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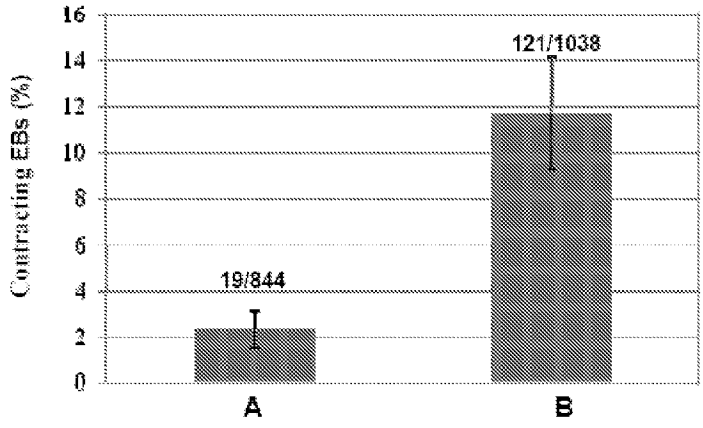
(54) **Title**: COMPOSITIONS AND METHODS OF FORMING CARDIAC PROGENITOR CELLS

FIG. 9



(57) **Abstract**: Provided herein are compositions and methods for forming cardiac progenitor cells from progenitor cells, such as embryonic stem cells. One aspect provides a cardiogenic culture medium including interleukin-3, interleukin-1, insulin, or transferin. Another aspect provides a method for forming cardiac progenitor cells from progenitor cells by culturing in the cardiogenic culture medium. Another aspect provides a method for forming cardiac progenitor cells from progenitor cells by modulating levels of Oct-4.

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TITLE OF INVENTION

COMPOSITIONS AND METHODS OF FORMING CARDIAC PROGENITOR CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the benefit of U.S. Provisional Application Serial No. 61,388,744 filed October 1, 2010, which is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with government support under Grant EB006362 awarded by the National Institutes of Health. The Government has certain rights in the invention.

MATERIAL INCORPORATED-BY-REFERENCE

[0003] Not Applicable.

FIELD OF THE INVENTION

[0004] The present invention generally relates to cardiac progenitor cells.

BACKGROUND OF THE INVENTION

[0005] The POU domain transcription factor Oct-4 (also termed *Pou5f1*) is required for the maintenance of pluripotency of embryonic stem cells (ESCs). The POU domain transcription factor Oct-4 is developmentally regulated in mouse ontogeny. Maternal Oct-4 RNA and protein are present in fertilized oocytes until the two-cell stage. Zygotic *Oct-4* gene expression starts at the four- to eight-cell stage. During early cleavage, uniform amounts of *Oct-4* RNA are found in all blastomeres, but the level decreases in the outer cells of the morula as they polarize and form the trophectoderm. In the 3.5 day postcoitum (dpc) blastocyst, *Oct-4* RNA and protein levels are low in the epithelial cell

layer and become undetectable one day later. In contrast, Oct-4 protein expression is maintained in the inner cell mass (ICM) of the blastocyst.

[0006] During embryogenesis, differential expression of Oct-4 protein is observed when the ICM differentiates at 4.5 dpc into the epiblast (primitive ectoderm, embryonic ectoderm) and the hypoblast (primitive endoderm, embryonic endoderm). Oct-4 expression is maintained in the epiblast but, as hypoblast cells differentiate into visceral and parietal endoderm, Oct-4 protein levels transiently increase and then decrease to undetectable levels. During gastrulation, Oct-4 expression is progressively repressed in the epiblast, and by 7.5 dpc is confined exclusively to newly established primordial germ cells (PGCs).

[0007] Embryonic stem cells (ESCs) are derived from the ICM of the pre-implantation embryo, and are roughly equivalent to epiblast in vivo. Recent studies have shown that endogenous Oct-4 mRNA and protein expression continues for at least four days in ESCs upon induction of differentiation, which may mirror the expression of Oct-4 in epiblasts during early embryogenesis. In the same study, it was shown that the presence of Oct-4 during the early stage of differentiation ESCs (day 0 to day 2 post-differentiation) is crucial for successful hematopoiesis. Previous reports have shown that the precise level of Oct-4 tightly regulates the differentiation capacity of ESCs. It has been reported that changes in Oct-4 expression level in ESCs can re-direct the cell fate of ESCs toward either primitive endodermal and early mesodermal differentiation (Oct-4 over-expression) or trophectoderm differentiation (Oct-4 under-expression). Additional studies have reported effects of Oct-4 over-expression in cardiogenic development and hematopoietic development.

[0008] In ESCs, Oct-4 is transcriptionally regulated by itself or by Nanog, Sox2 and FoxD3 through various feedback regulatory loops. Other than regulation of transcription, Oct-4 is also regulated at the post-translational level. Oct-4 protein has been shown to be targeted by an E3 Ub ligase, Wwp2, for degradation.

[0009] Under convention culture conditions, it has been reported that Oct-4 has no effect on other non-cardiogenic mesodermal development.

[0010] It has also been reported that changes in Oct-4 expression can affect hematopoietic development when a cytokine-supplemented culture condition is applied. Activation of TGFβ signaling pathway in ESCs by addition of TGFβ1, BMP2 or Nodal has been shown to promote expression of cardiogenic-specific markers, but there was low expression levels of other mesoderm lineage-specific markers (tPA/parietal endoderm, α-feto protein/visceral endoderm, nestin/neuronal and Tal1/hematopoietic lineages). This report showed that activation of TGFβ signaling increased expression of Oct-4, which in turn increased expression of cardiac-specific markers in the absence of LIF. Another study found that addition of TGFβ1 to ESCs would suppress early chondrogenic induction, when these cells were induced to chondrogenic differentiation in serum-free chondrogenic basal medium without growth factor supplement. In that study, Oct-4 and Nanog expression were sustained in the presence of TGFβ1. Others showed that down-regulation of stem cell genes and activation of epiblast/primitive streak genes is similar in serum and serum-free medium, but subsequent mesoderm differentiation is strongly influenced by the composition of the medium.

SUMMARY OF THE INVENTION

[0011] Among the various aspects of the present invention is the provision of a compositions and methods for generating cardiac progenitor cells via a novel culture medium, manipulation of Oct-4 levels, or a combination thereof.

[0012] On aspect provides a culture medium for generating cardiac progenitor cells. In some embodiments, the culture medium includes at least one of interleukin-3, interleukin-1, insulin, and transferin. In some configurations, the culture medium includes interleukin-3, interleukin-1, insulin, and transferin. In some configurations, the culture medium contains interleukin-3 at a concentration of at least about 0.5 ng/ml up to about 50 ng/ml. In one embodiment, the culture medium contains interleukin-3 at a concentration of about 5 ng/ml. In some configurations, the culture medium contains

interleukin-1 at a concentration of at least about 0.5 ng/ml up to about 50 ng/ml. In one embodiment, the culture medium contains interleukin-1 at a concentration of about 5 ng/ml. In some configurations, the culture medium contains insulin at a concentration of at least about 1 ng/ml up to about 100 ng/ml. In one embodiment, the culture medium contains insulin at a concentration of about 10 ng/ml. In some configurations, the culture medium contains transferin at a concentration of at least about 20 ng/ml up to about 2 µg/ml. In one embodiment, the culture medium contains transferin at a concentration of about 200 ng/ml. In some embodiments, the culture medium includes FBS or monothioglycerol. In some configurations the culture medium includes FBS. In some configurations the culture medium includes monothioglycerol. In some configurations the culture medium includes FBS and monothioglycerol.

[0013] Another aspect provides a method of forming a cardiac progenitor cell. In some embodiments, the method includes contacting a progenitor cell and a culture medium described herein; and culturing the progenitor cell so as to form a cardiac progenitor cell. In some configurations, the method includes increasing a level of Oct-4 in the progenitor cell.

[0014] In some embodiments, the method includes increasing a level of Oct-4 in a progenitor cell; and culturing the progenitor cell so as to form a cardiac progenitor cell.

[0015] In some embodiments, increasing the level of Oct-4 in the progenitor cell includes introducing exogenous Oct-4 to the progenitor cell. In some embodiments, increasing the level of Oct-4 in the progenitor cell includes increasing expression of endogenous Oct-4 in the progenitor cell. In some embodiments, increasing the level of Oct-4 in the progenitor cell includes introducing exogenous Oct-4 to the progenitor cell and increasing expression of endogenous Oct-4 in the progenitor cell.

[0016] In some embodiments, the method includes detecting a level of Oct-4 in the progenitor cell. In some embodiments of the method, the level of Oct-4 in the progenitor cell is increased to be about the level of Oct-4 in embryonic stem cell line CGR8 cultured under equivalent conditions. In some embodiments of the method, the level of

Oct-4 in the progenitor cell is increased so as promote expression of early mesodermal marker *Brachyury*. In some embodiments of the method, the level of Oct-4 in the progenitor cell is increased prior to 11 days of culturing the progenitor cell.

[0017] In some embodiments of the method, the number of formed cardiac progenitor cells is at least about 100% greater than the number of cardiac progenitor cells formed under culture conditions not comprising at least one of interleukin-3, interleukin-1, insulin, and transferin. In some embodiments of the method, the number of formed cardiac progenitor cells is at least about 100% greater than the number of cardiac progenitor cells formed in the absence of increasing the level of Oct-4. In some embodiments of the method, the number of formed cardiac progenitor cells is at least about 100% greater than the number of cardiac progenitor cells formed under culture conditions not comprising at least one of interleukin-3, interleukin-1, insulin, and transferin and the number of cardiac progenitor cells formed in the absence of increasing the level of Oct-4.

[0018] In some embodiments of the method, the cardiac progenitor cell displays one or more cellular markers selected from the group consisting of Nkx2.5, Tnnt2, Myh7, Myh6, and Brachyury.

[0019] In some embodiments of the method, the progenitor cell is an embryonic progenitor cell. In some embodiments of the method, the progenitor cell is an induced pluripotent stem cell. In some embodiments of the method, progenitor cells include embryonic progenitor cells and induced pluripotent stem cells. In some embodiments of the method, the progenitor cell is a human embryonic progenitor cell. In some embodiments of the method, the progenitor cell is a human induced pluripotent stem cell. In some embodiments of the method, progenitor cells include human embryonic progenitor cells and human induced pluripotent stem cells.

[0020] In some embodiments of the method, a first progenitor cell and a second progenitor cell are co-cultured. In some embodiments of the method, the first progenitor cell comprising a embryonic progenitor cell and the second progenitor cell comprising

an endothelial stem/progenitor cell (HUVEC). In some embodiments of the method, embryonic progenitor cells are present at about 90% to 99% and the HUVECs are present at about 10% to about 1% in the progenitor cell culture.

[0021] In some embodiments of the method, the cardiac progenitor cell comprises a contracting embryoid body.

[0022] Other objects and features will be in part apparent and in part pointed out hereinafter.

DESCRIPTION OF THE DRAWINGS

[0023] Those of skill in the art will understand that the drawings, described below, are for illustrative purposes only. The drawings are not intended to limit the scope of the present teachings in any way.

[0024] FIG. 1 is a series of images showing Formation of contracting cardiogenic progenitors from day 9 CGR8 and ZHBTc4 EBs when cultured in cardiogenic differentiating liquid medium. FIG. 1A and FIG. 1B shows single frames taken from videos showing that beating cells/focus (white arrow heads) derived from wt CGR8 ESCs when cultured in previously established cardiogenic differentiating liquid medium. FIG. 1A: 20X objective; FIG. 1B: 10X objective. FIG. 1C shows RT-PCR analyses of cardiogenic gene expression markers in cells collected from day 9 EBs derived from wt CGR8 or ZHBTc4 ESCs, with or without tet-treatment. 18S was used as an internal control. EBs- embryoid bodies. WT- wildtype. ESCs- embryonic stem cells. ANF-atrial natriuretic factor; Nkx 2.5- cardiac specific homoebox protein (Csx); Myh6- cardiac α-myosin heavy chain; Myh7- cardiac β-myosin heavy chain. Tet- tetracycline. Untx- untreated. TD0- tet-treated at day 0, before differentiation induction. TD2- tet-treated at day 2 of differentiation. TD4- tet-treated at day 4 of differentiation.

[0025] FIG. 2 is a pair of images and a bar graph showing formation of contracting cardiogenic progenitors from day 11 CGR8 EBs when cultured in semi-solid methycellulose medium. FIG. 2A and FIG. 2B shows single frames taken from videos

showing that beating EBs developed from wt CGR8 ESCs when cultured in condition A (left panel) or with modified condition B (right panel). Scale bar = 100 μm.

[0026] FIG. 3 is a series of images and a bar graph showing Oct-4 protein expression levels. FIG. 3A shows immunoblot analysis of Oct-4 expression in CGR8, tet-treated and untreated ZHTc6 ESCs before and after induction for differentiation. β-actin protein expression is shown to demonstrate equal loading of lanes. FIG. 3B shows quantification of Oct-4 protein expression levels in undifferentiated (D0, with LIF) or differentiating (D1, 24 hrs without LIF) CGR8 and ZHT6 ESCs. Levels of Oct-4 protein in wt CGR8 cells were set at 100% for comparison against Oct-4 protein levels in ZHTc6 cells. Oct-4 expression levels were normalized by the expression of β-actin in the same sample. ZHTc6 Tet- ZHTc6 cultured with tet before and after induction for differentiation. ZHTc6 24h- ZHTc6 ESCs removed from tet 24 hr before induction of differentiation and remained tet-free. ZHTc6 48h- ZHTc6 ESCs removed from tet 48 hr before induction of differentiation and remained tet-free.

[0027] FIG. 4 is a pair of bar graphs showing formation of contracting cardiogenic EBs from day 11 CGR8 and ZHTc6 EBs when cultured in the modified cardiogenic differentiating condition B. FIG. 4A shows ZHTc6 when cultured in cardiogenic differentiating condition B, tet-treated, differentiation induced. FIG. 4B shows contracting EBs formed from wt CGR8 and ZHTc6 ESCs, with or without tet-treatment, when cultured in cardiogenic differentiation culture condition B. Percentages of beating EBs were calculated to compare untreated (CGR8, ZHTc6 24h and ZHTc6 48h) and tet-treated (CGR8 TD0, ZHTc6 Tet) ESCs. Actual EB number count from each culture condition is shown (number of beating EBs/total number of EBs). Data are expressed as the average +/- SEM of 4 experiments. ZHTc6 Tet- ZHTc6 ESCs cultured with tet before and after induction of differentiation. ZHTc6 24h- ZHTc6 Tet ESCs removed from tet-treatment 24 hr before induction of differentiation and remained tet-free. ZHTc6 48h- ZHTc6 ESCs removed from tet-treatment 48 hr before induction for differentiation and

remained tet-free. TD0- tet-treatment began at day of differentiation. TD2- tet-treatment began at day 2 post-differentiation.

[0028] FIG. 5 is a series of bar graphs showing qRT-PCR analyses of cardiogenic marker expression from CGR8 and ZHTc6 ESCs. FIG. 5A-D show a summary of the relative expression of cardiac markers Nkx2.5, Tnnt2, Myh7 and Myh6 from day 0 ESCs and day 18 EBs; FIG. 5E-G show day 10 EBs. Data are expressed as the average +/- SEM of at least 3 experiments. ZHTc6 Tet- ZHTc6 ESCs cultured with tet before and after induction for differentiation. ZHTc6 24h- ZHTc6 ESCs released from tet 24 hr before induction of differentiation and remained tet-free. ZHTc6 48h- ZHTc6 Tet ESCs released from tet-treatment 48 hr before induction of differentiation and remained tet-free. TD0- tet-treatment began at day of differentiation. TD2- tet-treatment at day 2 post-differentiation. Nkx 2.5- cardiac specific homoebox protein (Csx); Tnnt2- cardiac-specific troponin T isoform 2; Myh6- cardiac α-myosin heavy chain; Myh7- cardiac β-myosin heavy chain.

[0029] FIG. 6 is a cartoon depicting culturing methods applied to generate contracting cells or EBs. Conventional protocol for cardiac differentiation of ESCs in liquid medium (condition A, left panel) and modified protocol for cardiac differentiation of ESCs in 3-dimensional methylcellulose (condition B, right panel).

[0030] FIG. 7 is a series of scatter plots showing flow cytometry analyses of formation of hematopoietic progenitors from day 12 EBs derived from CRG8 and ZHTc6 ESCs. FIG. 7A shows representative flow cytometric analyses on wt untreated CGR8, TD0 CGR8, tet-treat ZHTc6 (ZHTc6 Tet) and untreated ZHTc6 (ZHTc6 24h and ZHTc6 48h). CD45 marker was analyzed on the FL4 channel (y-axis) and CD11b marker was analyzed by the FL2 channel (x-axis). FIG. 7B shows calculation of the CD45/CD11b double positive cell populations from each clone. Data are expressed as the average +/- SEM of 3 experiments. A total number of 100,000 cells were counted from each sample. EBs- embryoid bodies; wt- wildtype; ESCs- embryonic stem cells; Untx- untreated. TD0- tet-treated at day 0.

[0031] FIG. 8 is a bar graph showing CD45+CD11b+ cell population (%) for wt untreated CGR8, TD0 CGR8, tet-treat ZHTc6 (ZHTc6 Tet) and untreated ZHTc6 (ZHTc6 24h and ZHTc6 48h).

[0032] FIG. 9 shows percentages of beating EBs when wt CGR8 ESCs were cultured in condition A (CGR8A) or condition B (CGR8B). Data are expressed as the average +/- SEM of 4 experiments (beating EBs/total number of EBs).

[0033] FIG. 10A is a western blot analysis on Oct-4 protein expression in CGR8 or Oct-4 knock-in ZHTc6 ESCs before (D0) and after induction for differentiation (D1). FIG. 10B is a bar graph of Oct-4 protein expression level before (D0) and after induction for differentiation (D1). FIG. 10C is a table describing Oct-4 protein level expression in CGR8 or Oct-4 knock-in ZHTc6 ESCs before (D0) and after induction for differentiation (D1).

[0034] FIG. 11A-G are bar graphs showing relative expression of cardiac-specific markers, Nkx2.5, Tnnt2, Myh7, and Myh6 in CGR8- and ZHTc6-derived EBs measured by Real Time PCR.

[0035] FIG. 12A-L are fluorescence microscopy images of immunohistochemically-stained samples of NBEBs and BEBs. The cardiac-specific marker targeted for staining was Myh6/7.

[0036] FIG. 13 shows a pair of bar graphs showing a percentage of Myh6/7 positive cells in BEBs and NBEBs. In FIG. 13A, beating EBs (BEBs) and non-beating EBs (NBEBs) were collected from CGR8 ESC-derived EBs or ZHTc6 24h ECS-derived EBs at day 12. In FIG. 13B, areas of myosin heavy chain staining (Myh6/7) and DAPI staining were measured from each EB. Percentages of myosin heavy chain +ve cell population was calculated from each EB by dividing Myh6/7+ve area with DAPI +ve area (n=5)

[0037] FIG. 14A-C shows immunofluorescence microscopy images of cardiac-specific marker Myh6/7 in BEBs from CGR8 24h ESCs. FIG. 14C is the zoomed-in area of FIG. 14A.

[0038] FIG. 15A-D shows immunofluorescence microscopy images of cardiac-specific marker Myh6/7 in BEBs from ZHTc6 24h ESCs. FIG. 15C-D are the zoomed-in areas of FIG. 15A-B.

[0039] FIG. 16 shows immunofluorescence microscopy images of cardiac-specific marker, Myh6/7, and vascular-specific marker, CD31, in CGR8-derived BEBs (FIG. 16A-F) and NBEBs (FIG. 16G-L).

[0040] FIG. 17A-B shows a pair of bar graphs showing cardiac potential in samples treated with Endothelial stem/progenitor cells (HUVECs).

DETAILED DESCRIPTION OF THE INVENTION

[0041] The present disclosure is based, at least in part, on the observation of a 5-fold increase in the number of contracting embryoid bodies when cultured in a novel cardiogenic culture medium containing interleukin-3, interleukin-1, insulin, transferring, and monothioglycerol as compared to conventional medium.

[0042] The present disclosure is also based, at least in part, on the observation that an about 20% to 50% decrease of Oct-4 expression in undifferentiated and early differentiating ESCs can severely cripple cardiogenesis and formation of functional cardiogenic progenitors, but this developmental defect can be rescued if Oct-4 levels are increased so as to approximate wild-type levels. In addition, although an up-regulation of *brachyury* is seen in Oct-4 over-expressing ESCs (about 50% higher than wild-type), this over-expression does not promote later stage cardiogenesis (or hematopoiesis) as much as the wild-type level expression of Oct-4 (wildtype biallelic expression).

[0043] Previous reports have shown that the precise level of Oct-4 tightly regulates the differentiation capacity of ESCs. In prior studies, it was unclear whether Oct-4 was

specifically required for the development of only hematopoietic cells, or for multiple mesodermal lineages, which would indicate that it is required for general mesodermal tissue specification. Consequently, the inventors investigated the role of Oct-4 levels on another mesodermal lineage, cardiogenesis, as shown herein. As shown herein, tight regulation of Oct-4 expression during early differentiation is also necessary for successful late stage multi-lineage mesodermal development. This represents a paradox for Oct-4, because it also is essential for maintaining pluripotentiality.

[0044] By manipulating an engineered ES cell line (ZHTc6), the inventors were able to induce Oct-4 protein concentration from an under-expressed level (~50% of wild-type) to an over-expressed level (~150%) compared to wild type CGR8 ESCs (see e.g., Example 3), and use such model system to demonstrate the effects of Oct-4 on mesoderm cardiogenic development.

[0045] Results described herein show that suboptimal expression of Oct-4 in undifferentiated and differentiating ESCs severely compromises cardiogenic development, but the defect can be rescued by restoring Oct-4 expression to a level that is comparable to wild type (see Example 4). In addition, a higher expression of Oct-4 (~150% of wildtype) in undifferentiated or early differentiating ESCs does not promote cardiac differentiation (see Example 5), although it does promote the expression of the early mesodermal marker *brachyury* (see Example 5), as shown previously. Thus is demonstrated that Oct-4 levels must be maintained that a specific level of Oct-4 must be maintained at particular levels for progression to post-brachyury+ differentiation of general mesodermal lineages, for example, when inducing pluripotent stem cells from adult tissues to differentiation into mature tissues for organ replacement. This is in contrast to the role of Oct-4 in sustaining ESC pluripotentiality, where there is more tolerance to Oct-4 levels.

[0046] Results presented herein show that, depending on the induction mechanism(s) and culture conditions, change in Oct-4 protein levels in ESCs does not necessarily target cardiac differentiation specifically, as has been previously reported.

The requirement for Oct-4 appears to be earlier in differentiation, perhaps in the specification of lateral plate mesoderm, which is the precursor for cardiac, hematopoietic and vascular cells. Therefore, not only is the presence of Oct-4 required for both cardiogenesis and hematopoiesis, but a specific concentration of Oct-4 is required for both pre- and post-*brachyury* positive mesodormal development. The present disclosure shows that specific Oct-4 levels are key regulators for mesodermal lineage specification.

[0047] ESCs are more tolerant of varying Oct-4 levels to maintain pluripotentiality than they are for mesodermal linage differentiation. ZHTc6 ESCs are able to remain undifferentiated despite decreased level of Oct-4. In contrast, as shown herein, even a small decrease in Oct-4 markedly reduced cardiogenic differentiation (see e.g., Example 2). Such finding implicates using Oct-4 to induce pluripotent stem cells from adult tissues. Thus, using induced pluripotent stem cells to engineer organ replacement from mesodermal lineages requires precise levels of Oct-4 for proper mesodermal differentiation.

[0048] Experiments described herein showed the effects of Oct-4 under-expression on cardiogenic differentiation from wt CGR8 and ZHTc6 ESCs. Data presented herein show that even a about a 20% decrease in Oct-4 expression in undifferentiated ESCs and a about a 50% decrease in early differentiating ESCs can severely compromise both cardiogenic and hematopoietic differentiation in tet-treated ZHTc6 ESCs (see Example 3; Example 5). Unlike previously reported, studies herein shows that higher expression of Oct-4 does not promote later stages of cardiogenesis (see Example 5). Similar to previous reports, studies herein shows that an overproduction of Oct-4 does not promote formation of later stage hematopoietic progenitors.

[0049] While being under no obligation to supply a mechanistic description, and in no way limited the scope of the invention, the following discussion addresses how Oct-4 maintains pluripotentiality, and yet is necessary for mesodermal lineage differentiation.

Partners of Oct-4 may switch as differentiation is induced, thus altering the genes transcriptionally regulated by Oct-4. The epigenetic structure of Oct-4 target promoters may be altered during the early differentiation process, and this alters Oct-4's ability to interact with those promoters. But experiments reported herein show that normal levels of Oct-4 are required not only for hematopoietic differentiation, but also for cardiogenic differentiation. This does not necessarily imply that Oct-4 is required for all types of mesodermal differentiation, since there is evidence that hematopoietic, vascular and cardiac tissue may derive from a common precursor. It does, however, imply that Oct-4-induced pluripotent stem cells require appropriate maintenance of Oct-4 expression to form certain mesodermal tissues.

CARDIOGENIC CULTURE MEDIUM

[0050] One aspect provides a cardiogenic culture medium that increases cardiogenesis or increases differentiation of cardiac progenitor cells from progenitor cells, such as stem cells or embryonic stem cells. Various embodiments of the cardiogenic culture medium promote both cardiogenesis and hematopoiesis simultaneously. As shown herein, the cardiogenic culture medium employed in studies reported herein generated cardiogenic EBs at a significantly higher rate than conventional culture conditions (see Example 2). Findings with respect the cardiogenic culture medium described herein supports the view of others that conventional culturing conditions for cardiogenic differentiation are not optimized.

[0051] In some embodiments, the cardiogenic culture medium can affect cardiogenesis and hematopoiesis. Such dual effect has not been previously reported in a culture medium.

[0052] By promoting cardiogenesis and hematopoiesis simultaneously, both *Tal1* and *Nkx 2.5* can be activated in the differentiating ESCs. It has be reported that in *Tal1* over-expressing ESCs, hematopoietic lineage specification became more favorable when cultured with cytokine supplementation, whereas cardiac genes were down-

regulated. In contrast, the inventors have discovered that both cardiogenic and hematopoietic genes can be co-expressed in cytokine-supplemented culture medium such as the cardiogenic culture medium described herein.

[0053] In the absence of cytokine supplementation, or in serum-free medium, cardiac gene expression would be enhanced when Oct-4 is over-expressed. But in the absence of cytokine supplementation, the cardiogenic promoting effect of over-expression of Oct-4 can become an inhibitory factor for cardiogenesis if hematopoietic genes are also activated.

[0054] The cardiogenic culture medium can include one or more of interleukin-3, interleukin-1, insulin, and transferin. For example, cardiogenic culture medium can include two or more, three or more, or all of interleukin-3, interleukin-1, insulin, and transferin. The cardiogenic culture medium can be a conventional culture medium, such as DMEM or MethoCult methylcellulose, supplemented with the above components. The cardiogenic culture medium can include further include components such as FBS and monothioglycerol.

[0055] In some embodiments, the cardiogenic culture medium can include a DMEM based medium supplemented with interleukin-3, interleukin-1, insulin, or transferin. For example, the cardiogenic culture medium can include a DMEM based medium supplemented with FBS, interleukin-3, interleukin-1, insulin, transferin, or monothioglycerol. As another example, the cardiogenic culture medium can be a DMEM medium supplemented with 15% FBS, interleukin-3 (5 ng/ml), interleukin-1 (5 ng/ml) insulin (10 ng/ml), transferrin (200 ng/ml) or monothioglycerol (100 μM).

[0056] In some embodiments, the cardiogenic culture medium can include a methylcellulose medium supplemented with interleukin-3, interleukin-1, insulin, or transferin. An exemplary methylcellulose medium is MethoCult medium. For example, the cardiogenic culture medium can include a methylcellulose based medium supplemented with FBS, interleukin-3, interleukin-1, insulin, transferin, or monothioglycerol.

[0057] In some embodiments, the cardiogenic culture medium includes interleukin-3. The cardiogenic culture medium can include interleukin-3 at a concentration of at least about 0.5 ng/ml. The cardiogenic culture medium can include interleukin-3 at a concentration of up to about 50 ng/ml. The cardiogenic culture medium can include interleukin-3 at a concentration of at least about 0.5 ng/ml up to about 50 ng/ml. For example, the cardiogenic culture medium can include interleukin-3 at a concentration of about 0.5 ng/ml, about 0.6 ng/ml, about 0.7 ng/ml, about 0.8 ng/ml, about 0.9 ng/ml, about 1 ng/ml, about 2 ng/ml, about 3 ng/ml, about 4 ng/ml, about 5 ng/ml, about 6 ng/ml, about 7 ng/ml, about 8 ng/ml, about 9 ng/ml, about 10 ng/ml, about 20 ng/ml, about 30 ng/ml, about 40 ng/ml, or about 50 ng/ml. For example, the cardiogenic culture medium can include interleukin-3 at a concentration of about 5 ng/ml.

[0058] In some embodiments, the cardiogenic culture medium includes interleukin-1. The cardiogenic culture medium can include interleukin-1 at a concentration of at least about 0.5 ng/ml. The cardiogenic culture medium can include interleukin-1 at a concentration of up to about 50 ng/ml. The cardiogenic culture medium can include interleukin-1 at a concentration of at least about 0.5 ng/ml up to about 50 ng/ml. For example, the cardiogenic culture medium can include interleukin-1 at a concentration of about 0.5 ng/ml, about 0.6 ng/ml, about 0.7 ng/ml, about 0.8 ng/ml, about 0.9 ng/ml, about 1 ng/ml, about 2 ng/ml, about 3 ng/ml, about 4 ng/ml, about 5 ng/ml, about 6 ng/ml, about 7 ng/ml, about 8 ng/ml, about 9 ng/ml, about 10 ng/ml, about 20 ng/ml, about 30 ng/ml, about 40 ng/ml, or about 50 ng/ml. For example, the cardiogenic culture medium can include interleukin-1 at a concentration of about 5 ng/ml.

[0059] In some embodiments, the cardiogenic culture medium includes insulin. The cardiogenic culture medium can include insulin at a concentration of at least about 1 ng/ml. The cardiogenic culture medium can include insulin at a concentration of up to about 100 ng/ml. The cardiogenic culture medium can include insulin at a concentration of at least about 1 ng/ml up to about 100 ng/ml. For example, the cardiogenic culture medium can include insulin at a concentration of about 1 ng/ml, about 2 ng/ml, about 3

ng/ml, about 4 ng/ml, about 5 ng/ml, about 6 ng/ml, about 7 ng/ml, about 8 ng/ml, about 9 ng/ml, about 10 ng/ml, about 11 ng/ml, about 12 ng/ml, about 13 ng/ml, about 14 ng/ml, about 15 ng/ml, about 16 ng/ml, about 17 ng/ml, about 18 ng/ml, about 19 ng/ml, about 20 ng/ml, about 30 ng/ml, about 40 ng/ml, about 50 ng/ml, about 60 ng/ml, about 70 ng/ml, about 80 ng/ml, about 90 ng/ml, or about 100 ng/ml. For example, the cardiogenic culture medium can include insulin at a concentration of about 10 ng/ml.

[0060] In some embodiments, the cardiogenic culture medium includes transferin. The cardiogenic culture medium can include transferin at a concentration of at least about 20 ng/ml. The cardiogenic culture medium can include transferin at a concentration of up to about 2 µg/ml. The cardiogenic culture medium can include transferin at a concentration of at least about 20 ng/ml up to about 2 µg/ml. For example, the cardiogenic culture medium can include transferin at a concentration of about 20 ng/ml, about 30 ng/ml, about 40 ng/ml, about 50 ng/ml, about 60 ng/ml, about 70 ng/ml, about 80 ng/ml, about 90 ng/ml, about 100 ng/ml, about 110 ng/ml, about 120 ng/ml, about 130 ng/ml, about 140 ng/ml, about 150 ng/ml, about 160 ng/ml, about 170 ng/ml, about 180 ng/ml, about 190 ng/ml, about 200 ng/ml, about 210 ng/ml, about 220 ng/ml, about 230 ng/ml, about 240 ng/ml, about 250 ng/ml, about 260 ng/ml, about 270 ng/ml, about 280 ng/ml, about 290 ng/ml, about 300 ng/ml, about 400 ng/ml, about 500 ng/ml, about 600 ng/ml, about 700 ng/ml, about 800 ng/ml, about 900 ng/ml, about 1 µg/ml, about 1.5 µg/ml, or about 2 µg/ml. For example, the cardiogenic culture medium can include transferin at a concentration of about 200 ng/ml.

[0061] In some embodiments, the cardiogenic culture medium includes monothioglycerol. The cardiogenic culture medium can include monothioglycerol at a concentration of at least about 10 µM. The cardiogenic culture medium can include monothioglycerolat a concentration of up to about 1 mM. The cardiogenic culture medium can include monothioglycerolat a concentration of at least about 10 µM up to about 1 mM. For example, the cardiogenic culture medium can include monothioglycerolat a concentration of about 10 µM, about 20 µM, about 30 µM, about

40 μM, about 50 μM, about 60 μM, about 70 μM, about 80 μM, about 90 μM, about 100 μM, about 110 μM, about 120 μM, about 130 μM, about 140 μM, about 150 μM, about 160 μM, about 170 μM, about 180 μM, about 190 μM, about 200 μM, about 300 μM, about 400 μM, about 500 μM, about 600 μM, about 700 μM, about 800 μM, about 900 μM, or about 1 mM.

[0062] In some embodiments, the cardiogenic culture medium includes FBS. The cardiogenic culture medium can include FBS at a concentration of at least about 1%. The cardiogenic culture medium can include FBS at a concentration of up to about 30%. The cardiogenic culture medium can include FBS at a concentration of at least about 1% up to about 30%. For example, the cardiogenic culture medium can include FBS at a concentration of about 1%, about 2%, about 3%, about 4%, about 5%, about 6%, about 7%, about 8%, about 9%, about 10%, about 11%, about 12%, about 13%, about 14%, about 15%, about 16%, about 17%, about 18%, about 19%, about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 26%, about 27%, about 28%, about 29%, or about 30%.

[0063] As an example, the cardiogenic culture medium can be a DMEM medium supplemented with about 15% FBS, interleukin-3 (about 5 ng/ml), interleukin-1 (about 5 ng/ml) insulin (about 10 ng/ml), transferrin (about 200 ng/ml) or monothioglycerol (about 100 μM). As another example, the cardiogenic culture medium can be a methylcellulose medium supplemented with about 15% FBS, interleukin-3 (about 5 ng/ml), interleukin-1 (about 5 ng/ml) insulin (about 10 ng/ml), transferrin (about 200 ng/ml) or monothioglycerol (about 100 μM).

[0064] As described above, a cardiogenic culture medium described herein can increase differentiation of cardiac progenitor cells from progenitor cells. For example, a cardiogenic culture medium described herein can increase differentiation of cardiac progenitor cells from progenitor cells by at least about 10%. For example, a cardiogenic culture medium described herein can increase differentiation of cardiac progenitor cells from progenitor cells by at least about 50%. For example, a cardiogenic culture medium

described herein can increase differentiation of cardiac progenitor cells from progenitor cells by at least about 100%. As another example, a cardiogenic culture medium described herein can increase differentiation of cardiac progenitor cells from progenitor cells by at least about 150%, at least about 200%, at least about 250%, at least about 300%, at least about 350%, at least about 400%, at least about 450%, at least about 500%, at least about 550%, at least about 600%, at least about 650%, at least about 700%, at least about 750%, at least about 800%, at least about 850%, at least about 900%, at least about 950%, or at least about 1,000%.

PROGENITOR CELL AND CARDIAC PROGENITOR CELL

[0065] The compositions and methods of the invention generally employ progenitor cells so as to form a cardiac progenitor cell. The cardiac progenitor cell is understood to be a more differentiated cell than the starting progenitor cell.

[0066] A progenitor cell, as that term is used herein, can differentiate into, or otherwise form, a cardiac progenitor cell. A progenitor cell can be a pluripotent cell. A progenitor cell can be a multipotent cell. A progenitor cell can be self-renewing. For example, a progenitor cell can be an embryonic stem cell (ESC), such as a human embryonic stem cell (hESC). ESCs can be derived from epiblast tissue of the inner cell mass (ICM) of a blastocyst or earlier morula stage embryo. As another example, a progenitor cell can be an induced pluripotent stem cell, such as a human induced pluripotent stem cell (hiPSC).

[0067] Preferably, the progenitor cell is substantially less differentiated than a cardiac progenitor cell. For example, a progenitor cell can be freshly isolated or not pre-treated with growth factors before being further cultured with a cardiogenic culture medium described herein or exposed to increased levels of Oct-4.

[0068] In various embodiments, a progenitor cell is a precursor to a cardiac progenitor cell and differentiates under culture conditions including the cardiogenic culture medium described herein. In various embodiments, a progenitor cell is a

precursor to a cardiac progenitor cell and differentiates under culture conditions including certain levels of exogenous or endogenous Oct-4 at one or more stages of differentiation. In some embodiments, the progenitor cell is an Oct-4-induced pluripotent stem cell. In some embodiments, the progenitor cell is an Oct-4-induced pluripotent embryonic stem cell. In some embodiments, the progenitor cell does not display a cardiac-specific marker, such as Nkx2.5, Tnnt2, Myh7, and Myh6.

[0069] Progenitor cells can be isolated, purified, or cultured by a variety of means known to the art Methods for the isolation and culture of progenitor cells are discussed in, for example, Vunjak-Novakovic and Freshney (2006) Culture of Cells for Tissue Engineering, Wiley-Liss, ISBN-10 0471629359.

[0070] A progenitor cell can be derived from an animal, including, but not limited to, mammals, reptiles, and avians, more preferably horses, cows, dogs, cats, sheep, pigs, and chickens, and most preferably human. A progenitor cell can be derived from the same or different species as an intended transplant recipient.

[0071] A cardiac progenitor cell is understood to be more differentiated than a progenitor cell but less differentiated than a cardiac cell. A cardiac progenitor cell can be a multipotent cell. A cardiac progenitor cell can be an oligopotent cell. A cardiac progenitor cell can be unipotent. A cardiac progenitor cell can have limited self-renewal ability.

[0072] A cardiac progenitor cell can be a cell that displays one or more cardiac-specific markers. Cardiac-specific markers include, but are not limited to, Nkx2.5, Tnnt2, Myh7, and Myh6 (see Example 6). Nkx 2.5, Tnnt2 and Myh7 are understood to be early or intermediate cardiogenic markers. Myh6 is understood to be a late cardiac marker.

[0073] A cardiac progenitor cell can be a hematopoietic embryoid bodies (EBs). For example, a cardiac progenitor cell can be a contracting embryoid body or a beating embryoid body. As another example, a cardiac progenitor cell can be a spontaneously

beating embryoid body. As another example, a cardiac progenitor cell can be a hematopoietic embryoid body.

[0074] In some embodiments, a progenitor cell or a cardiac progenitor cell can be transformed with a heterologous nucleic acid so as to express a bioactive molecule, or heterologous protein or to overexpress an endogenous protein. As an example, a progenitor cell or a cardiac progenitor cell can be genetically modified to expresses a fluorescent protein marker. Exemplary markers include GFP, EGFP, BFP, CFP, YFP, and RFP. As another example, a progenitor cell or a cardiac progenitor cell can be genetically modified to express an angiogenesis-related factor, such as activin A, adrenomedullin, aFGF, ALK1, ALK5, ANF, angiogenin, angiopoietin-1, angiopoietin-2, angiopoietin-3, angiopoietin-4, angiostatin, angiotropin, angiotensin-2, AtT20-ECGF, betacellulin, bFGF, B61, bFGF inducing activity, cadherins, CAM-RF, cGMP analogs, ChDI, CLAF, claudins, collagen, collagen receptors $\alpha_1\beta_1$ and $\alpha_2\beta_1$, connexins, Cox-2, ECDGF (endothelial cell-derived growth factor), ECG, ECI, EDM, EGF, EMAP, endoglin, endothelins, endostatin, endothelial cell growth inhibitor, endothelial cell-viability maintaining factor, endothelial differentiation shpingolipid G-protein coupled receptor-1 (EDG1), ephrins, Epo, HGF, TNF-alpha, TGF-beta, PD-ECGF, PDGF, IGF, IL8, growth hormone, fibrin fragment E, FGF-5, fibronectin and fibronectin receptor α5β1, Factor X, HB-EGF, HBNF, HGF, HUAF, heart derived inhibitor of vascular cell proliferation, IFN-gamma, IL1, IGF-2 IFN-gamma, integrin receptors, K-FGF, LIF, leiomyoma-derived growth factor, MCP-1, macrophage-derived growth factor, monocyte-derived growth factor, MD-ECI, MECIF, MMP 2, MMP3, MMP9, urokiase plasminogen activator, neuropilin (NRP1, NRP2), neurothelin, nitric oxide donors, nitric oxide synthases (NOSs), notch, occludins, zona occludins, oncostatin M, PDGF, PDGF-B, PDGF receptors, PDGFR-β, PD-ECGF, PAI-2, PD-ECGF, PF4, P1GF, PKR1, PKR2, PPAR-gamma, PPAR-gamma ligands, phosphodiesterase, prolactin, prostacyclin, protein S, smooth muscle cell-derived growth factor, smooth muscle cell-derived migration factor, sphingosine-1-phosphate-1 (S1P1), Syk, SLP76, tachykinins, TGF-beta, Tie 1, Tie2, TGF-β, and TGF-β receptors, TIMPs, TNF-alpha, TNF-beta,

transferrin, thrombospondin, urokinase, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF, VEGF.sub.164, VEGI, EG-VEGF, VEGF receptors, PF4, 16 kDa fragment of prolactin, prostaglandins E1 and E2, steroids, heparin, 1-butyryl glycerol (monobutyrin), and/or nicotinic amide. As another example, a progenitor cell or a cardiac progenitor cell can be transfected with genetic sequences that are capable of reducing or eliminating an immune response in a host (*e.g.*, expression of cell surface antigens such as class I and class II histocompatibility antigens can be suppressed). This can allow the transplanted cells to have reduced chance of rejection by the host.

CULTURE

[0075] Provided herein is a method of forming a cardiac progenitor cell from a progenitor cell.

[0076] Processes for the culture and differentiation of progenitor cells are well known (see e.g., Vunjak-Novakovic and Freshney (2006) Culture of Cells for Tissue Engineering, Wiley-Liss, ISBN-10 0471629359; Lanza et al., 2009 Essentials of Stem Cell Biology, 2d Ed., Essentials of Stem Cell Biology, ISBN-10: 0123747295; Lanza and Klimanskaya 2009 Essential Stem Cell Methods, Academic Press, ISBN-10: 012375061X; Loring et al. 2007 Human Stem Cell Manual: A Laboratory Guide, Academic Press, ISBN-10: 0123704650). Except as otherwise noted herein, therefore, the process of the present disclosure can be carried out in accordance with such processes.

[0077] An exemplary embodiment is described below (see e.g., FIG. 6). Embryoid bodies (EBs) can be harvested at about day 2 of development. Harvested EBs can be transferred to a plate. For example, about 100 to about 300 EB can be transferred to a 35 mm plate. The plate can contain a suitable medium, such as a semi-solid methycelluse medium. After culture, about 100 to about 300 well-separated and suspended EBs per plate are provided.

[0078] The method of forming a cardiac progenitor cell can include culturing a progenitor cell in a cardiogenic culture medium described herein so as to form a cardiac progenitor cell.

[0079] The method of forming a cardiac progenitor cell can include increasing a level of Oct-4 in a progenitor cell and culturing the progenitor cell so as to form a cardiac progenitor cell. The method of forming a cardiac progenitor cell can include increasing a level of Oct-4 in a progenitor cell and culturing the progenitor cell in a cardiogenic culture medium described herein so as to form a cardiac progenitor cell. In some embodiments, the level of Oct-4 in the progenitor cell is detected. If the level of Oct-4 is lower than a pre-determined value, then Oct-4 can be increased accordingly. For example, if the level of Oct-4 is lower than about the level of Oct-4 in embryonic stem cell line CGR8 cultured under equivalent conditions, then Oct-4 can be increased accordingly.

[0080] The level of Oct-4 in the progenitor cell can be increased so as to increase the expression of cellular markers. For example, the level of Oct-4 in the progenitor cell can be increased so as to increase the expression of early or intermediate cardiogenic markers, such as Nkx 2.5, Tnnt2 or Myh7. As another example, the level of Oct-4 in the progenitor cell can be increased so as to increase the expression a late cardiac marker, such as Myh6. As another example, the level of Oct-4 in the progenitor cell can be increased so as to increase the expression an early mesodermal marker, such as *Brachyury*.

[0081] The level of Oct-4 in the progenitor cell can be increased at a developmentally suitable stage of development. For example, the level of Oct-4 in the progenitor cell can be increased prior to 11 days of culturing the progenitor cell. For example, the level of Oct-4 in the progenitor cell can be increased prior to expression of early mesodermal marker *Brachyury*.

[0082] Oct-4 can be increased in a progenitor cell by supplying exogenous Oct-4. Oct-4 can be increased in a progenitor cell by increasing expression of endogenous Oct-4 in the progenitor cell.

[0083] Methods described herein can increase the number of formed cardiac progenitor cells as compared to conventional methods. For example, culture methods described herein can increase differentiation of cardiac progenitor cells from progenitor cells by at least about 10%. For example, culture methods described herein can increase differentiation of cardiac progenitor cells from progenitor cells by at least about 50%. For example, culture methods described herein can increase differentiation of cardiac progenitor cells from progenitor cells by at least about 100%. As another example, culture methods described herein can increase differentiation of cardiac progenitor cells from progenitor cells by at least about 150%, at least about 200%, at least about 250%, at least about 300%, at least about 350%, at least about 400%, at least about 450%, at least about 500%, at least about 550%, at least about 600%, at least about 650%, at least about 700%, at least about 750%, at least about 800%, at least about 850%, at least about 900%, at least about 950%, or at least about 1,000%.

[0084] In some embodiments, a progenitor cell or a cardiac progenitor cell can be co-cultured with one or more additional cell types. Such additional cell types can include (but are not limited to) skin cells, liver cells, heart cells, kidney cells, pancreatic cells, lung cells, bladder cells, stomach cells, intestinal cells, cells of the urogenital tract, breast cells, skeletal muscle cells, skin cells, bone cells, cartilage cells, keratinocytes, hepatocytes, gastro-intestinal cells, epithelial cells, endothelial cells, mammary cells, skeletal muscle cells, smooth muscle cells, parenchymal cells, osteoclasts, or chondrocytes.

[0085] In some embodiments, a first progenitor cell can be cultured with a second progenitor cell under conditions described herein. For example, an embryonic progenitor cell can be cultured with an endothelial stem/progenitor cell (HUVEC). As shown herein, co-culture of ESCs and HUVECs can increase cardiogenic potential of

ESCs differentiating into cardiac progenitor cells (see Example 15). It is presently thought that vasculogenesis associated with HUVECs facilitates cardiogenesis associated with ESCs. A co-culture of ESCs and HUVECs can include about 99% ESCs, about 98% ESCs, about 97% ESCs, about 96% ESCs, about 95% ESCs, about 94% ESCs, about 93% ESCs, about 92% ESCs, about 91% ESCs, about 90% ESCs, about 85% ESCs, or about 80% ESCs. A co-culture of ESCs and HUVECs can include about 1% HUVECs, about 2% HUVECs, about 3% HUVECs, about 4% HUVECs, about 5% HUVECs, about 6% HUVECs, about 7% HUVECs, about 8% HUVECs, about 9% HUVECs, about 10% HUVECs, about 15% HUVECs, or about 20% HUVECs. For example, a co-culture of ESCs and HUVECs can include about 98% ESCs and about 2% HUVECs. As another example, a co-culture of ESCs and HUVECs can include about 95% ESCs and about 5% HUVECs.

MOLECULAR ENGINEERING

[0086] Design, generation, and testing of the variant nucleotides, and their encoded polypeptides, having the above required percent identities and retaining a required activity of the expressed protein is within the skill of the art. For example, directed evolution and rapid isolation of mutants can be according to methods described in references including, but not limited to, Link et al. (2007) Nature Reviews 5(9), 680-688; Sanger et al. (1991) Gene 97(1), 119-123; Ghadessy et al. (2001) Proc Natl Acad Sci USA 98(8) 4552-4557. Thus, one skilled in the art could generate a large number of nucleotide and/or polypeptide variants having, for example, at least 95-99% identity to the reference sequence described herein and screen such for desired phenotypes according to methods routine in the art. Generally, conservative substitutions can be made at any position so long as the required activity is retained.

[0087] Nucleotide and/or amino acid sequence identity percent (%) is understood as the percentage of nucleotide or amino acid residues that are identical with nucleotide or amino acid residues in a candidate sequence in comparison to a reference sequence

when the two sequences are aligned. To determine percent identity, sequences are aligned and if necessary, gaps are introduced to achieve the maximum percent sequence identity. Sequence alignment procedures to determine percent identity are well known to those of skill in the art. Often publicly available computer software such as BLAST, BLAST2, ALIGN2 or Megalign (DNASTAR) software is used to align sequences. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared. When sequences are aligned, the percent sequence identity of a given sequence A to, with, or against a given sequence B (which can alternatively be phrased as a given sequence A that has or comprises a certain percent sequence identity to, with, or against a given sequence B) can be calculated as: percent sequence identity = X/Y100, where X is the number of residues scored as identical matches by the sequence alignment program's or algorithm's alignment of A and B and Y is the total number of residues in B. If the length of sequence A is not equal to the length of sequence B, the percent sequence identity of A to B will not equal the percent sequence identity of B to A.

[0088] "Highly stringent hybridization conditions" are defined as hybridization at 65 °C in a 6 X SSC buffer (*i.e.,* 0.9 M sodium chloride and 0.09 M sodium citrate). Given these conditions, a determination can be made as to whether a given set of sequences will hybridize by calculating the melting temperature (T_m) of a DNA duplex between the two sequences. If a particular duplex has a melting temperature lower than 65°C in the salt conditions of a 6 X SSC, then the two sequences will not hybridize. On the other hand, if the melting temperature is above 65 °C in the same salt conditions, then the sequences will hybridize. In general, the melting temperature for any hybridized DNA:DNA sequence can be determined using the following formula: $T_m = 81.5\ °C + 16.6(\log_{10}[Na^+]) + 0.41(\text{fraction G/C content}) - 0.63(\%\ \text{formamide}) - (600/l)$. Furthermore, the T_m of a DNA:DNA hybrid is decreased by 1-1.5°C for every 1% decrease in nucleotide identity (see e.g., Sambrook and Russel, 2006).

[0089] Host cells can be transformed using a variety of standard techniques known to the art (*see*, *e.g.*, Sambrook and Russel (2006) Condensed Protocols from Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, ISBN-10: 0879697717; Ausubel et al. (2002) Short Protocols in Molecular Biology, 5th ed., Current Protocols, ISBN-10: 0471250929; Sambrook and Russel (2001) Molecular Cloning: A Laboratory Manual, 3d ed., Cold Spring Harbor Laboratory Press, ISBN-10: 0879695773; Elhai, J. and Wolk, C. P. 1988. Methods in Enzymology 167, 747-754). Such techniques include, but are not limited to, viral infection, calcium phosphate transfection, liposome-mediated transfection, microprojectile-mediated delivery, receptor-mediated uptake, cell fusion, electroporation, and the like. The transfected cells can be selected and propagated to provide recombinant host cells that comprise the expression vector stably integrated in the host cell genome.

[0090] Host strains developed according to the approaches described herein can be evaluated by a number of means known in the art (*see e.g.*, Studier (2005) Protein Expr Purif. 41(1), 207–234; Gellissen, ed. (2005) Production of Recombinant Proteins: Novel Microbial and Eukaryotic Expression Systems, Wiley-VCH, ISBN-10: 3527310363; Baneyx (2004) Protein Expression Technologies, Taylor & Francis, ISBN-10: 0954523253).

[0091] Methods of down-regulation or silencing genes are known in the art. For example, expressed protein activity can be down-regulated or eliminated using antisense oligonucleotides, protein aptamers, nucleotide aptamers, and RNA interference (RNAi) (e.g., small interfering RNAs (siRNA), short hairpin RNA (shRNA), and micro RNAs (miRNA) (see e.g., Fanning and Symonds (2006) Handb Exp Pharmacol. 173, 289-303G, describing hammerhead ribozymes and small hairpin RNA; Helene, C., et al. (1992) Ann. N.Y. Acad. Sci. 660, 27-36; Maher (1992) Bioassays 14(12): 807-15, describing targeting deoxyribonucleotide sequences; Lee et al. (2006) Curr Opin Chem Biol. 10, 1-8, describing aptamers; Reynolds et al. (2004) Nature Biotechnology 22(3), 326 – 330, describing RNAi; Pushparaj and Melendez (2006)

Clinical and Experimental Pharmacology and Physiology 33(5-6), 504-510, describing RNAi; Dillon et al. (2005) Annual Review of Physiology 67, 147-173, describing RNAi; Dykxhoorn and Lieberman (2005) Annual Review of Medicine 56, 401-423, describing RNAi). RNAi molecules are commercially available from a variety of sources (*e.g.*, Ambion, TX; Sigma Aldrich, MO; Invitrogen). Several siRNA molecule design programs using a variety of algorithms are known to the art (*see e.g.*, Cenix algorithm, Ambion; BLOCK-iT™ RNAi Designer, Invitrogen; siRNA Whitehead Institute Design Tools, Bioinofrmatics & Research Computing). Traits influential in defining optimal siRNA sequences include G/C content at the termini of the siRNAs, Tm of specific internal domains of the siRNA, siRNA length, position of the target sequence within the CDS (coding region), and nucleotide content of the 3' overhangs.

[0092] Definitions and methods described herein are provided to better define the present invention and to guide those of ordinary skill in the art in the practice of the present invention. Unless otherwise noted, terms are to be understood according to conventional usage by those of ordinary skill in the relevant art.

[0093] In some embodiments, numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth, used to describe and claim certain embodiments of the invention are to be understood as being modified in some instances by the term “about.” In some embodiments, the term “about” is used to indicate that a value includes the standard deviation of the mean for the device or method being employed to determine the value. In some embodiments, the numerical parameters set forth in the written description and attached claims are approximations that can vary depending upon the desired properties sought to be obtained by a particular embodiment. In some embodiments, the numerical parameters should be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. Notwithstanding that the numerical ranges and parameters setting forth the broad scope of some embodiments of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as

practicable. The numerical values presented in some embodiments of the invention may contain certain errors necessarily resulting from the standard deviation found in their respective testing measurements. The recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein.

[0094] In some embodiments, the terms “a” and “an” and “the” and similar references used in the context of describing a particular embodiment (especially in the context of certain of the following claims) can be construed to cover both the singular and the plural, unless specifically noted otherwise. In some embodiments, the term “or” as used herein, including the claims, is used to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive.

[0095] The terms “comprise,” “have” and “include” are open-ended linking verbs. Any forms or tenses of one or more of these verbs, such as “comprises,” “comprising,” “has,” “having,” “includes” and “including,” are also open-ended. For example, any method that “comprises,” “has” or “includes” one or more steps is not limited to possessing only those one or more steps and can also cover other unlisted steps. Similarly, any composition or device that “comprises,” “has” or “includes” one or more features is not limited to possessing only those one or more features and can cover other unlisted features.

[0096] All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (*e.g.* “such as”) provided with respect to certain embodiments herein is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the invention.

[0097] Groupings of alternative elements or embodiments of the invention disclosed herein are not to be construed as limitations. Each group member can be referred to and claimed individually or in any combination with other members of the group or other elements found herein. One or more members of a group can be included in, or deleted from, a group for reasons of convenience or patentability. When any such inclusion or deletion occurs, the specification is herein deemed to contain the group as modified thus fulfilling the written description of all Markush groups used in the appended claims.

[0098] All publications, patents, patent applications, and other references cited in this application are incorporated herein by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application or other reference was specifically and individually indicated to be incorporated by reference in its entirety for all purposes. Citation of a reference herein shall not be construed as an admission that such is prior art to the present invention.

[0099] Having described the invention in detail, it will be apparent that modifications, variations, and equivalent embodiments are possible without departing the scope of the invention defined in the appended claims. Furthermore, it should be appreciated that all examples in the present disclosure are provided as non-limiting examples.

EXAMPLES

[0100] The following non-limiting examples are provided to further illustrate the present invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent approaches the inventors have found function well in the practice of the invention, and thus can be considered to constitute examples of modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

[0101] Embryonic Stem cells.

[0102] Embryonic stem cell (ESCs) lines used were CGR8, ZHBTc4 and ZHTc6. ZHBTc4 is an ES cell line that has had both endogenous *Pou5f1* (Oct-4) alleles deleted, and an exogenous tetracycline-repressible Oct-4 vector introduced, along with an expression vector producing the tetracycline (tet) activator. In the absence of tet, these cells express Oct-4 and maintain an undifferentiated state. In the presence of 10 μg/ml tet (Sigma, St. Louis, MO), which binds to and represses the tet activator, Oct-4 expression is completely repressed in these cells within 24 hours. ZHTc6 is an ES cell line that had one allele of *Pou5f1* inactivated by targeted integration of an IRESzeopA cassette, and contains a tet-repressed Oct-4 transgene. This transgene can be fully activated when removed from tet for 48 hours, resulting in over-expression of Oct-4. CGR8 cells are the parental wild-type (wt) ES cell line from which ZHBTc4 and ZHTc6 cells were derived.

[0103] All ESCs (CGR8, ZHTc6 and ZHBTc4) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Hyclone, Thermo Fisher Scientific, Pittsburgh, PA) containing 15% fetal bovine serum (FBS, embryonic stem cell grade, Hyclone), 1% sodium pyruvate (Invitrogen, Carlsbad, CA), 2mM L-glutamine (StemCell Technologies, Vancouver, BC), 0.1 mM nonessential amino acids (NEAA) (Invitrogen), 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen), leukemia inhibitory factor (LIF) (1000U/ml) (Chemicon, Temecula, CA) and 55 μM β-mercaptoethanol (βME, Sigma).

[0104] Cardiogenic Differentiation of Embryonic Stem Cells in Liquid Medium.

[0105] ESCs were cultured either with the hanging drop method in DMEM supplemented with 20% fetal calf serum (FCS, Hyclone), 2 mM L-glutamine, 0.1 mM NEAA and 0.1 mM βME, or directly cultured in 10 cm bacteriological culture plates with 10 ml DMEM supplemented with 15% FBS, 2mM L-glutamine, 0.1 mM NEAA, 50 U/ml penicillin, 50 μg/ml streptomycin and 0.1 mM βME for 2 days. Embryoid Bodies (EBs)

were collected and placed into fresh 10 cm bacteriological plates supplied with fresh differentiation medium. On the fifth day, single EBs were seeded onto 0.1% gelatin pre-coated 24-well plates and medium was changed every other day until cells were ready for analysis.

[0106] Cardiac Differentiation of ESCs in Semi-solid Methylcellulose Medium.

[0107] One million CGR8 or ZHTc6 ESCs were cultured in 10 cm bacteriological culture plates (Fisher) with 10 ml of (i) DMEM supplemented with 15% FBS (differentiation grade, StemCell Technologies), 1% nonessential amino acids (NEAA, Invitrogen), 1% L-glutamine (StemCell Technologies), 1X penicillin/streptomycin (Pen/Strep, Invitrogen) and 0.1 mM βME; or (ii) DMEM supplemented with 15% FBS (Differentiation Grade), murine interleukin-3 (IL-3, 5 ng/ml, R & D Systems, Minneapolis, MN), murine interleukin-1 (α-IL-1, 5 ng/ml, R & D Systems), insulin (10 ng/ml, Sigma), transferrin (200 ng/ml, Calbiochem, EMD Chemicals, Darmstadt, Germany) and monothioglycerol (MTG, 100 μM, Sigma) for 2 days at 37^0C, 5% CO_2. EBs were collected and subsequently seeded onto 35 mm culture plates (StemCell Technologies) containing 1.5 ml MethoCult methylcellulose (StemCell Technologies) supplemented with either (iii) 20% FBS (Differentiation Grade, StemCell Technologies), 1% nonessential amino acids (NEAA) (Invitrogen), 1% L-glutamine (StemCell Technologies), 1X penicillin/streptomycin (Pen/Strep) (Invitrogen) and 0.1 mM βME; or (iv) 15% FBS (Differentiation Grade, StemCell Technologies), murine interleukin-3 (IL-3) (5 ng/ml) (R & D Systems), murine interleukin-1 (α-IL-1) (5 ng/ml) (R & D Systems), insulin (10 ng/ml) (Sigma), transferrin (200 ng/ml) (Calbiochem) and monothioglycerol (100 μM, Sigma). Cells were incubated at 37^0C, 5% CO_2 until EBs were ready for further cardiogenic differentiation analyses. To control for the possibility that culture conditions produced the phenotypes observed, two distinct cardiogenic differentiation culture conditions were tested. Cardiogenic differentiating condition A used culture reagents (i) + (iii), and condition B used (ii) + (iv).

[0108] Reverse Transcription PCR (RT-PCR) Analysis.

[0109] Total RNA was extracted from undifferentiated (day 0) or differentiated (day 9) wt CGR8 or ZHBTc4 ESCs/EBs, with and without tetracycline treatment, using the RNeasy kit from Qiagen. First strand cDNA was synthesized using the Invitrogen First-Strand cDNA Synthesis kit according to the manufacturer's instructions. PCR was for 30 cycles at 94° C for 30 seconds, 58°C for 40 seconds, and 68°C for 40 seconds. Primers used for the experiments were: Myh6 For - CTG CTG GAG AGG TTA TTC CTC G, Myh6 Rev - GGA AGA GTG AGC GGC GCA TCA AGG, Myh7 For - TGC AAA GGC TCC AGG TCT GAG GGC, Myh7 Rev - GCC AAC ACC AAC CTG TCC AAG TTC, ANF For - TGA TAG ATG AAG GCA GGA AGC CGC, ANF Rev - AGG ATT GGA GCC CAG AGT GGA CTA G, Nkx 2.5 For - CGA CGG AAG CCA CGC GTG CT, Nkx 2.5 Rev - CCG CTG TCG CTT GCA CTT G, 18S For - AAACGGCTACCACATCCAAG, 18S Rev - CCTCCAATGGATCCTCGTTA. Myh6- cardiac-specific α-myosin heavy chain, Myh7- cardiac-specific β-myosin heavy chain, ANF- atrial natriuretic factor.

[0110] Immunoblots.

[0111] Total protein was extracted from undifferentiated ESCs or differentiating EBs using 1x lysis buffer (50 mM Tris-Cl, pH 7.8, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% NP-40, and 1x protease inhibitor cocktail). Briefly, 3 volumes of lysis buffer (cell pellet size equals one volume) was added to each sample and was pipetted multiple times on ice before being subjected to centrifugation at 12,000 RPM at 4°C for 15 minutes. Supernatants were collected as total protein extracts. Thirty μg of each protein sample was used for PAGE, and separated proteins were transferred to a nitrocellulose membrane for immunoblotting. After blocking, primary antibodies used were anti-Oct-4 monoclonal antibody (1:400, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-actin monoclonal antibody (1:10,000, Sigma). Secondary antibodies were donkey anti-mouse IgG conjugated with horse radish peroxidase (HRP, 1:10,000, GE Healthcare, Pittsburgh, PA) and sheep anti-rabbit IgG HRP-conjugated (1:10,000, GE Healthcare).

[0112] Photomicroscopy and videos.

[0113] Photomicrographs and videos of cardiogenic EBs formed at day 9 cultured in liquid medium or at day 11-13 cultured in methylcellulose medium were taken by a Hamamatsu Orca-HR camera (Hamamatsu Photonics, Bridgewater, NJ) attached to a Nikon TE-2000-U microscope. Single and sequence of images were processed by Wasabi software version 1.5 (Hamamatsu Photonics).

[0114] Quantitative Real-Time PCR (qRT-PCR).

[0115] Total RNA was isolated from differentiated cardiogenic EBs using Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA) as described above from the indicated cell populations. First strand cDNA was synthesized using the Invitrogen cDNA synthesis kit according to the manufacturer's instructions. Quantitative expression analysis was performed using SYBR Green PCR Master Mix reagents (Applied Biosystems, Foster City, CA). Primers used to target cardiac-specific mRNAs and internal control were purchased from Qiagen's QuantiTech Primer Assays. Relative expression was calculated using the comparative $2^{(\Delta\Delta Ct)}$ method. Murine GAPDH expression was used to normalize mRNA expression across different RNA preparations. Results are represented as means +/- SEM of at least three independent experiments.

[0116] Hematopoietic Embryoid Body Differentiation and Flow Cytometry.

[0117] Hematopoietic embryoid bodies (EBs) were generated from ESCs by plating $1x10^4$ ESCs in 1.5 ml 0.9% methylcellulose-base medium (Stem Cell Technologies) supplemented with 10% fetal bovine serum (differentiation grade, Stem Cell Technologies), 5% protein-free hybridoma medium-II (Invitrogen), murine IL-1 (5ng/ml), murine IL-3 (5 ng/ml), SCF (10 ng/ml), GM-CSF (5 ng/ml) and EPO (3U/ml) at 37°C and 5% CO_2 for 12-14 days. Differentiated cells and EBs from 12d cultures were collected in phosphate-buffered saline (PBS) and spun down at 1,200 rpm for 10 minutes. EBs were disaggregated by the addition of 3 ml 0.25% collagenase to each sample and incubated at 37°C for one hour. Single-cell suspensions were collected and washed with PBS and pellets were resuspended with 100 μl of antibody cocktail (CD45-

PE and CD11b-APC, 1:100, BD Biosciences, San Jose, CA), and incubated at ambient temperature in the dark for 30 minutes. Cells were washed with PBS supplemented with 0.5% BSA once and resuspended in 250 μl PBS for FACS analysis (FACSCalibur, BD Biosciences). Data collected from all samples were analyzed by FlowJo software (version 6.4.1, Tree Star Inc., Ashland, OR).

[0118] Immunofluorescence staining

[0119] Five beating EBs (BEBs) and five non-beating EBs (NBEBs) were collected (a total of ten) from day 12 wt CGR8 differentiating EBs cultured in cardiogenic culture condition B. Each EB was placed into separate eppendorf tube with pre-warmed PBS. After centrifugation, supernatant was aspirated and the single EB was resuspended into 0.3 ml collagenase (CalBiochem) (0.25% collagenase in PBS supplemented with 20% FBS). EBs in collagenase were incubated at 37°C for an hour with one swirling at the halfway time point. After incubation, EB was dissociated into single cell suspension using a 21 ga needle. Single cell suspension derived from each EB (BEB or NBEB) was then plated onto laminin-coated glass coverslips (Sigma, used at a concentration of 50μg/ml in PBS) and cultured for 2-3 days in cardiac differentiation culture condition B with liquid medium base. Cells were prepared for immunofluorescence using standard procedures as previously described (Sussman et al., 1994a). Primary antibody used was anti-cardiac MHC6/7 monoclonal antibody (1:250) (Abcam). Secondary antibodies used were FITC-conjugated anti-rabbit IgG (Sigma) (1:80), FITC-conjugated anti-mouse IgG (Sigma) (1:100), and TRITC-conjugated anti-mouse IgG (Sigma) (1:100). Immunostained cardiomyocytes were mounted in Vectashield antibleaching medium (Vector) and sealed with nail polish (Revivánail™, European Touch). Digital images were taken with a Axioskop fluorescence microscope equipped with filters for fluorescein and rhodamine epifluoresence.

EXAMPLE 2

[0120] This example demonstrates a 5-fold increase in the number of contracting embryoid bodies when cultured in a modified cardiogenic culture condition (condition B). This example shows that CGR8 mESCs cultured in condition A results in a low-level of beating cells. This example also demonstrates that *Oct-4* knock-in ZHTc6 ESCs failed to generate contracting EBs upon induction of differentiation. Methods are according to Example 1, unless otherwise specified.

[0121] Previously published protocols for generating contracting embryoid bodies were found to be inefficient at generating cardiogenic differentiation (see e.g., FIG. 1). CGR8 mESCs were cultured in conventional cardiac differentiation medium (non-cytokine-supplemented condition A). Low-level of beating cells are formed under this culture condition (see e.g., white arrowheads in FIG. 1A and FIG. 1B). RT-PCR analysis show low- to moderate-expression levels of cardiac-specific markers in cells cultured in condition A (see e.g., FIG. 1C).

[0122] Therefore, a modified protocol to increase the efficiency of contracting EB generation was used in this study (see Example 1, FIG. 6). The conventional culturing condition was designated condition A, and the modified culture condition as B (see Example 1). Contracting EBs can be seen forming with both conditions (see e.g., FIG. 2A and FIG. 2B). But less than 3 percent of contracting EBs were seen in day 11 CGR8 EBs when cultured in condition A (2.3$\pm$0.83%) whereas there was a 5-fold increase in the number of contracting EBs seen in day 11 CGR8 EBs when cultured in condition B (11.7$\pm$2.4%; see e.g., FIG. 9). A significant number of contracting EBs were not observed to develop from day 11 ZHTc6 EBs (with additional daily monitoring for 20 days) when cultured in conditions A or B (data not shown).

[0123] ZHTc6 is a tet-regulated, heterozygous *Oct-4* knock-in ES cell line. In these cells, one copy of endogenous *Pou5f1* was replaced by a tet-regulated *Oct-4* transgene. Without activation of the *Oct-4* transgene, Oct-4 protein expression level in this cell line is ~ 70-80% of that seen in wt bi-allelic ESCs (CGR8)[6]. To address the possibility that a

suboptimal expression level of Oct-4 protein in the undifferentiated ESCs contributed to the lack of contracting cardiogenic EB formation, the experiments were repeated and included conditions that restored Oct-4 protein expression to a level similar to wt expression, or to ~50% higher than wt in the ZHTc6 cells.

EXAMPLE 3

[0124] This example demonstrates that Oct-4 expression can be restored in differentiating ZHTc6 ESCs. Methods are according to Example 1, unless otherwise specified.

[0125] To show that wt levels of Oct-4 are required for proper cardiogenic differentiation, ZHTc6 ESCs were removed from tet either 24 hrs or 48 hrs before they were induced to differentiate, which would allow the tet-repressed *Oct-4* transgene to be expressed. Immunoblot analyses confirmed that four days of continuous expression of Oct-4 protein can be detected in all differentiating ESC lines (CGR8, tet-treated ZHTc6 and untreated ZHTc6) (see e.g., FIG. 3A). Quantification using ImageJ software (v. 1.42q, NIH, USA) showed that Oct-4 protein expression levels from undifferentiated ZHTc6 cells in tet and ZHTc6 cells 24 hrs off tet (day 0, in the presence of LIF) was ~ 20% lower than wt CGR8, which was similar to previously reported[6] (see e.g., FIG. 3B). However, after 1 day of differentiation out of LIF, expression of Oct-4 protein in ZHTc6 cells 24 hrs off tet increased back to wt level, whereas Oct-4 protein levels dropped further in ZHTc6 cells with tet (~50% of wt; see e.g., 3B). In ZHTc6 cells off tet for 48 hrs, Oct-4 protein levels increased from ~130% (in LIF, Day 0 of differentiation) to ~150% after one day of differentiation without LIF (see e.g., FIG. 3B).

[0126] The much lower number of contracting EBs in conventional condition supports using the modified culture condition B for optimization of cardiac development.

EXAMPLE 4

[0127] This example demonstrates that a significant increase in cardiogenic EB formation is seen in day 11 ZHTc6 EBs when Oct-4 expression is restored back to normal levels. Methods are according to Example 1, unless otherwise specified.

[0128] When cultured in tet, no contracting EBs could be detected from day 9 to day 20 of differentiation in ZHTc6 EBs (Oct-4 under-expression; see e.g., FIG. 4A). When ZHTc6 cells were removed from tet for 24 hours (ZHTc6 24h) before differentiation by removal from LIF, and cultured without tet, a significant number of cardiogenic EBs (9.7±2.5%) were seen forming in these cells, which had Oct-4 levels comparable to wt CGR8 (see e.g., FIG. 3B). After cardiogenic differentiation, CGR8 cells formed contracting EBs at 11.7±2.4% of the time.

[0129] These results show that a decrease of Oct-4 expression in undifferentiated or early differentiating ESCs can severely compromise cardiogenic development, but this defective phenotype can be reversed if Oct-4 expression is restored back to wt levels early in differentiation (see e.g., FIG. 4A).

EXAMPLE 5

[0130] This example demonstrates that while Oct-4 expression after formation of brachyury+ mesoderm is required for successful cardiogenic development, over-expression of Oct-4 does not promote cardiogenic differentiation. Methods are according to Example 1, unless otherwise specified.

[0131] To exclude the possibility of tet cytotoxicity in ZHTc6 ESCs and to investigate whether full expression of Oct-4 after the formation of brachyury^{+} mesodermal tissues (day 2 EBs) is necessary for successful cardiogenesis, the experiments were repeated with the addition of CGR8 TD0 (tet-treated at day 0, at removal of LIF), ZHTc6 48h (out of tet for 48 hrs prior to induction of differentiation by removal of LIF, Oct-4 expression ~50% higher than wild-type CGR8), ZHTc6 24h TD2

(removed tet 24 hours prior for differentiation, and tet added back to differentiating ESCs at day 2 after differentiation induction), and ZHTc6 48h TD2 (removed from tet 48 hrs prior to differentiation, tet added back to differentiating ESCs at day 2 after differentiation).

[0132] Similar high levels of contracting EB formation were seen from differentiated untreated CGR8, CGR8 TD0 and ZHTc6 24h cells (7.54±1.43%, 10.8±2.6%, and 12.7±1.6% respectively; see e.g., FIG. 4B). A higher percentage of contracting EBs were not observed to be generated from ZHTc6 48h ESCs (which have Oct-4 expression ~50% higher than wild-type CGR8). Instead, a significant decrease of contracting EB formation (~75% less) was seen from ZHTc6 48h ESCs when compared to ZHTc6 24h ESCs (Oct-4 expression similar to wildtype; see e.g., FIG. 4A and FIG. 4B) or wt CGR8 ESCs. Reducing Oct-4 expression after formation of brachyury$^+$ mesodermal tissues also repressed the development of contracting cardiogenic progenitors (ZHTc6 24h TD2, and ZHTc6 48h TD2; see e.g., FIG. 4B).

[0133] A showing that overproduction of Oct-4 does not promote formation of later stage hematopoietic progenitors is similar to previous reports. That study reported an increase of the hemangioblast with a 2-fold increase of Oct-4, but there was no increase in later stage hematopoietic progenitors. Indeed, they found that higher levels of Oct-4 (4-fold increase) markedly inhibited hematopoietic development. Their data are consistent with observations reported herein that an increase of Oct-4 in ESCs can promote the expression of early mesodermal marker, *Brachyury* (unpublished data), but is not necessarily beneficial to the later stages of hematopoietic differentiation.

EXAMPLE 6

[0134] This example shows high expression of cardiac-specific markers in EBs expressing wild-type levels of Oct-4, but not decreased levels of Oct-4. Methods are according to Example 1, unless otherwise specified.

[0135] To validate results from the microscopic analyses (see e.g., FIG. 4), undifferentiated ESCs (day 0), day 10, and 18 differentiated EBs were harvested from CGR8 and ZHTc6 (cultured with or without tet) real-time PCR (qRT-PCR) was performed to examine the expression of cardiac-specific markers. Day 0 ESCs and the well-differentiated day 18 EBs were examined. High expression of cardiac markers can be seen in these late-stage differentiating CRG8, ZHTc6 24h and ZHTc6 48h EBs (see e.g., FIG. 5A-D). However, except for Myh6, day 18 CGR8 EBs did not show as strong expression signal of the cardiac markers than day 18 ZHTc6 24h EBs (see e.g., FIG. 5A-D). Experiments were repeated with an earlier time point EBs (day 10), confirming that expression of early and intermediate cardiogenic markers (Nkx 2.5, Tnnt2 and Myh7) peaked earlier in wildtype CGR8 EBs (untreated or tet-treated) than in the genetically altered ZHTc6 EBs (see e.g., FIG. 5E-G). Except for ZHTc6 24h, expression of the Nkx 2.5, Tnnt2 and Myh7 had subsided significantly by day 18 post-differentiation (see e.g., FIG. 5A-C). Expression of the late cardiac marker, Myh6, remained strong in untreated CGR8, CGR8 TD0 and ZHTc6 24h day 18 EBs (see e.g., FIG. 5D).

[0136] The qRT-PCR results reinforce microscopic observations that full expression of Oct-4 in undifferentiated and early differentiating (wild-type CGR8 and ZHTc6 24h) ESCs had the highest yield in producing cardiogenic progenitors (see e.g., FIG. 2 and FIG. 4). The cardiac-specific markers, Nkx 2.5, Tnnt2, Myh6 and Myh7 are only highly expressed in untreated CGR8, CGR8 TD0, and ZHTc6 24h EBs, albeit at different time points (see e.g., FIG. 5). Since both wt (CGR8) and a manipulated ES cell line (ZHTc6) were used in this study, it is reasonable to compare the gene expression time points within subgroups (i.e., untreated CGR8 vs. tet-treated CGR8; tet-treated ZHTc6 vs. induced ZHTc6) (see e.g., FIG. 5). Accordingly, qRT-PCR data have shown a delayed induction of cardiogenic differentiation in ZHTc6 24h ESCs.

EXAMPLE 7

[0137] This example shows that generation of myeloid progenitors increased at a dose-dependent manner corresponding to Oct-4 protein expression levels in ZHTc6 ESCs. Methods are according to Example 1, unless otherwise specified.

[0138] Previous reports have shown that EB differentiation was arrested prematurely when Oct-4 protein expression was completely repressed in undifferentiated or early differentiating ESCs, which in turn led to unsuccessful hematopoietic differentiation. Here, the effect of partial expression or over-expression of Oct-4 protein on hematopoiesis was investigated using the ZHTc6 ESCs. Flow cytometric analysis showed that generation of hematopoietic progenitors from tet-treated ZHTc6 EBs was 27-33% of that seen from wt CGR8 EBs (see e.g., FIG. 7). When Oct-4 protein expression was increased in ZHTc6 ESCs by relieving the cells from tet-treatment prior to differentiation (ZHTc6 24h and ZHTc6 48h ESCs), hematopoietic progenitor formation increased in a dose-dependent manner (see e.g., FIG. 7).

[0139] As full a rescue of hematopoietic progenitor subpopulations from ZHTc6 24h EBs was not observed as it was in cardiogenic differentiation (see e.g., FIG. 4 and FIG. 5). It may be that ZHTc6 cells may be predisposed to lower hematopoietic development efficiency or the induction mechanism for hematopoietic differentiation is more susceptible to suboptimal level of Oct-4 protein in undifferentiated ESCs than the cardiogenic differentiation mechanism. This may suggest that hematopoietic progenitors are distinguished from cardiogenic progenitors at an early stage of mesodermal development, and have distinct requirements for Oct-4.

EXAMPLE 8

[0140] This example shows that contracting EBs derived from CGR8 mESCs cultured in condition B demonstrate a significant increase in generation of contracting EBs.

[0141] Contracting EBs derived from CGR8 mESCs cultured in conventional cardiac differentiation medium (condition A); or cytokine-supplemented cardiac differentiation medium (condition B). An about 4 to 6-fold increase in contracting EBs were generated from CGR8 when cells were differentiated in culture condition B (see e.g., FIG. 9).

EXAMPLE 9

[0142] Further experiments were directed at the determination of Oct-4 protein expression in CGR8 or Oct-4 knock-in ZHTc6 ESCs.

[0143] Western blot analysis was conducted on Oct-4 protein expression in wt CGR8 or Oct-4 knock-in ZHTc6 ESCs before (D0) and after induction for differentiation (D1) (see e.g., FIG. 10A-C).

EXAMPLE 10

[0144] Further experiments were directed at measuring cardiac-specific marker expression in undifferentiated ESCs and D18 or D10 wt CGR8- or Oct4 Knock-in ZHTc6-derived EBs.

[0145] RT-qPCR analysis was performed on cardiac-specific marker expression in D0 undifferentiated ESCs or D18 wt CGR8- or Oct4 Knock-in ZHTc6-derived EBs (see e.g., FIG. 11A-D).

[0146] RT-qPCR analysis was performed on cardiac-specific marker expression in D0 undifferentiated ESCs or D10 wt CGR8- or Oct4 Knock-in ZHTc6-derived EBs (see e.g., FIG 11E-G).

EXAMPLE 11

[0147] Further experiments were directed at immunofluorescent microscopy of cardiac-specific marker Myh6/7.

[0148] The cardiac-specific marker, Myh6/7, was immunofluorescently stained in samples of NBEBs and BEBs with CGR8 stem cell lines or ZHTc6 stem cell lines. Fluorescence microscopy illustrates that Myh6/7 is not expressed in the NBEBs regardless of ES cell line (see e.g., FIG. 12A-L).

EXAMPLE 12

[0149] This example shows that BEBs possess a significantly higher percentage of Myh6/7+ve cells compared to NBEBs.

[0150] Percent of Myh6/7+ve cells in BEBs and NBEBs was determined by immunofluorescence analysis of cardiac myosin heavy chain expression from CGR8 and ZHTc6 EBs. Beating EBs (BEBs) and non-beating EBs (NBEBs) were collected from CGR8 ESC-derived EBs or ZHTc6 24h ECS-derived EBs at day 12 (see e.g., FIG. 13A). Areas of myosin heavy chain staining (Myh6/7) and DAPI staining were measured from each EB (see e.g., FIG. 13B). Percentages of myosin heavy chain +ve cell population was calculated from each EB by dividing Myh6/7+ve area with DAPI +ve area (n=5).

EXAMPLE 13

[0151] Further experiments were directed at immunofluorescent microscopy of cardiac-specific marker Myh6/7.

[0152] Immunofluorescence analysis of cardiac myosin heavy chain expression from beating EBs derived from CGR8 24h ESCs (see e.g., FIG. 14A-C) show that well-organized, striated sarcomeric structures (green fluorescence, white arrowheads) were observed from CGR8-derived BEBs but not from ZHTc6 24h ESC-derived BEBs (green fluorescence) (see e.g., FIG. 15A-D, where C and D are zoomed in images of the insets in A and B).

EXAMPLE 14

[0153] Further experiments were directed at determining if vasculogenesis has a synergic effect on cardiogenesis during development.

[0154] BEBs and BEBs derived from CGR8 were stained using MHC6/7 staining (cardiac) and CD31 (vascular) staining (see e.g., FIG. 16A-L).

EXAMPLE 15

[0155] Further experiments were directed at examining the effect of vasculogenesis during cardiogenic differentiation. Co-culturing with VEGF (5 and 10 ng/ml) showed a very mild effect, which was considered inconclusive. However, co-culturing with Endothelial stem/progenitor cells (HUVECs) showed an increase cardiogenic potential in mESCs (see e.g., FIG. 17A-B).

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[0191] Park IH, Zhao R, West JA, et al. Reprogramming of human somatic cells to pluripotency with defined factors. Nature. 2008;451:141-146.

CLAIMS

What is claimed is:

Claim 1. A culture medium for generating cardiac progenitor cells comprising at least one of interleukin-3, interleukin-1, insulin, and transferin.

Claim 2. The culture medium of claim 1 comprising interleukin-3, interleukin-1, insulin, and transferin.

Claim 3. The culture medium of any one of claims 1-2 wherein:

interleukin-3 has a concentration of at least about 0.5 ng/ml up to about 50 ng/ml;
interleukin-1 has a concentration of at least about 0.5 ng/ml up to about 50 ng/ml;
insulin has a concentration of at least about 1 ng/ml up to about 100 ng/ml; or
transferin has a concentration of at least about 20 ng/ml up to about 2 µg/ml.

Claim 4. The culture medium of any one of claims 1-3, wherein:

interleukin-3 has a concentration of about 5 ng/ml;
interleukin-1 has a concentration of about 5 ng/ml;
insulin has a concentration of about 10 ng/ml; or
transferin has a concentration of about 200 ng/ml.

Claim 5. The culture medium of any one of claims 1-4, further comprising one or more of FBS or monothioglycerol.

Claim 6. A method of forming a cardiac progenitor cell comprising:

contacting a progenitor cell and the culture medium of any one of claims 1-5; and
culturing the progenitor cell so as to form a cardiac progenitor cell.

Claim 7. A method of forming a cardiac progenitor cell comprising:
increasing a level of Oct-4 in a progenitor cell; and
culturing the progenitor cell so as to form a cardiac progenitor cell.

Claim 8. The method of claim 6 comprising:
increasing a level of Oct-4 in the progenitor cell.

Claim 9. The method of any one of claims 6-7, wherein increasing the level of Oct-4 in the progenitor cell comprises (i) introducing exogenous Oct-4 to the progenitor cell; or (ii) increasing expression of endogenous Oct-4 in the progenitor cell.

Claim 10. The method of any one of claims 6-9, comprising:
detecting a level of Oct-4 in the progenitor cell.

Claim 11. The method of any one of claims 7-10, wherein the level of Oct-4 in the progenitor cell is increased to be about the level of Oct-4 in embryonic stem cell line CGR8 cultured under equivalent conditions.

Claim 12. The method of any one of claims 7-11, wherein the level of Oct-4 in the progenitor cell is increased so as promote expression of early mesodermal marker *Brachyury*.

Claim 13. The method of any one of claims 7-12, wherein the level of Oct-4 in the progenitor cell is increased prior to 11 days of culturing the progenitor cell.

Claim 14. The method of any one of claims 6-13, wherein the number of formed cardiac progenitor cells is at least about 100% greater than the number of cardiac progenitor cells formed (i) under culture conditions not comprising at least one of interleukin-3,

interleukin-1, insulin, and transferin; or (ii) in the absence of increasing the level of Oct-4.

Claim 15. The method of any one of claims 6-13, wherein the cardiac progenitor cell displays one or more cellular markers selected from the group consisting of Nkx2.5, Tnnt2, Myh7, Myh6, and Brachyury.

Claim 16. The method of any one of claims 6-15, wherein the progenitor cell is an embryonic progenitor cell or an induced pluripotent stem cell.

Claim 17. The method of claim 16, wherein the progenitor cell is a human embryonic progenitor cell or a human induced pluripotent stem cell.

Claim 18. The method of any one of claims 6-15, comprising a first progenitor cell and a second progenitor cell, the first progenitor cell comprising a embryonic progenitor cell and the second progenitor cell comprising an endothelial stem/progenitor cell (HUVEC).

Claim 19. The method of claim 18, wherein embryonic progenitor cells are present at about 90% to 99% and the HUVECs are present at about 10% to about 1% in the progenitor cell culture.

Claim 20. The method of any one of claims 6-19, wherein the cardiac progenitor cell comprises a contracting embryoid body.

FIG. 1A-B

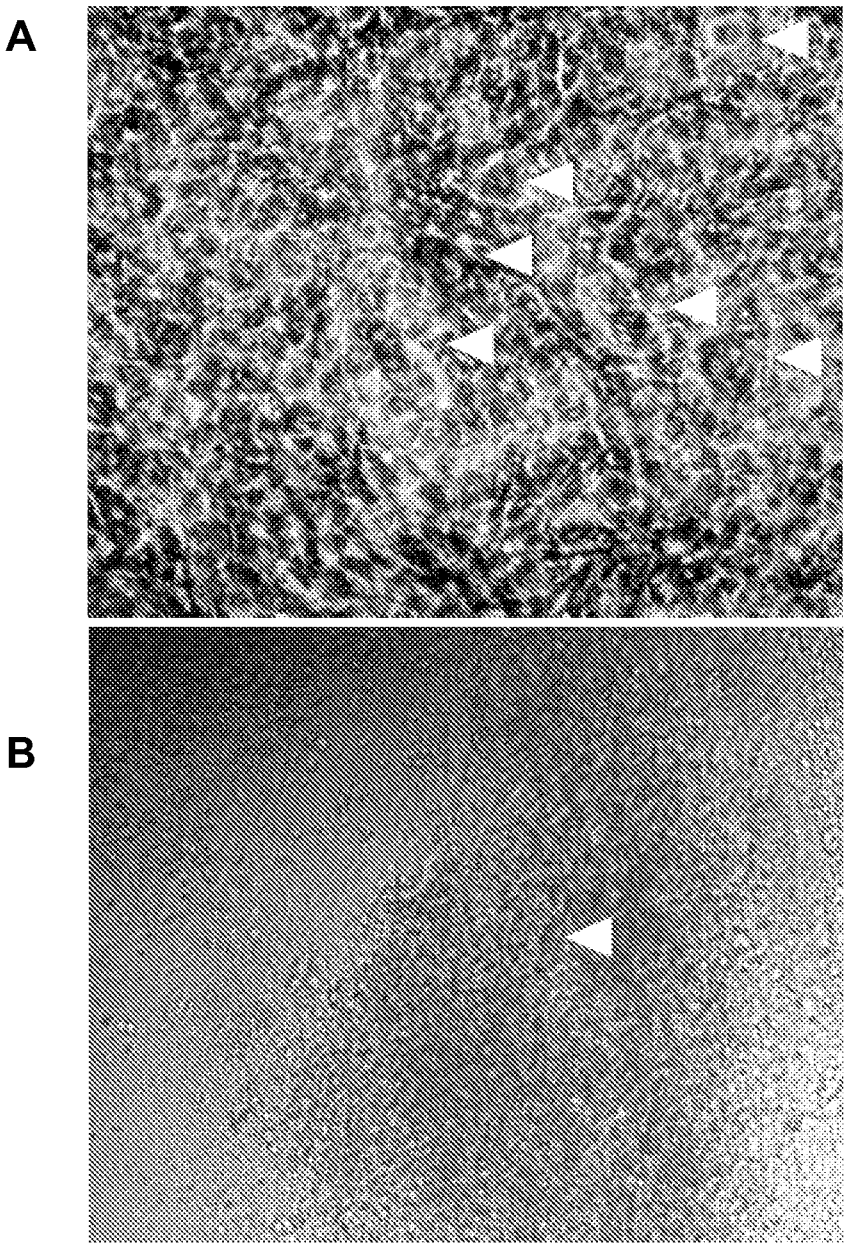


FIG. 1C

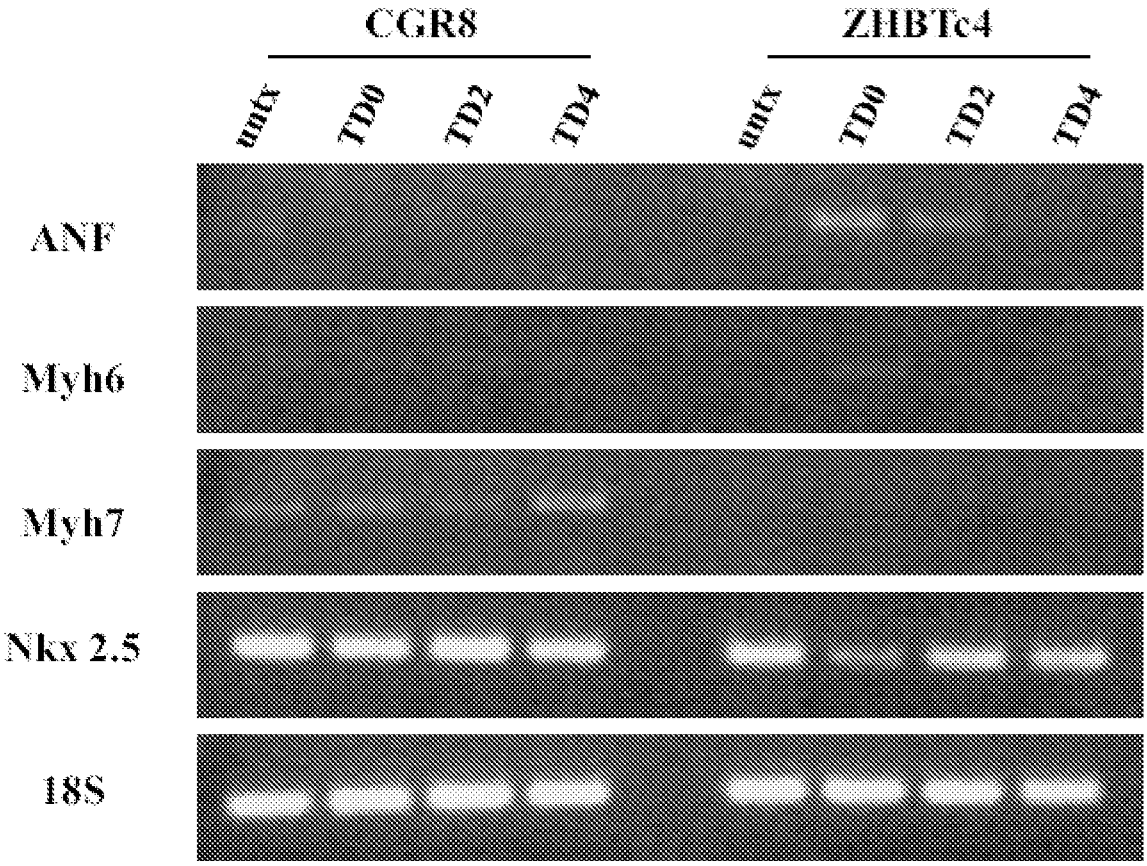

CGR8
ZHBTc4
mtx
TD0
TD2
TD4
mtx
TD0
TD2
TD4
ANF
Myh6
Myh7
Nkx 2.5
18S

FIG. 2

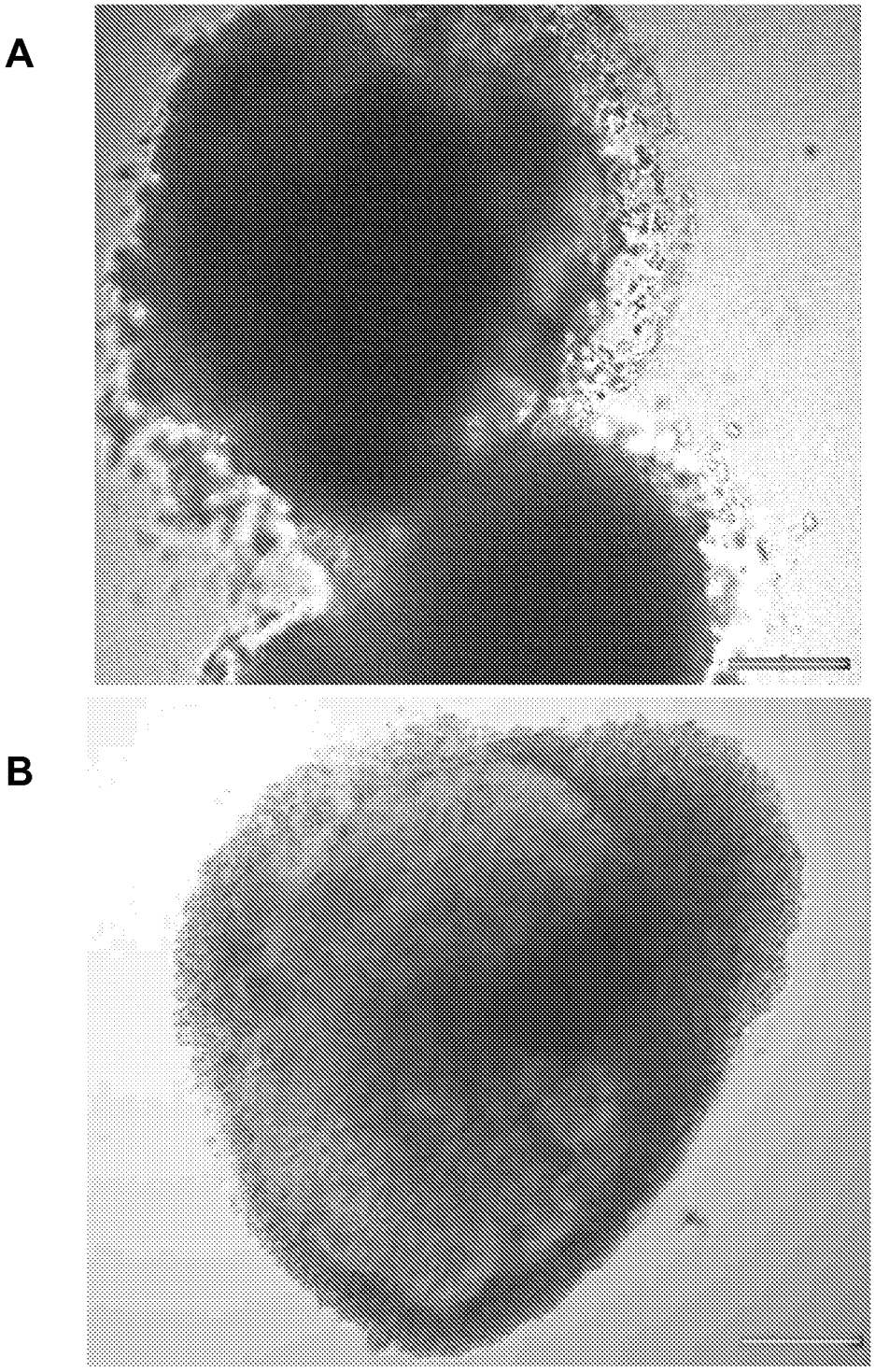


FIG. 3A

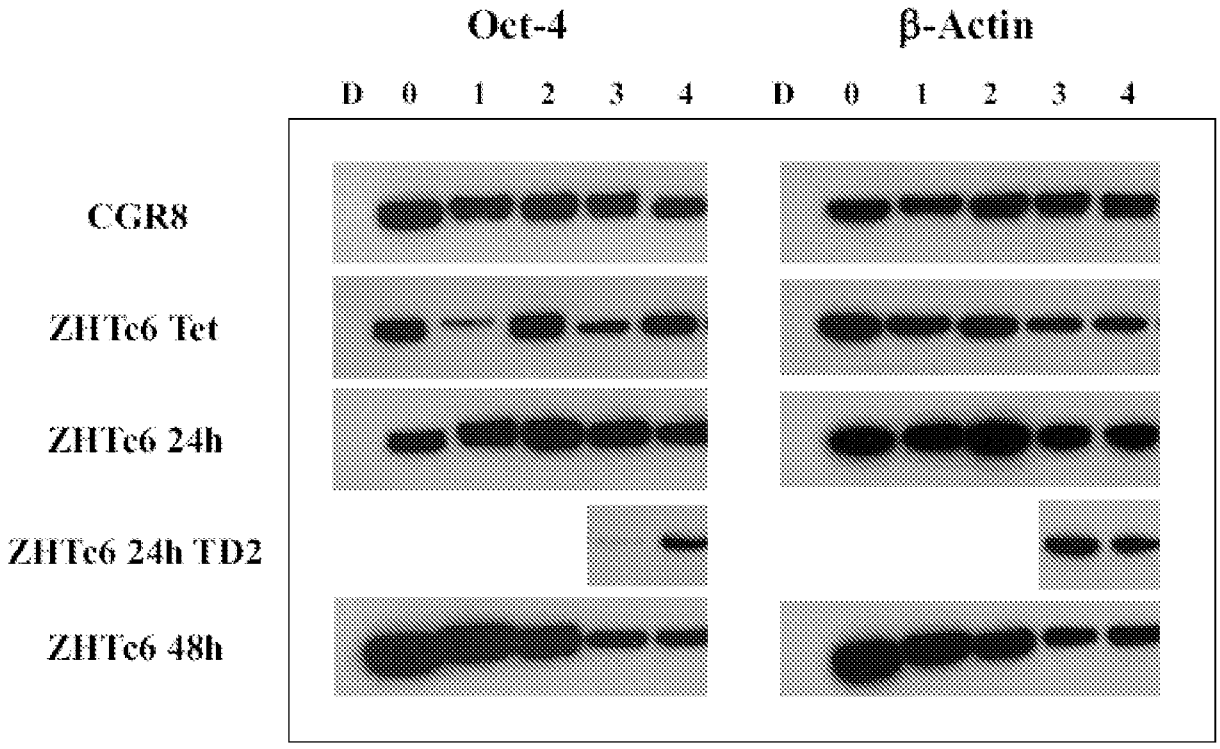

Oct-4
β-Actin
D 0 1 2 3 4
D 0 1 2 3 4
CGR8
ZHTc6 Tet
ZHTc6 24h
ZHTc6 24h TD2
ZHTc6 48h

FIG. 3B

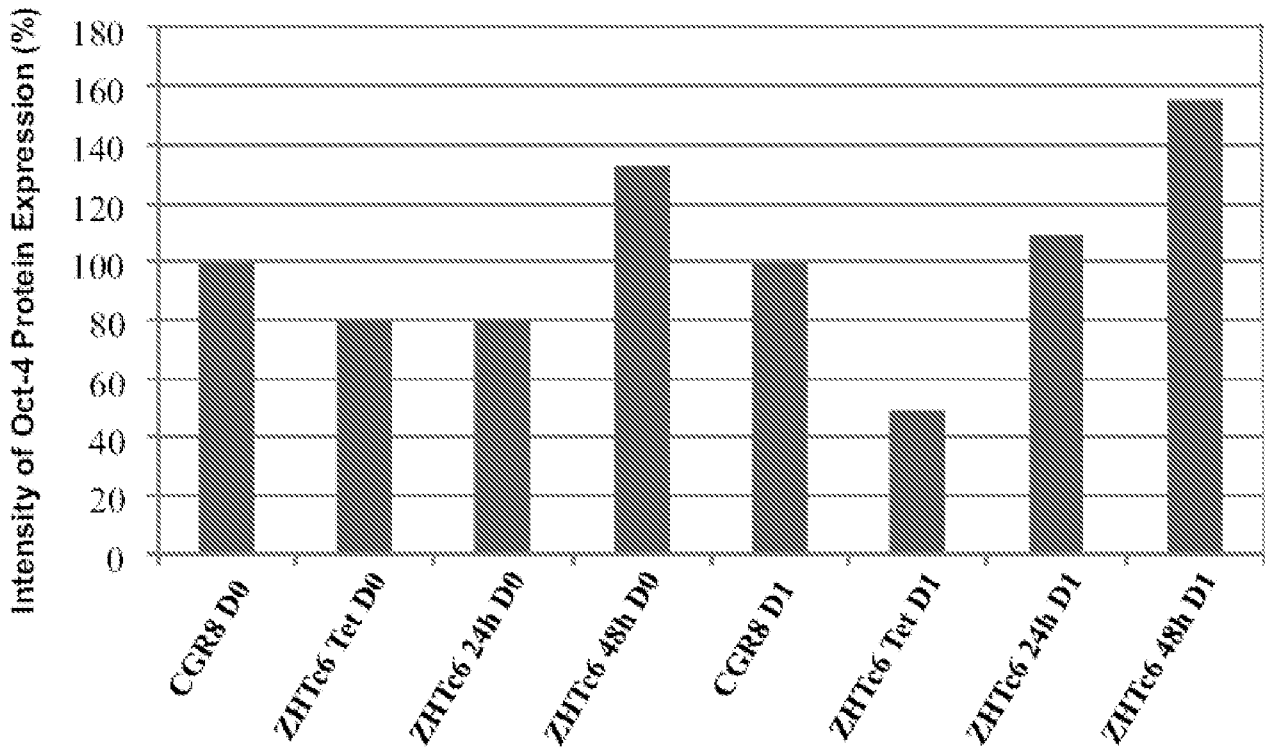

Intensity of Oct-4 Protein Expression (%)
180
160
140
120
100
80
60
40
20
0
CGR8 D0
ZHTc6 Tet D0
ZHTc6 24h D0
ZHTc6 48h D0
CGR8 D1
ZHTc6 Tet D1
ZHTc6 24h D1
ZHTc6 48h D1

FIG. 4A

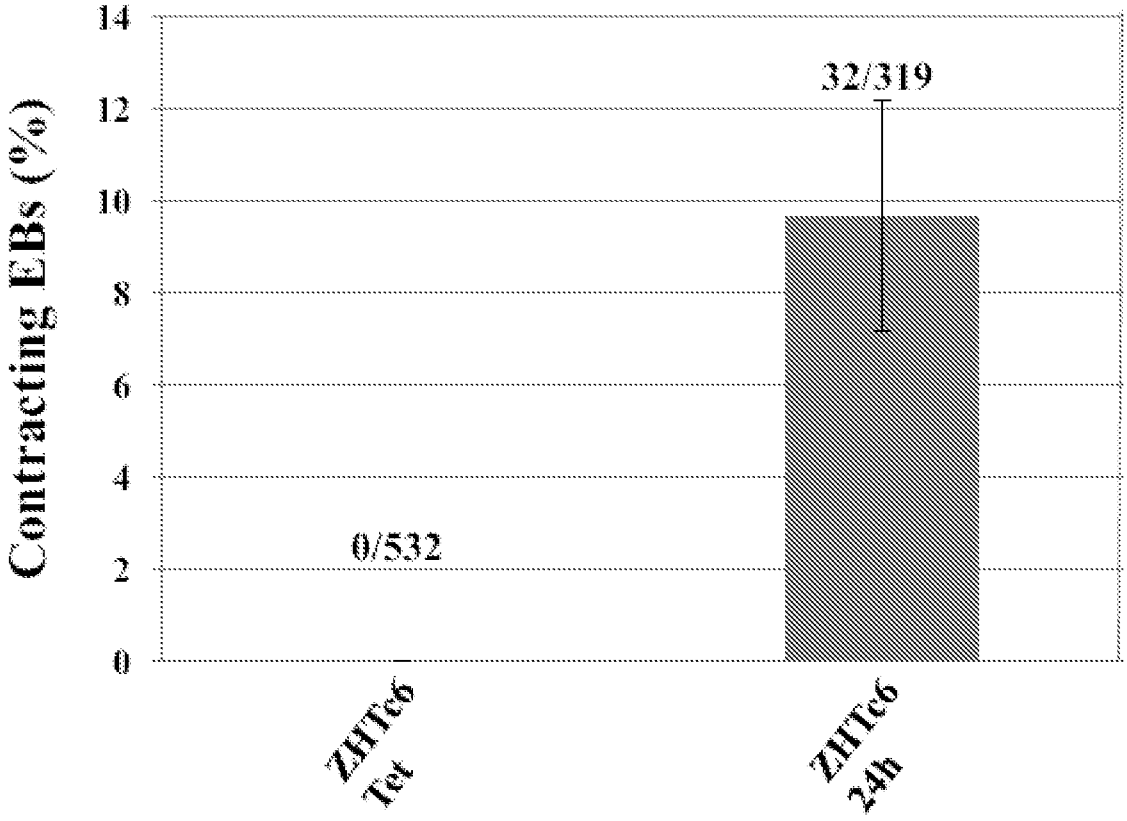

Contracting EBs (%)
14
12
10
8
6
4
2
0
32/319
0/532
ZHTc6 Tet
ZHTc6 24h

FIG. 4B

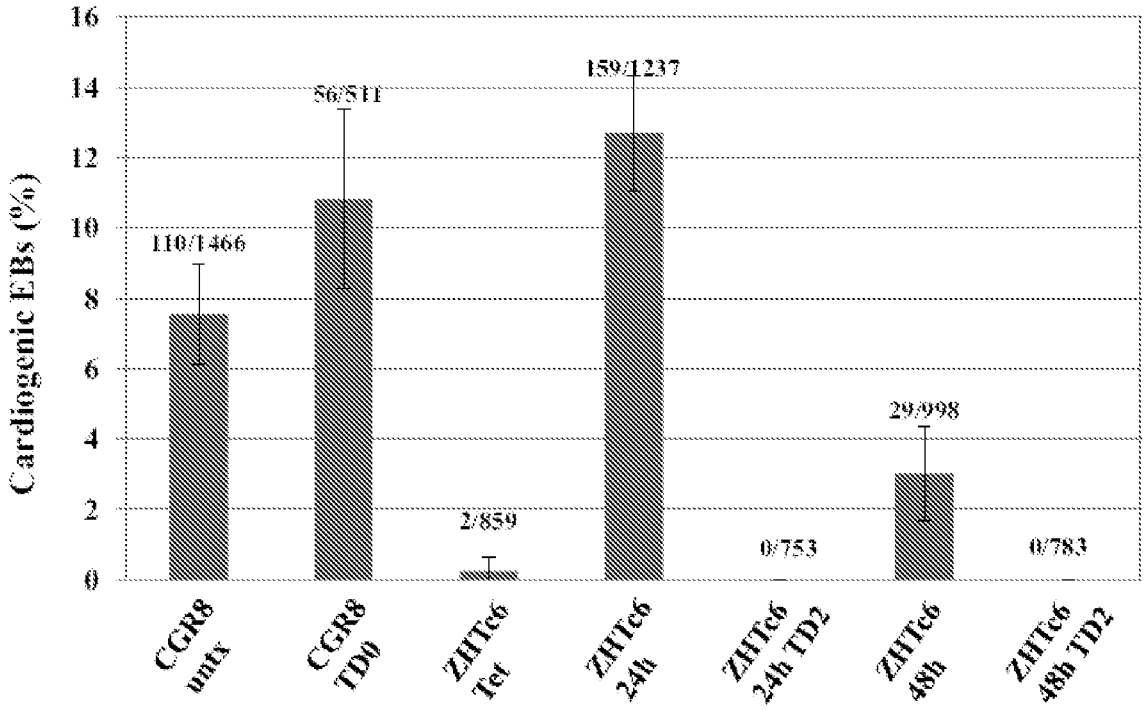

Cardiogenic EBs (%)
16
14
12
10
8
6
4
2
0
110/1466
56/511
2/859
159/1237
0/753
29/998
0/783
CGR8 untx
CGR8 TD0
ZHTc6 Tet
ZHTc6 24h
ZHTc6 24h TD2
ZHTc6 48h
ZHTc6 48h TD2

FIG. 5A

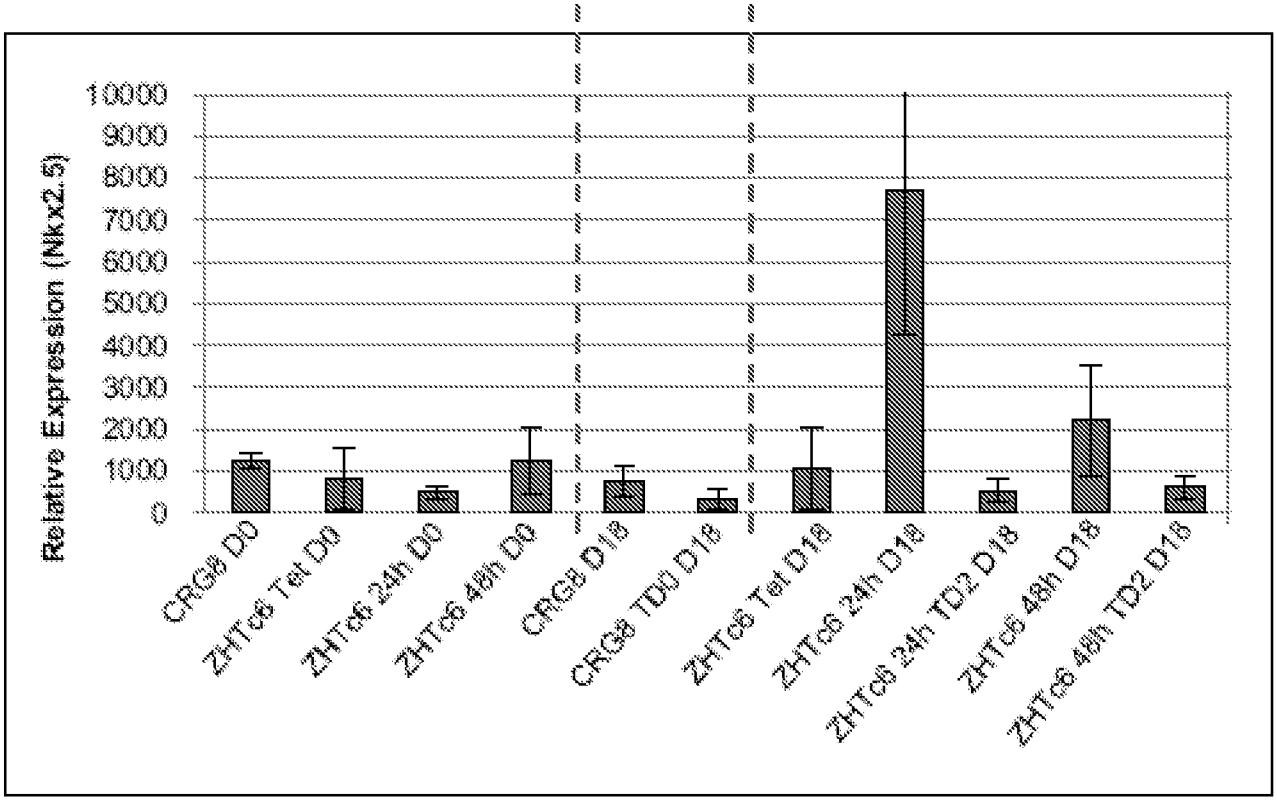

Relative Expression (Nkx2.5)
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
CRG8 D0
ZHTc6 Tet D0
ZHTc6 24h D0
ZHTc6 48h D0
CRG8 D18
CRG8 TD0 D18
ZHTc6 Tet D18
ZHTc6 24h D18
ZHTc6 24h TD2 D18
ZHTc6 48h D18
ZHTc6 48h TD2 D18

FIG. 5B

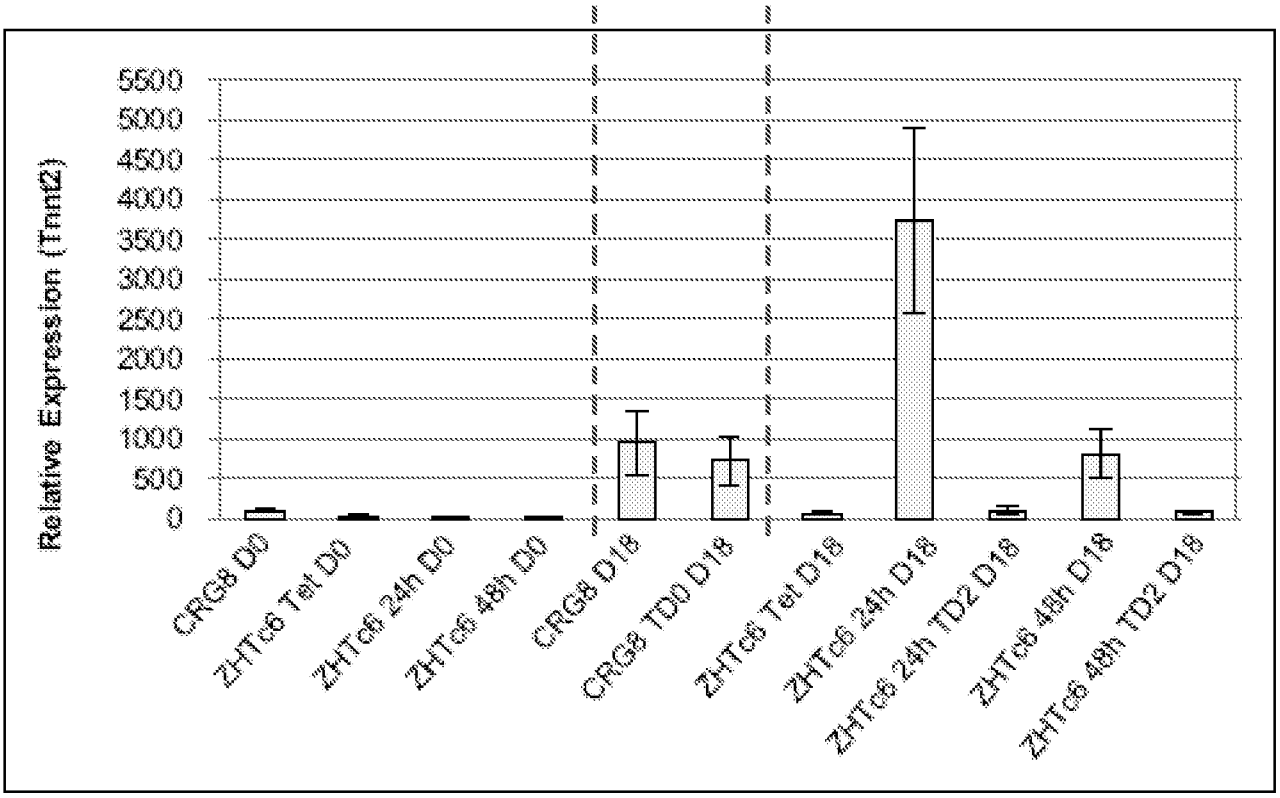

Relative Expression (Tnnt2)
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
CRG8 D0
ZHTc6 Tet D0
ZHTc6 24h D0
ZHTc6 48h D0
CRG8 D18
CRG8 TD0 D18
ZHTc6 Tet D18
ZHTc6 24h D18
ZHTc6 24h TD2 D18
ZHTc6 48h D18
ZHTc6 48h TD2 D18

FIG. 5C

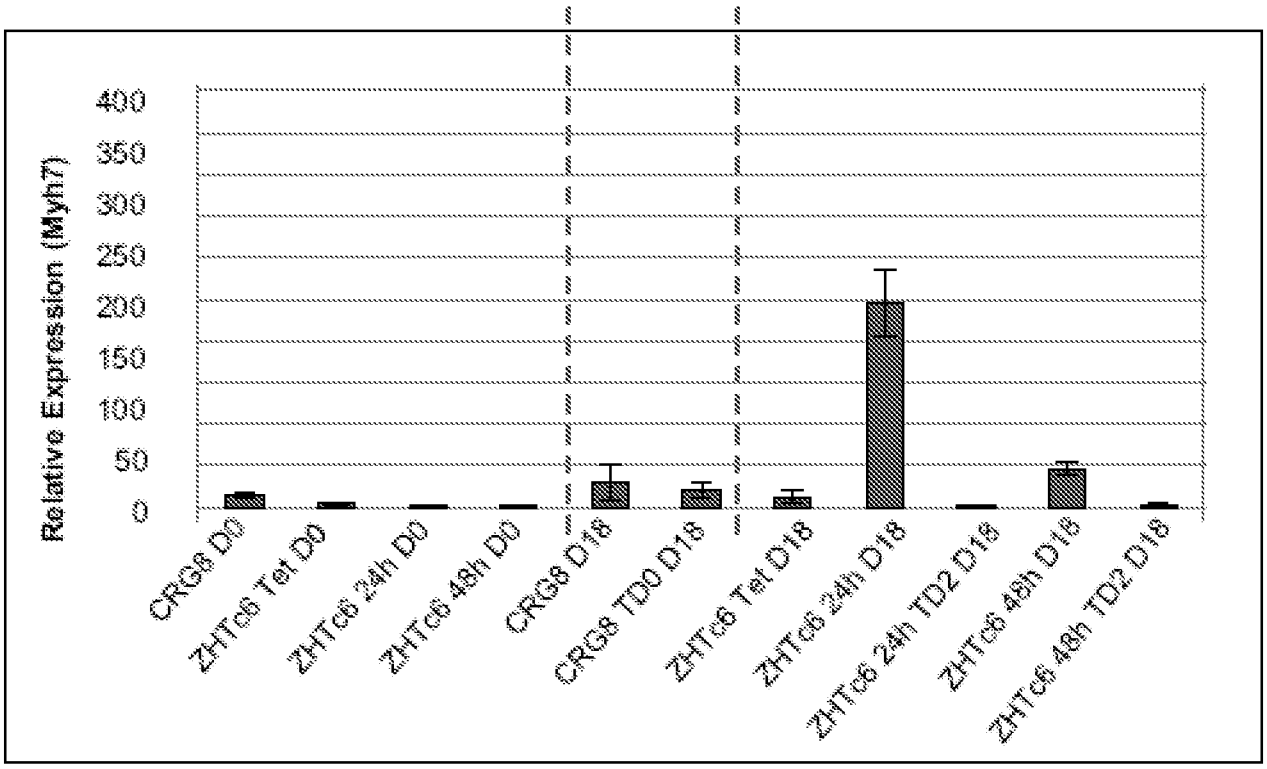

Relative Expression (Myh7)
400
350
300
250
200
150
100
50
0
CRG8 D0
ZHTc6 Tet D0
ZHTc6 24h D0
ZHTc6 48h D0
CRG8 D18
CRG8 TD0 D18
ZHTc6 Tet D18
ZHTc6 24h D18
ZHTc6 24h TD2 D18
ZHTc6 48h D18
ZHTc6 48h TD2 D18

FIG. 5D

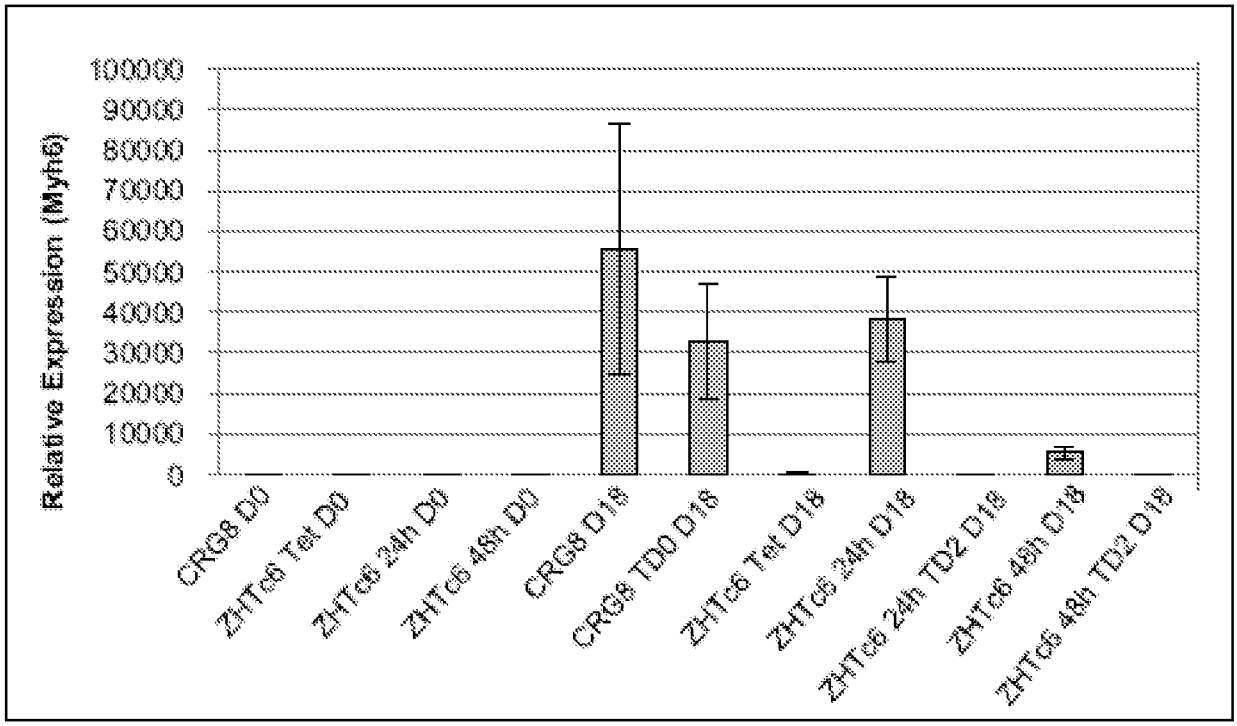

Relative Expression (Myh6)
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
CRG8 D0
ZHTc6 Tet D0
ZHTc6 24h D0
ZHTc6 48h D0
CRG8 D18
CRG8 TD0 D18
ZHTc6 Tet D18
ZHTc6 24h D18
ZHTc6 24h TD2 D18
ZHTc6 48h D18
ZHTc6 48h TD2 D18

FIG. 5E

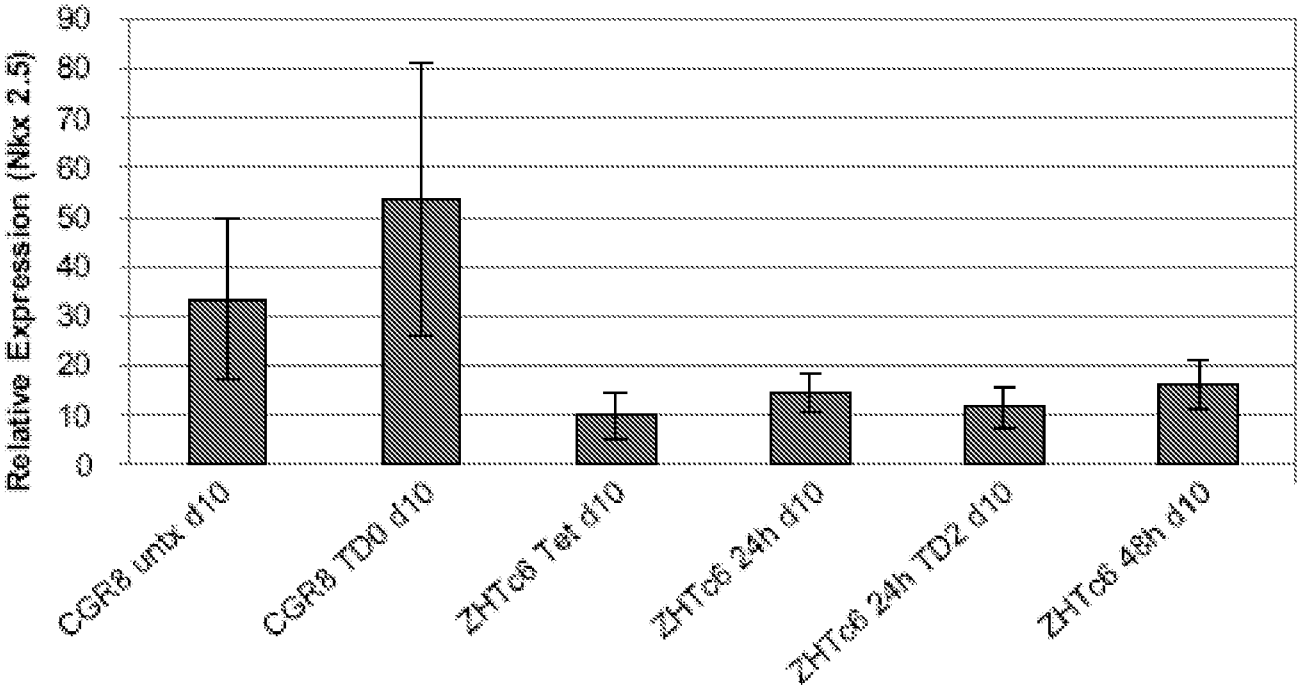

Relative Expression (Nkx 2.5)
0
10
20
30
40
50
60
70
80
90
CGR8 unfx d10
CGR8 TD0 d10
ZHTc6 Tet d10
ZHTc6 24h d10
ZHTc6 24h TD2 d10
ZHTc6 48h d10

FIG. 5F

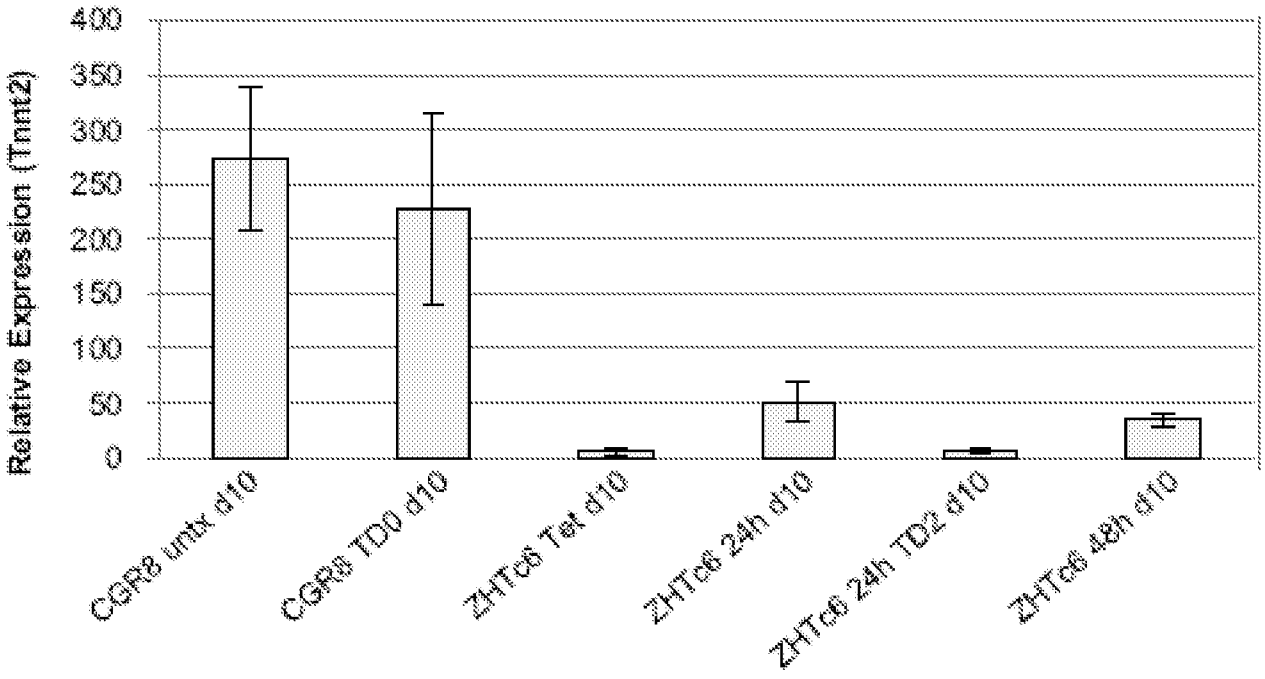

Relative Expression (Tnnt2)
400
350
300
250
200
150
100
50
0
CGR8 untx d10
CGR8 TD0 d10
ZHTc6 Tet d10
ZHTc6 24h d10
ZHTc6 24h TD2 d10
ZHTc6 48h d10

FIG. 5G

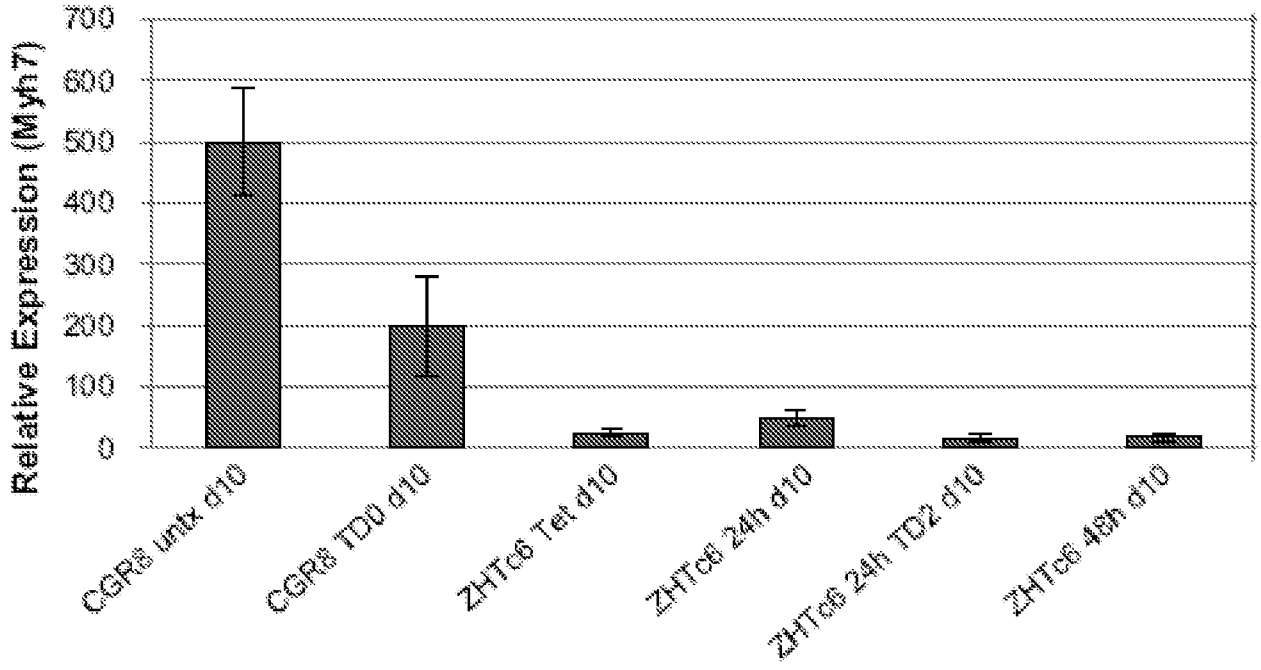

Relative Expression (Myh7)
700
600
500
400
300
200
100
0
CGR8 untx d10
CGR8 TD0 d10
ZHTc6 Tet d10
ZHTc6 24h d10
ZHTc6 24h TD2 d10
ZHTc6 48h d10

FIG. 6

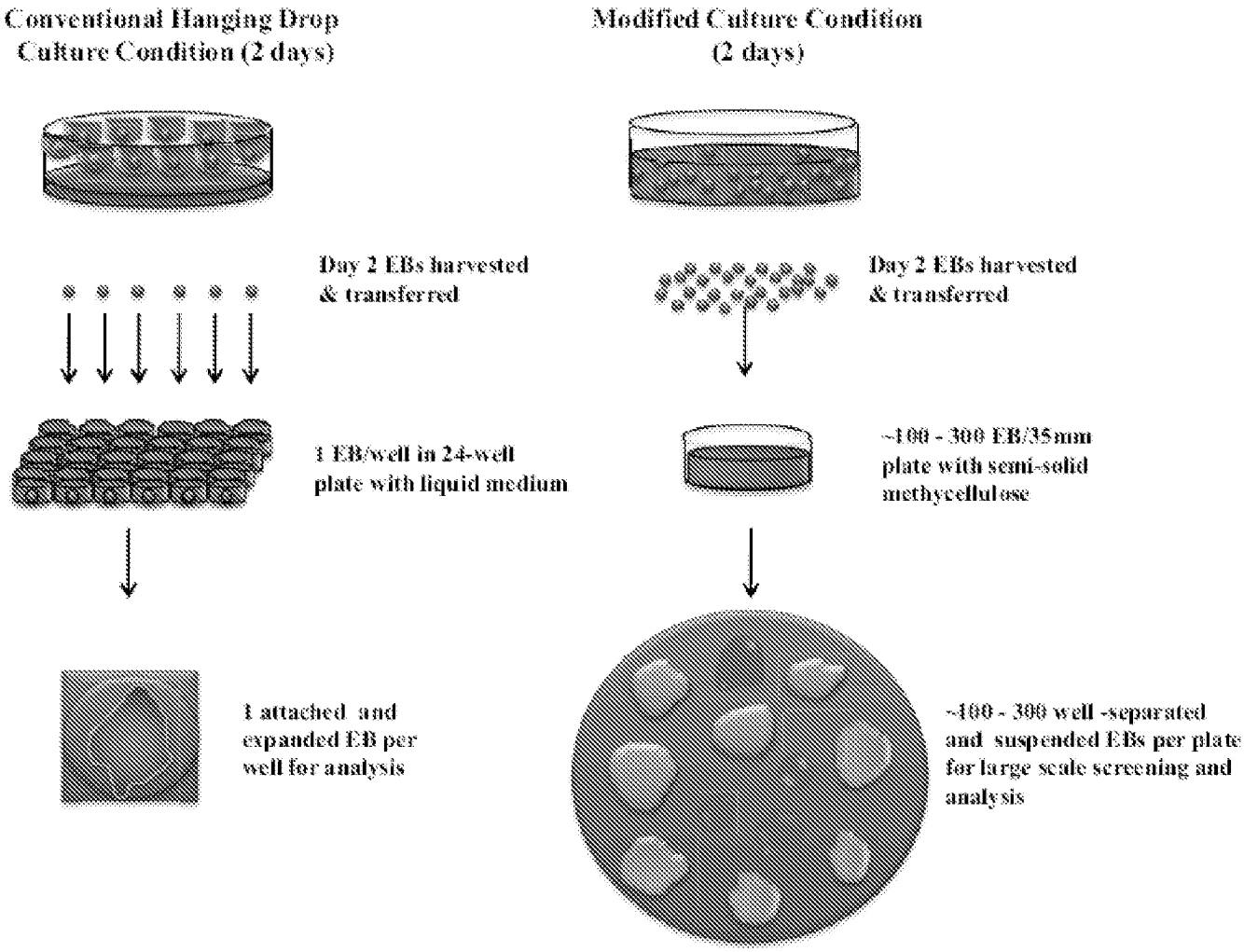

Conventional Hanging Drop Culture Condition (2 days)
Modified Culture Condition (2 days)
Day 2 EBs harvested & transferred
Day 2 EBs harvested & transferred
1 EB/well in 24-well plate with liquid medium
~100 - 300 EB/35mm plate with semi-solid methycellulose
1 attached and expanded EB per well for analysis
~100 - 300 well -separated and suspended EBs per plate for large scale screening and analysis

FIG. 7

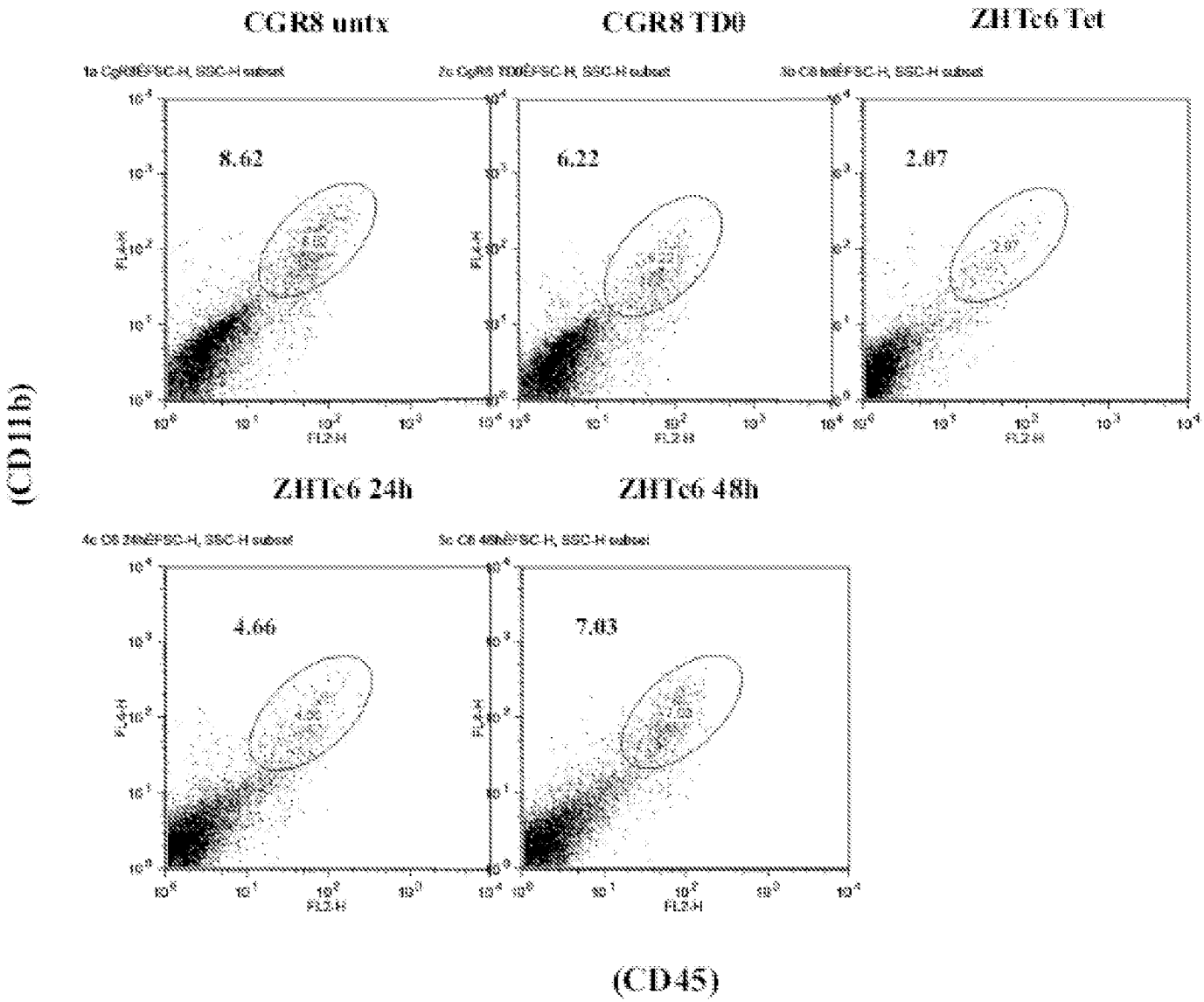

CGR8 untx
CGR8 TD0
ZHTc6 Tet
8.62
6.22
2.07
ZHTc6 24h
ZHTc6 48h
4.66
7.03
(CD11b)
(CD45)

FIG. 8

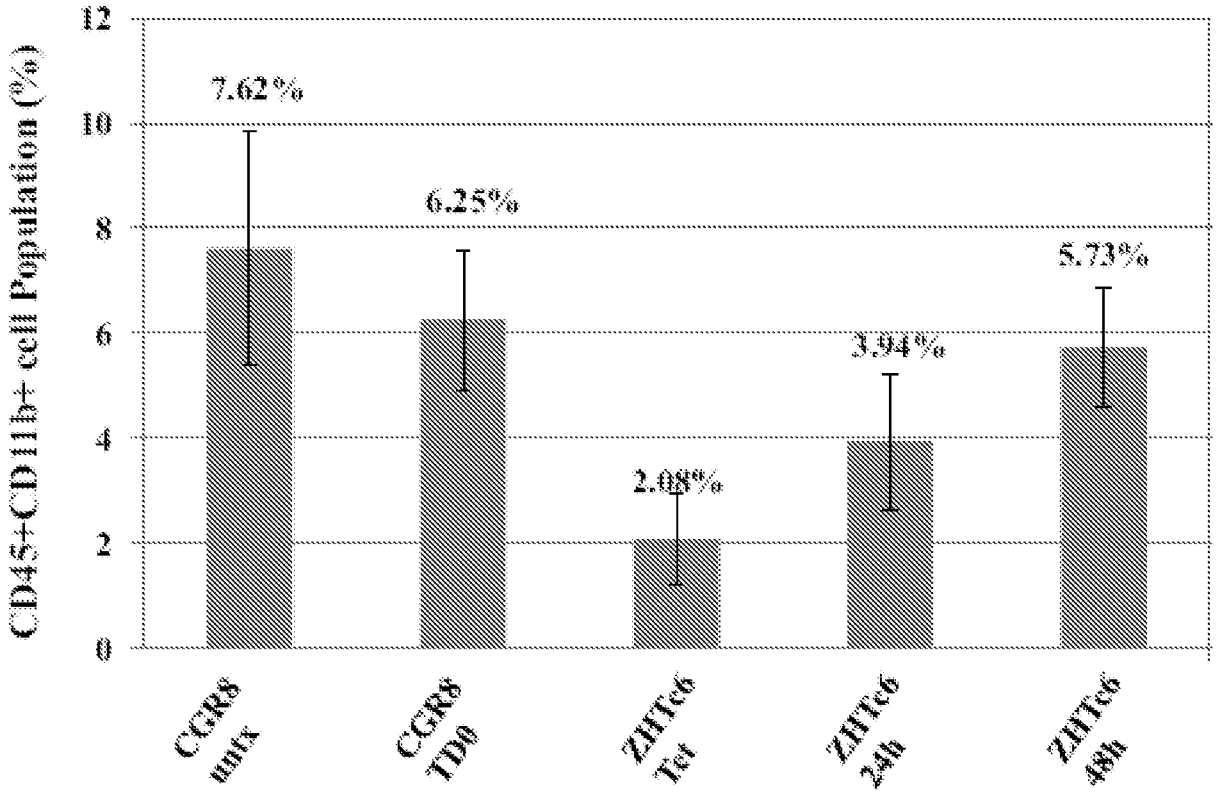

CD45+CD11b+ cell Population (%)
12
10
8
6
4
2
0
7.62%
6.25%
2.08%
3.94%
5.73%
CGR8 mix
CGR8 TD0
ZHTc6 Tet
ZHTc6 24h
ZHTc6 48h

FIG. 9

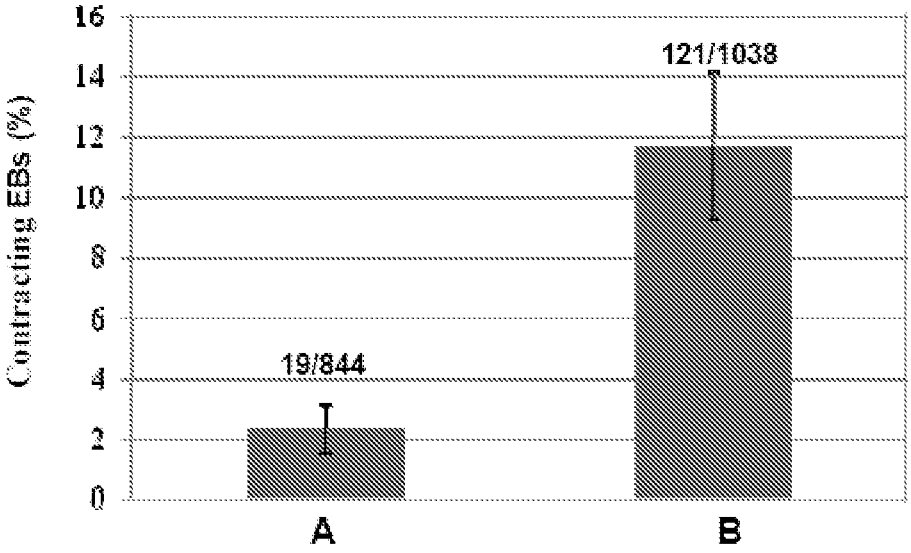
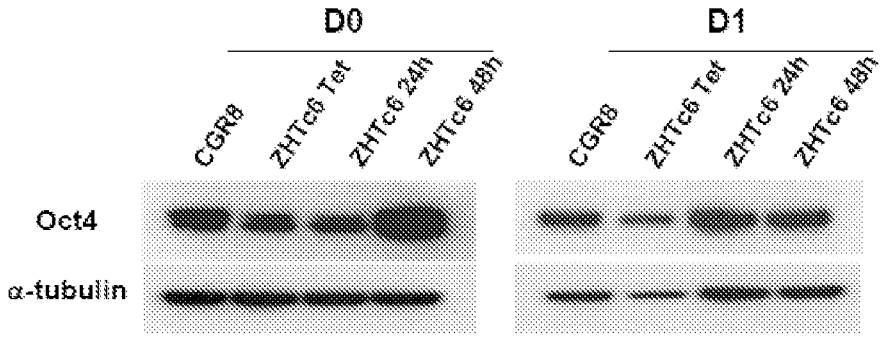
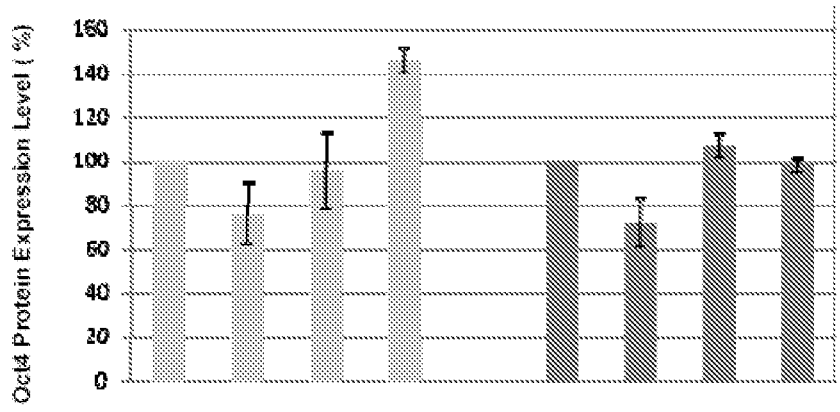


FIG. 10

A



B



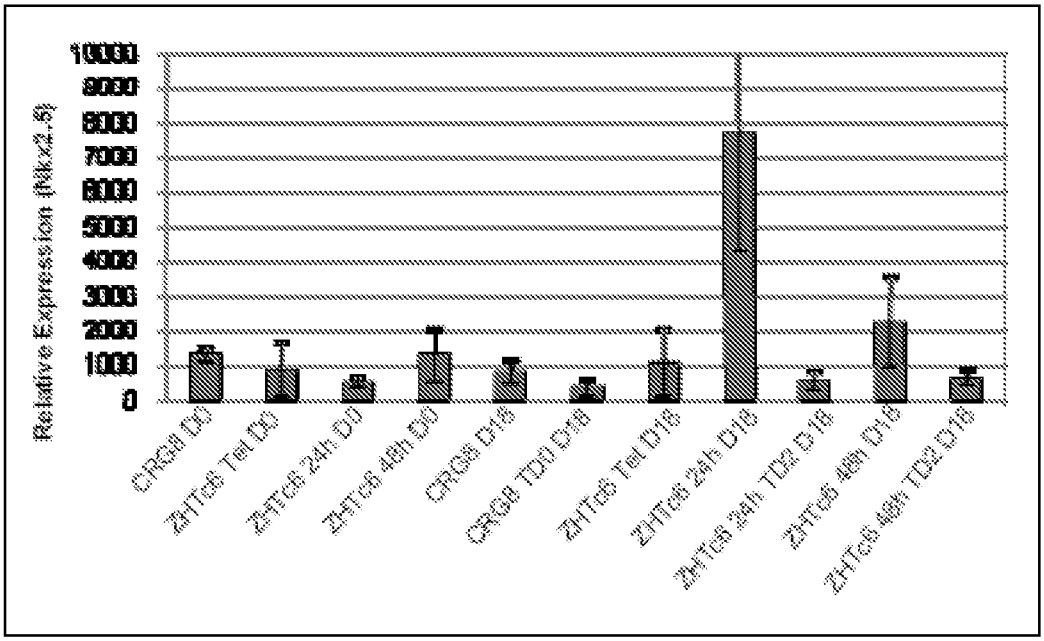
C

Oct4 protein expression level in CGR8 and ZHTc6 ESCs

ESC	Oct4 protein expression level (D0)	Oct4 protein expression level (D1)
CGR8	100% (biallelic)	100% (biallelic)
ZHTc6 tet	75.6% (monoallelic)	71.5% (monoallelic)
ZHTc6 24h	95.3% (biallelic)	106.4% (biallelic)
ZHTc6 48h	146.7% (biallelic)	97.5% (biallelic)

FIG. 11A-B

A


Relative Expression (Nkx2.5)
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
CRG8 D0
ZHTc6 Tet D0
ZHTc6 24h D0
ZHTc6 48h D0
CRG8 D16
CRG8 TD0 D16
ZHTc6 Tet D16
ZHTc6 24h D16
ZHTc6 24h TD2 D16
ZHTc6 48h D16
ZHTc6 48h TD2 D16

B

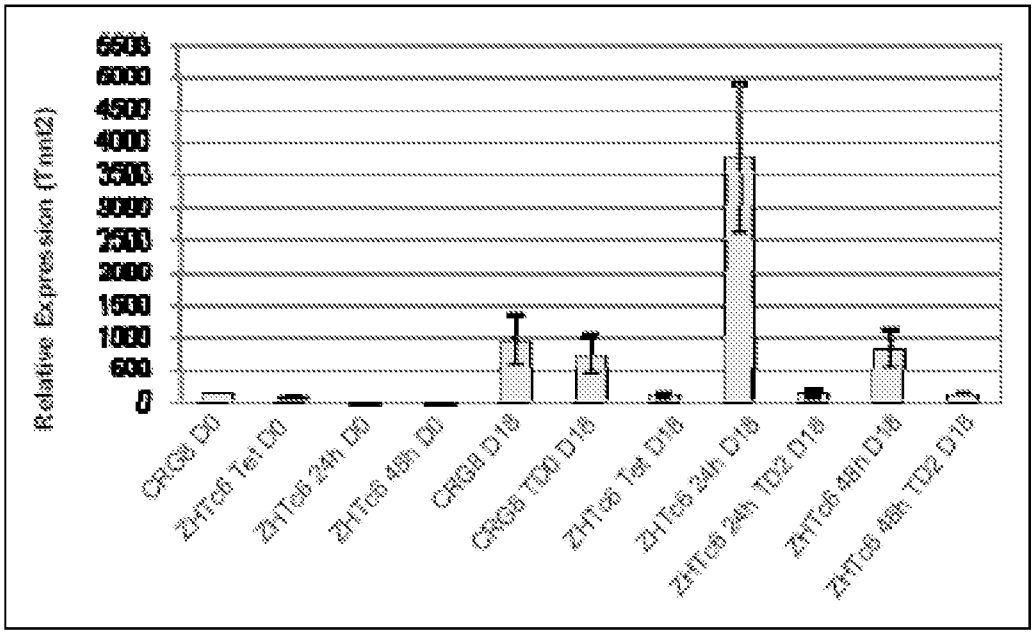
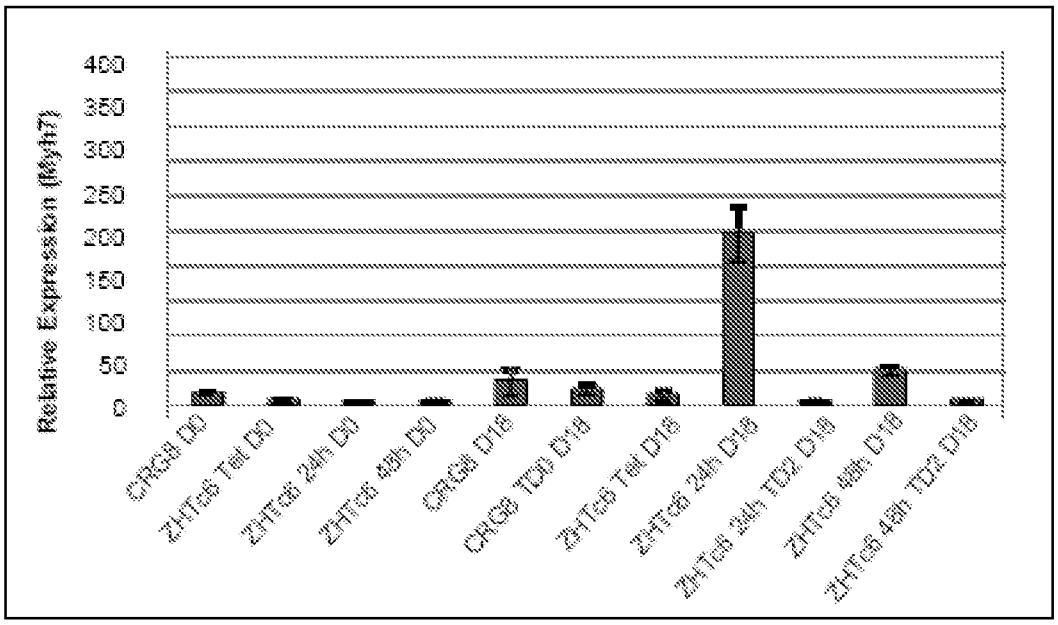

Relative Expression (Tnnt2)
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
CRG8 D0
ZHTc6 Tet D0
ZHTc6 24h D0
ZHTc6 48h D0
CRG8 D16
CRG8 TD0 D16
ZHTc6 Tet D16
ZHTc6 24h D16
ZHTc6 24h TD2 D16
ZHTc6 48h D16
ZHTc6 48h TD2 D16

FIG. 11C-D

C


Relative Expression (Myh7)
400
350
300
250
200
150
100
50
0
CRG8 D0
Z4Tc6 Tet D0
Z4Tc6 24h D0
Z4Tc6 48h D0
CRG8 D16
CRG8 TD0 D16
Z4Tc6 Tet D16
Z4Tc6 24h D16
Z4Tc6 24h TD2 D16
Z4Tc6 48h D16
Z4Tc6 48h TD2 D16

D

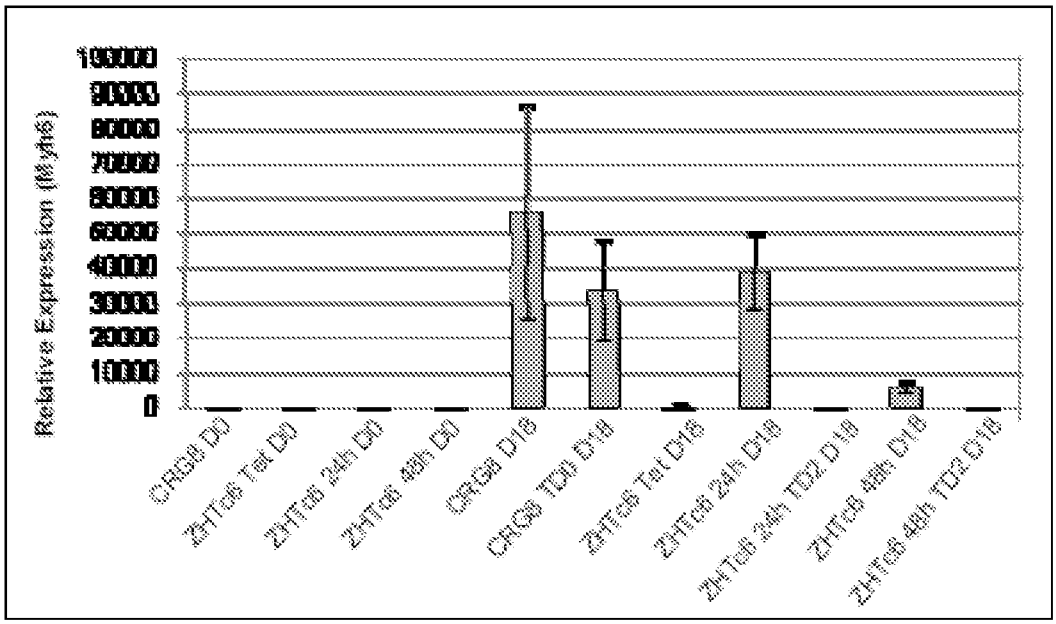
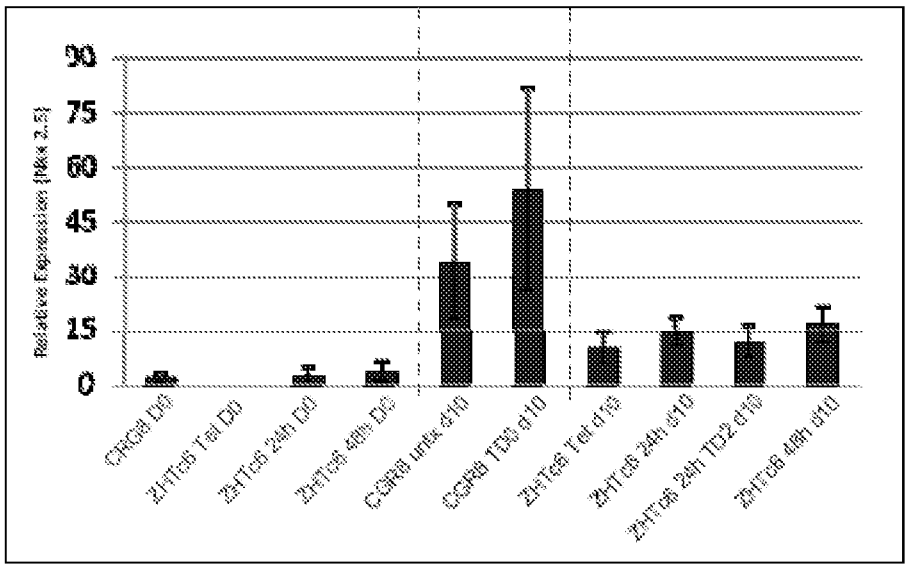

Relative Expression (Myh6)
CRG8 D0
Z4Tc6 Tet D0
Z4Tc6 24h D0
Z4Tc6 48h D0
CRG8 D16
CRG8 TD0 D16
Z4Tc6 Tet D16
Z4Tc6 24h D16
Z4Tc6 24h TD2 D16
Z4Tc6 48h D16
Z4Tc6 48h TD2 D16

FIG. 11E-F

E


90
75
60
45
30
15
0
Relative Expression (Nkx 2.5)
CRCB4 D0
ZhTc6 Tet D0
ZhTc6 24h D0
ZhTc6 48h D0
CGR8 untx d10
CGR8 TD0 d10
ZhTc6 Tet d10
ZhTc6 24h d10
ZhTc6 24h TD2 d10
ZhTc6 48h d10

F

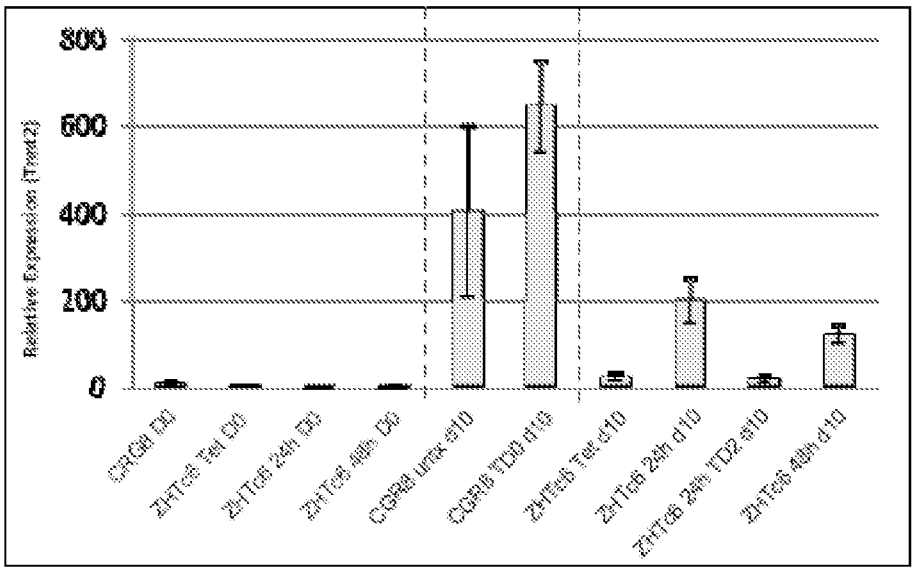

800
600
400
200
0
Relative Expression (Tnnt2)
CRCB4 D0
ZhTc6 Tet D0
ZhTc6 24h D0
ZhTc6 48h D0
CGR8 untx d10
CGR8 TD0 d10
ZhTc6 Tet d10
ZhTc6 24h d10
ZhTc6 24h TD2 d10
ZhTc6 48h d10

FIG. 11G

G

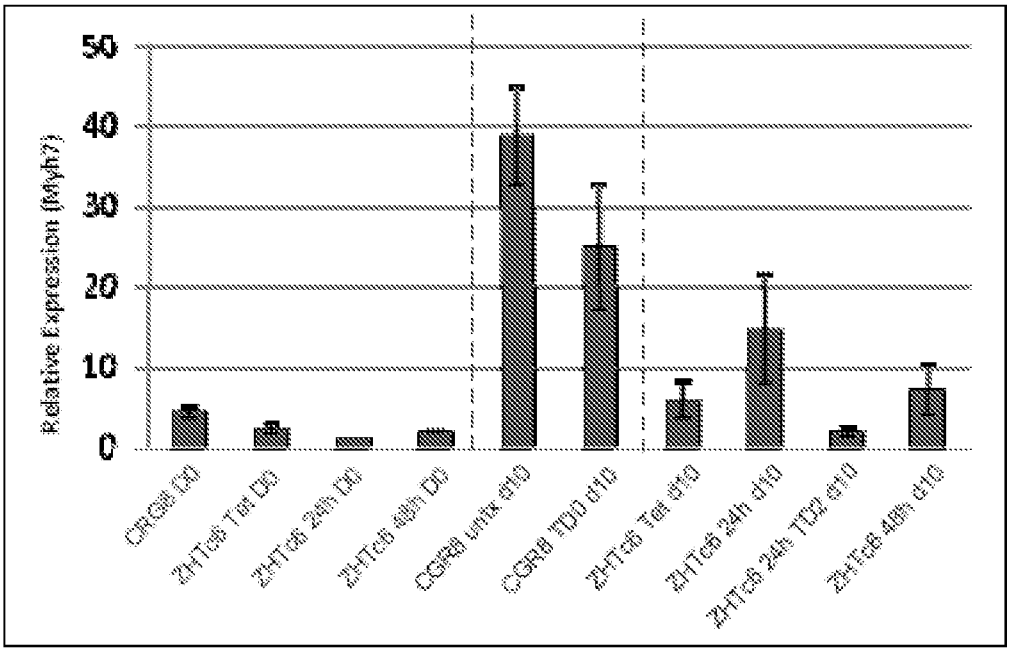

Relative Expression (Myh7)
50
40
30
20
10
0
CR-CSR (D0)
ZHTc6 Tet D0
ZHTc6 24h D0
ZHTc6 48h D0
CSR8 unTx d10
CSR8 TD0 d10
ZHTc6 Tet d10
ZHTc6 24h d10
ZHTc6 24h TD2 d10
ZHTc6 48h d10

FIG. 12

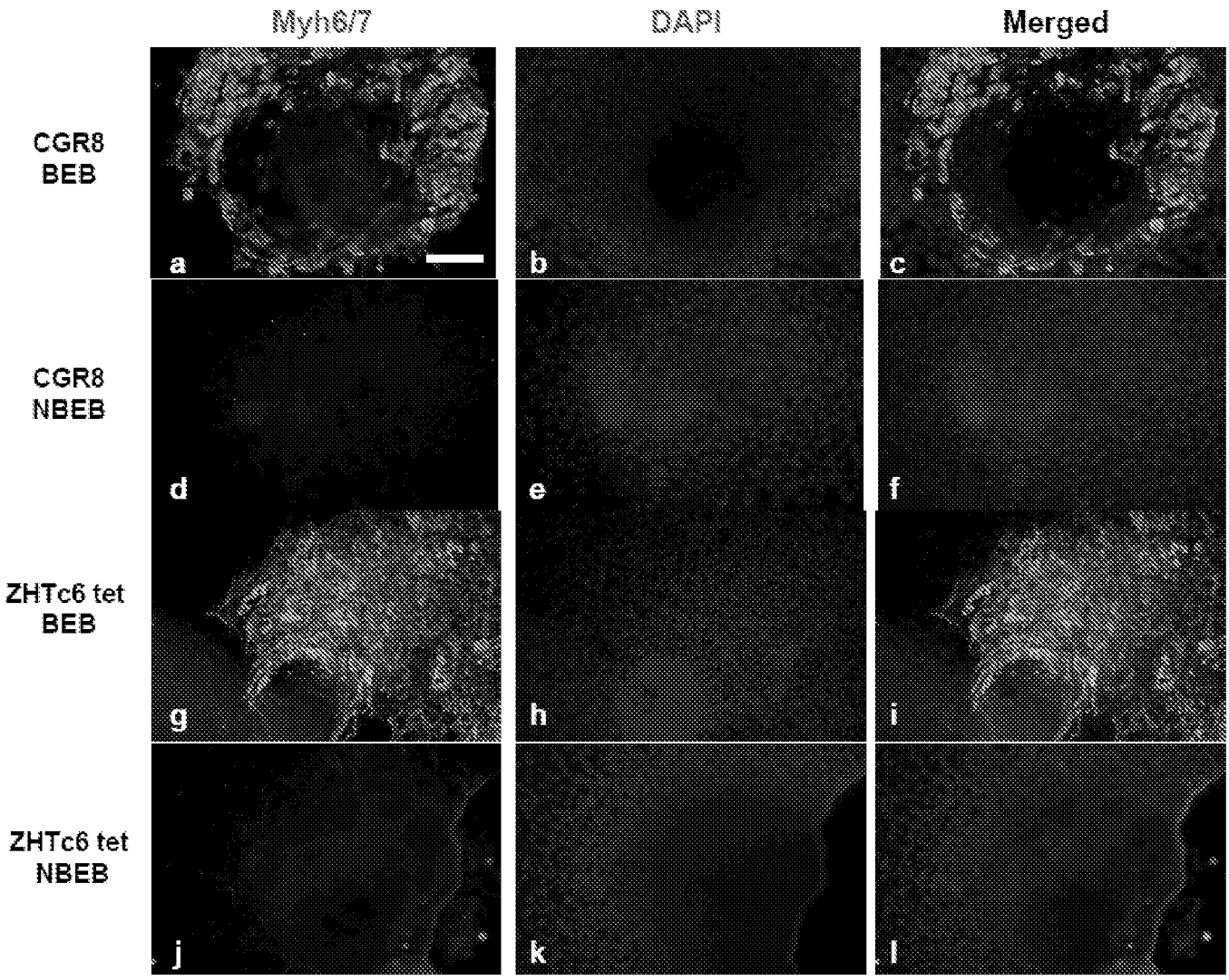

Myh6/7
DAPI
Merged
CGR8 BEB
CGR8 NBEB
ZHTc6 tet BEB
ZHTc6 tet NBEB
a
b
c
d
e
f
g
h
i
j
k
l

FIG. 13

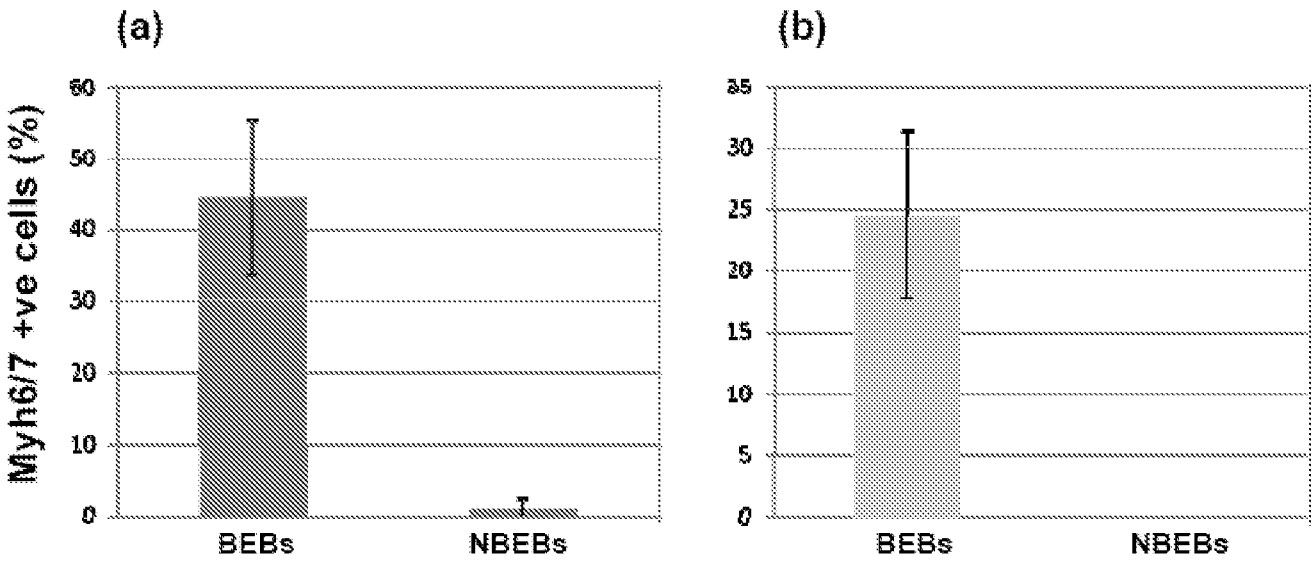

(a)
Myh6/7 +ve cells (%)
60
50
40
30
20
10
0
BEBs
NBEBs
(b)
35
30
25
20
15
10
5
0
BEBs
NBEBs

FIG. 14A-B

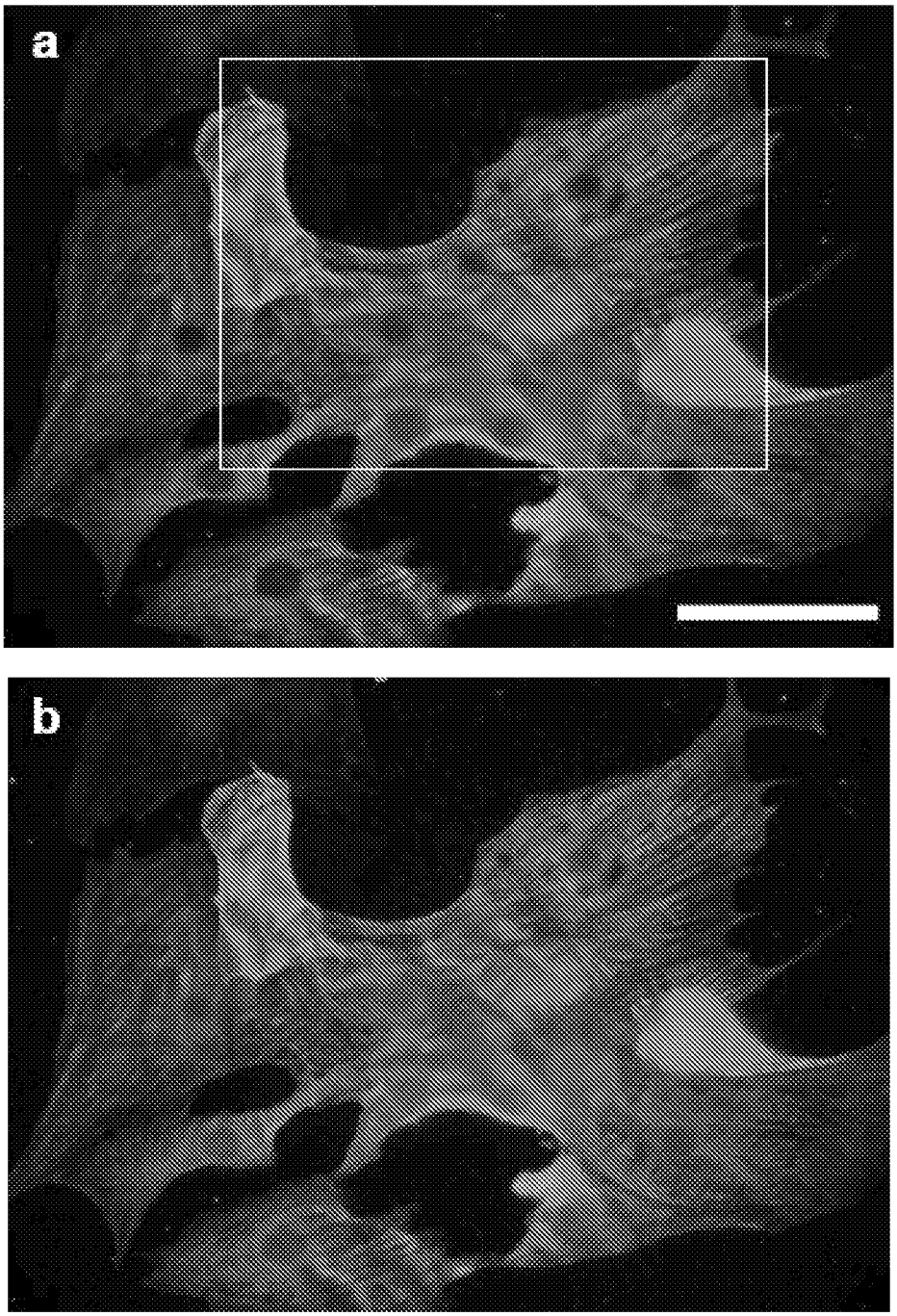


FIG. 14C

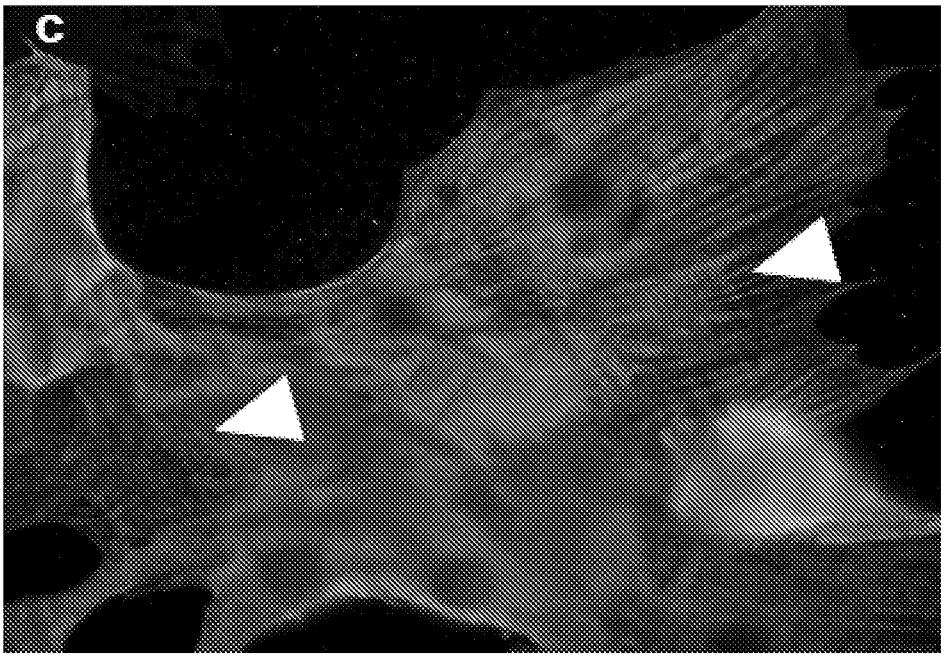


FIG. 15

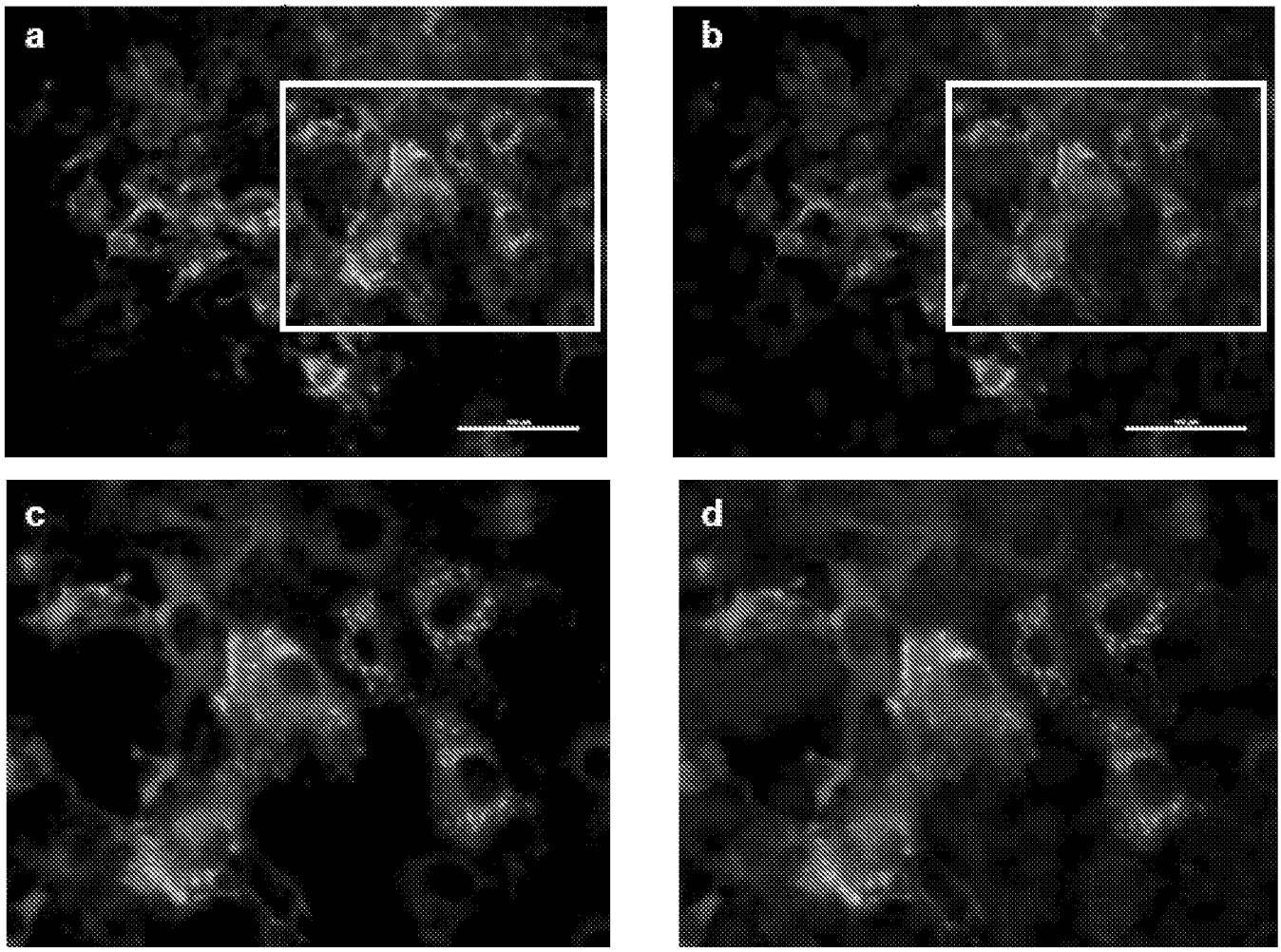


FIG. 16

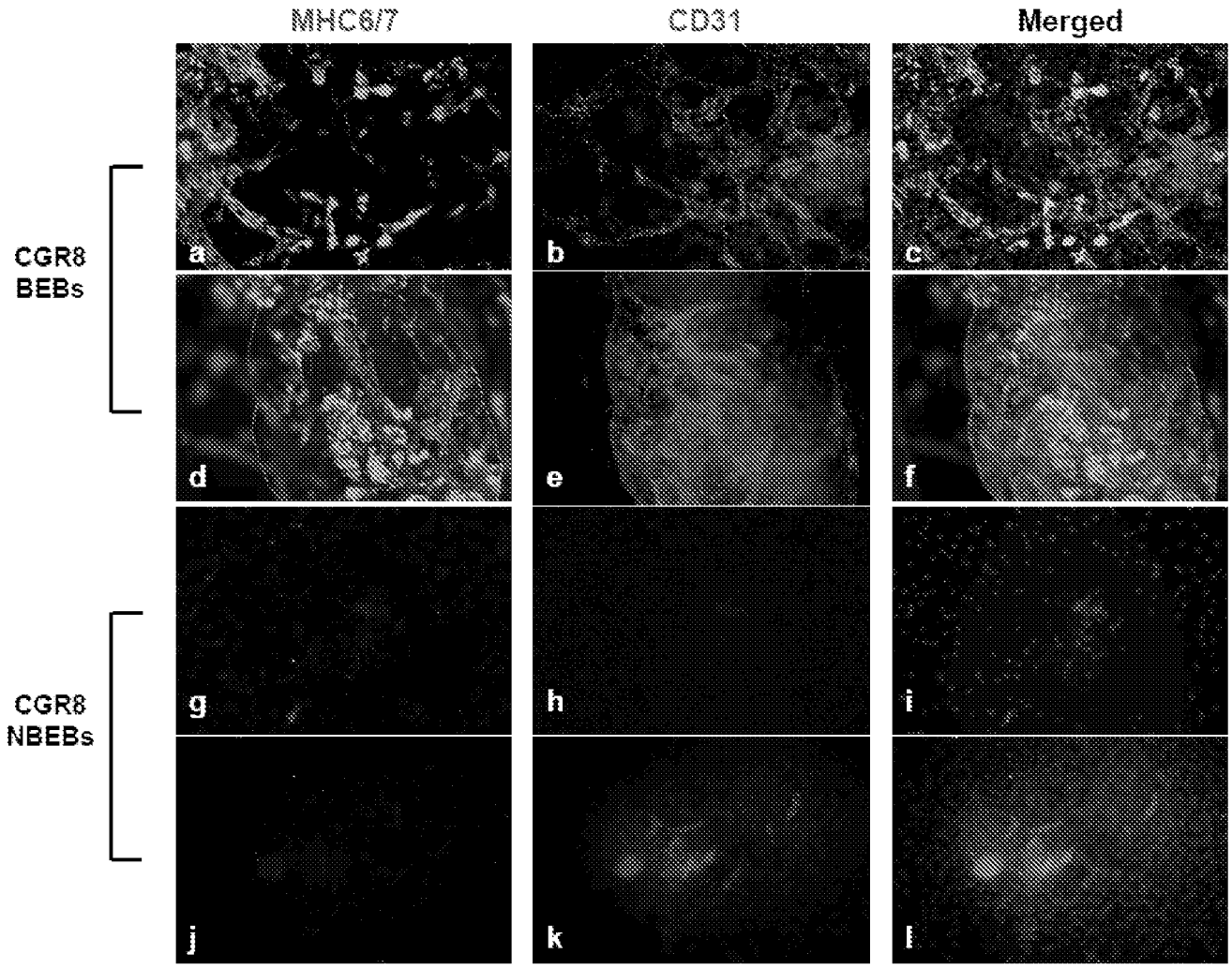
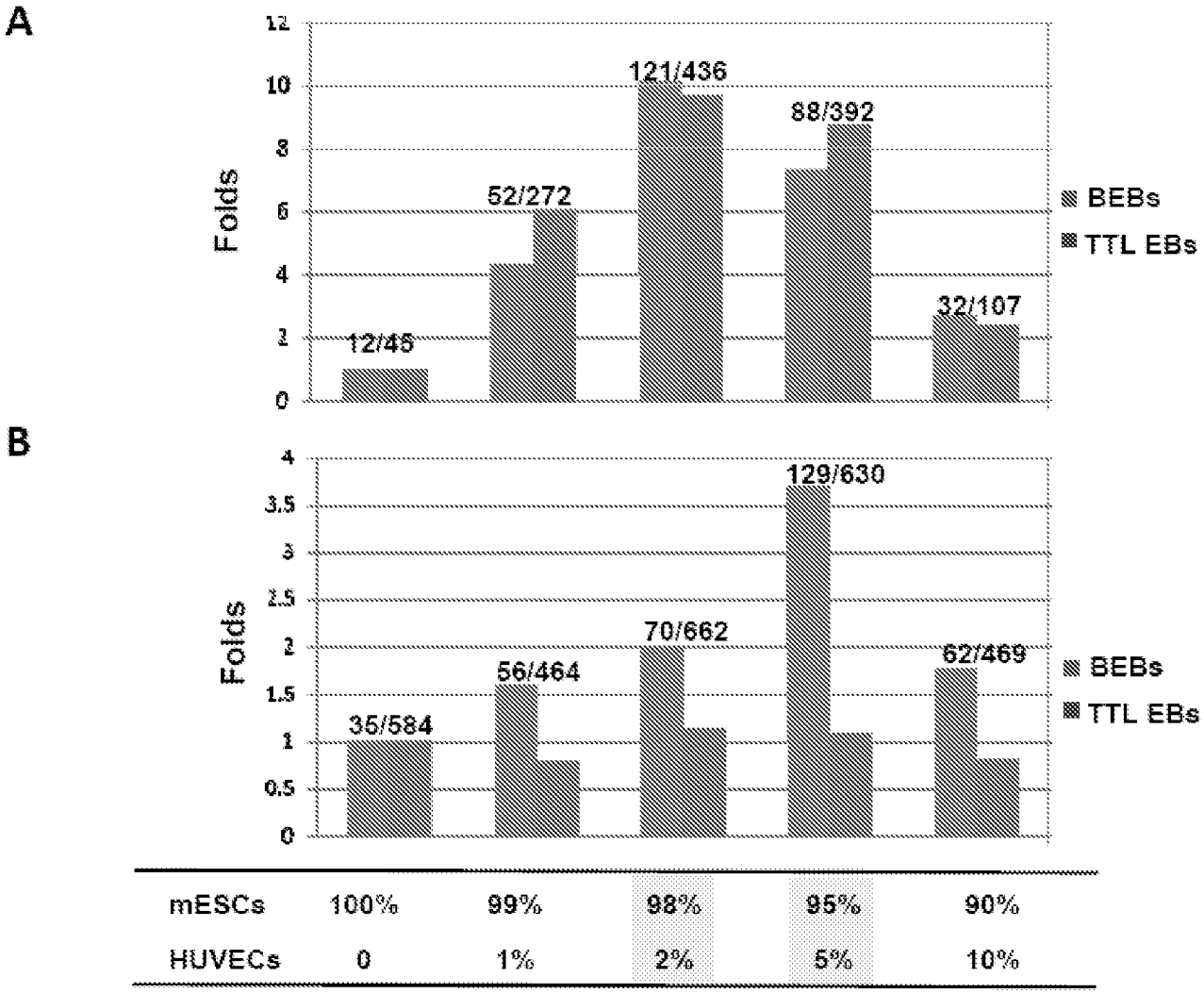


FIG. 17



mESCs	100%	99%	98%	95%	90%
HUVECs	0	1%	2%	5%	10%