adult stem cells. Based on these findings, the inventors have completed the present invention.

[0015] More specifically, in one embodiment, the present invention provides a method for selecting cardiomyocytes from a cell mixture containing cardiomyocytes and non-cardiomyocytes derived from embryonic stem cells, adult stem cells or fetuses, wherein said cell mixture is cultured in the culture medium under the following conditions: (i) a low- sugar -supplemented condition; and (ii) one or more conditions selected from the group consisting of a low calcium condition, a low-nutritional condition, a lactic acid-supplemented condition, an aspartic acid/glutamic acid-supplemented condition, and a pyruvic acid-supplemented condition. In this embodiment, the cell mixture can be prepared by inducing differentiation of embryonic stem cells, forming embryoid bodies comprising programmed cardiomyocytes (undifferentiated mesoblast cell), culturing the embryoid bodies in the culture medium under a low-serum-supplemented condition and/or a mildly-acidic pH condition.

[0016] In another embodiment, the prenset invention provides a method for selecting cardiomyocytes derived from embryonic stem cells, wherein the cardiomyocytes are selected by the following steps of: inducing differentiation of embryonic stem cells to form embryoid bodies comprising

undifferentiated mesoblast cell, the programmed cardiomyocytes) are referred to as a "non-cardiomyocyte".

[0026] The present inventors studied effects of the culture conditions such as a low-serum-supplemented condition, a low-sugar-supplemented condition, a low-nutritional condition, a low calcium condition, and a mildly-acidic pH condition on selection of the cardiomyocytes, in order to specifically discriminating the cardiomyocytes or cells which differentiate into the cardiomyocytes in the future from non-cardiomyocytes contained within the thus prepared embryoid bodies.

[0027] As a result, the present inventors found that when a cell mixture containing the undifferentiated mesoblast cell, the programmed cardiomyocytes and the cardiomyocytes is cultured under one or more conditions which are selected from the group consisting of a low-serum-supplemented condition, a low-sugar-supplemented condition, a low-nutritional condition, a low calcium condition, a mildly-acidic pH condition, alone or in any combination, the cardiomyocytes or cells which differentiate into the cardiomyocytes in the future are less sensitive to cytotoxic effect as compares with the non-cardiomyocytes under such condition.

[0028] On the basis of this finding, in the present invention, cells which are viable even under the above conditions were able to be selected as programmed cardiomyocytes and cardiomyocytes from a cell mixture containing the undifferentiated mesoblast cell, the programmed cardiomyocytes and the cardiomyocytes, by culturing the cell

mixture under the condition of a low- sugar -supplemented condition in combination with any one or any combinations of a low-serum-supplemented condition, a low-nutritional condition, a low calcium condition and a mildly-acidic pH condition.

[0029] The above described selection method is to select the cells of interst by culturing the cell mixture under the condition to which the cardiomyocytes are physiologically resistant, and therefore, is referred to as "physiological resistance-based selection method". The physiological resistance-based selection method of the present invention is characterized by culturing the cell mixture containing the cardiomyocytes in the culture medium under the condition selected from a low- sugar -supplemented condition, a low-serum-supplemented condition, a low-nutritional condition, a low calcium condition, or a mildly-acidic pH condition.

[0030] In the present invention, the term "a low-glucose-supplemented condition" is defined to be a condition under which the cell mixture is cultured in the culture medium with reduced level of sugars (i.e., group of substances including polysaccharides and monosaccharides (such as glucose, galactose, fructose, mannose), which are biochemically dissolve and converted in vivo or intracellularly to finally be catabolized by the glycolytic system). In the preferred embodiment, the term "a low- sugar -supplemented condition" means that the cell mixture is cultured in the culture medium in the absence of the sugars above or in the culture medium in which the level of sugars is limited to less than 1% as compared to the level of sugars in the culture medium used for

the differentiation induction. In the present invention, it is desirable to eliminate glucose, among others, as much as possible from the culture medium. For example, the commercially aveirable culture mediums which are generally used in the art (such as α-MEM, MEM [Hank's BSS], DMEM) contain 1 g/L of D-glucose (5.56 mM), RPMI 1640 contains 2.0 g/L of D-glucose (11.12 mM), and Ham's F-12 contains 1.82 g/L of D-glucose (10.12 mM), respectively. Therefore, the culture medium with the level of sugars being reduced to 1% means the culture medium containing 55.60-111.20 μM of sugars.

[0031] The present inventors found that, when the cell mixture is cultured in the culture medium under the limited level of sugars to less than 1% as compared to the level of sugars generally used in the art, the non-cardiomyocytes undergo cell death, while the undifferentiated mesoblast cell, the programmed cardiomyocytes and the cardiomyocytes can survive in the culture medium under such condition. In the present invention, a low- sugar -supplemented condition can be achieved by using, for example, RPMI culture medium (sugar-free), and DMEM culture medium (sugar-free) (both from GIBCO).

[0032] As used herein, the term "serum component" includes serum itself, components of biologically active agents containind in animal or human serum, and recombinantly or artificially produced components of the biologically active agent. In the present specification, the term "a low-serum-supplemented condition" refers to the condition where the level of the serum or the serum component, or the

ability to exclusively differentiate into the cardiomyocytes without a need to supplement of any additional substances to the culture medium. The "cardiomyocytes" refers to autonomously pulsating cells when the cells are viable and also cells expressing some markers such as Nkx2.5, GATA4, and Actinin, which can be detected after fixation.

[0043] For example, when the mouse embryonic stem cells are used as an origin of the cardiomyocytes, the cell population which can suvive under these culture conditions can be selected as a cell population consisting of the programmed cardiomyocytes or the cardiomyocytes from cells consisting of the embryoid bodies by the following steps: inducing differentiation of the mouse embryonic stem cells by eliminating LIF from the culture medium, then incubating for 4-7 days the mouse embryonic stem cells to generate embryoid bodies, and culturing the embryoid bodies under the physiological resistance-based selection method and/or the metabolism-based selection method to select the viable cell population as the programmed cardiomyocytes or the cardiomyocytes.

[0044] By way of example, after 5 days from the start of the differentiation induction, cells were selected for additional 24 hours under a low-sugar -supplemented condition and any one of or any combinations of four types of the conditions (i.e., a low-serum-supplemented condition, a low-nutritional condition, a low calcium condition, and a mildly-acidic pH condition). Though the selected cells were those with no pulsating ability, the cell population with no

CLAIMS

[1] A method for selecting cardiomyocytes from a cell mixture containing cardiomyocytes and non-cardiomyocytes derived from stem cells having similar characteristics to those of embryonic stem cells, wherein the stem cells exclude embryonic stem cells, adult stem cells, or fetuses, wherein said cell mixture is cultured in the culture medium under the following conditions:

(i) a low-sugar-supplemented condition; and

(ii) one or more conditions selected from the group consisting of a low calcium condition, a low-nutritional condition, a lactic acid-supplemented condition, an aspartic acid/glutamic acid-supplemented condition,and a pyruvic acid-supplemented condition.

[2] The method for selecting cardiomyocytes according to Claim 1, further comprising a step of eliminating dead cells adhered to the cardiomyocytes using collagenase.

[3] The method for selecting cardiomyocytes according to Claim 2, wherein the dead cells are eliminated based on the fact that the specific gravity of the dead cells is higher than that of the cardiomyocytes.

[4] The method for selecting cardiomyocytes according to any one of Claims 1-3, wherein the condition (ii) of the culture medium is a lactic acid-supplemented condition.

[5] The method for selecting cardiomyocytes according to any one of Claims 1-3, wherein the condition (ii) of the culture medium is an aspartic acid/glutamic acid-supplemented condition.

[6] The method for selecting cardiomyocytes according to any one of Claims 1-3, wherein the condition (ii) of the culture medium is a pyruvic acid-supplemented condition.

[7] The method for selecting cardiomyocytes according to Claim 1, wherein the cell mixture is prepared by the following steps of:

inducing differentiation of the stem cells having similar characteristics to those of embryonic stem cells;

forming embryoid bodies comprising programmed cardiomyocytes (undifferentiated mesoblast cells) ;

preparing the cell mixture by culturing the embryoid bodies in the culture medium under a low-serum-supplemented condition and/or a mildly-acidic pH condition.

[8] The method for selecting cardiomyocytes according to any one of Claims 1-6, wherein the low-sugar-supplemented condition is a sugar-free condition, a condition where sugar content is decreased less than 1% as compared to the sugar condition of the culture medium used for differentiation induction, or a condition where sugar content is 111.20μM or less.

[9] The method for selecting cardiomyocytes according to any one of Claims 1-6, wherein the low calcium condition is a condition where the calcium concentration in the culture medium ranges between 0.3-1.3 mM.

[10] The method for selecting cardiomyocytes according to any one of Claims 1-6, wherein the low-nutritional condition is a condition where, when using a culture medium selected from the group consisting of RPMI culture medium, DMEM culture medium, MEM culture medium, F12 culture medium, and α-MEM culture medium as a culture medium, the contents of each nutritional components included in the culture medium are reduced to 10% or less as compared with the contents of each nutritional components originally included in these culture medium.

[11] The method for selecting cardiomyocytes according to any one of Claims 1-6, wherein the lactic acid-supplemented condition is a condition where 0.1-5 mM of lactic acid is supplemented to the culture medium.

[12] The method for selecting cardiomyocytes according to any one of Claims 1-6, wherein the aspartic acid/glutamic acid-supplemented condition is a condition where 20-100 mg/L of aspartic acid and 20-100 mg/L of glutamic acid are supplemented to the culture medium.

[13] The method for selecting cardiomyocytes according to any one of Claims 1-6, wherein the pyruvic acid-supplemented condition is a condition where 0.5-5 mM of pyruvic acid is supplemented to the culture medium.

[14] The method for selecting cardiomyocytes according to Claim 7, wherein the low-serum-supplemented condition is serum-free condition, or a condition where, when the concentration of serum or a serum components supplemented to the culture medium used in the process for obtaining undifferentiated mesoblast cells is considered as 100%, the concentration of serum or serum components is reduced to 0%-10%.

[15] The method for selecting cardiomyocytes according to Claim 7, wherein the mildly-acidic pH condition is a condition of pH6.5.