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Expression of ErbB receptors in ES cell-derived cardiomyocytes☆

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Abstract

To explore the role of ErbB-mediated signaling in cardiogenesis of ES cells, we examined the expression of ErbB receptors as well as effects of a ligand and inhibitors using Nkx2.5GFP ES cells, in which the GFP gene was knocked-in to the Nkx2.5 locus to monitor cardiac differentiation. Although all ErbB receptors were expressed in developing embryoid bodies, expression of ErbB4 was almost exclusively found in differentiated cardiomyocytes. Heregulin β1, a ligand of ErbB receptors, enhanced the generation of Nkx2.5/GFP(+) cardiomyocytes in embryoid bodies, while AG1478 and PD153035, inhibitors of ErbBs, drastically blocked the generation of Nkx2.5/GFP(+) cardiomyocytes. These results suggest that the signaling pathway mediated by ErbBs is important in the induction and differentiation of cardiomyocytes from ES cells.

Keywords: ES cell; Cardiomyocyte; ErbB; Neuregulin; Apoptosis

Neuregulin (NRG)-ErbB signaling has been shown to play an important role in heart development [1]. Among four closely related ErbB receptors, ErbB1 (epidermal growth factor receptor, EGF receptor), ErbB2, ErbB3, and ErbB4, the latter three ErbB receptors and one of their ligands, neuregulin 1 (NRG1), which is also known as heregulin (HRG), have been shown to play an essential role in heart development, especially in inducing trabeculation and valvuloseptal formation [2–5]. The effects of NRG-ErbB signaling have also been reported to involve the regulation of proliferation and survival of cardiomyocytes in vitro [6,7]. It is, however, still unclear whether NRG-ErbB signaling involves induction and differentiation of cardiomyocytes, in part, because no ideal system for in vitro differentiation has been established in cardiomyocytes.

Embryonic stem (ES) cells, which can differentiate into derivatives of all three primary germ layers, including cardiomyocytes, are considered one of the most promising sources of cells for transplantation therapy as well as a model system for elucidating mechanisms of cell differentiation in vitro [8–11]. Differentiation of ES cells is induced by formation of embryo-like structures called embryoid bodies (EBs). Following induction of mesodermal cells, a portion of cells develops into cardiomyocytes when ES cells are differentiated in the standard serum containing fetal calf serum. However, no growth factors have been specifically identified to induce cardiac differentiation or expand cardiomyocytes derived from ES cells, although BMP- and Wnt-mediated signaling pathways have been shown to play an important role in cardiogenesis in multipotent embryonal carcinoma cells [12–14].

Recently, we established the Nkx2.5GFP ES cell line, in which the GFP gene is knocked-in to the Nkx2.5 locus [15]. Nkx2.5, a homeobox-containing cardiac transcription factor, is expressed in cardiomyocytes throughout the course of heart development and plays a pivotal role in heart morphogenesis [16–19]. Nkx2.5/GFP(+) cells derived from the Nkx2.5GFP ES cell line provide a powerful tool to elucidate the mechanisms that control cardiac differentiation and proliferation.

☆ *Abbreviations:* ES cell, embryonic stem cell; EB, embryoid body; GFP, green fluorescent protein; NRG, neuregulin; HRG, heregulin; MHC, myosin heavy chain.

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Here, we have investigated the role of NRG-ErbB-mediated signaling in ES cell cardiac differentiation, taking advantage of the GFP marker on ES cell-derived cardiomyocytes to track the generation of cardiomyocytes in developing EBs.

Materials and methods

ES cell culture, differentiation, and ES cell-derived cardiomyocytes. Nkx2.5-GFP knock-in ES cells (Nkx2.5GFP ES cells) were maintained and differentiated as previously described [15]. To detect cardiac differentiation by flow cytometry, floating EBs were dissociated and analyzed by FACS Calibur (BD Biosciences, San Jose, CA). ES cell-derived cardiomyocytes were isolated from differentiated Nkx2.5GFP ES cells by collecting GFP(+) cells by FACS Vantage SE (BD Biosciences) at day 8.

RT-PCR. Total RNA was extracted from ES cell-derived cells using Trizol reagent (Invitrogen, Carlsbad, CA) and was reverse-transcribed to cDNA with SuperScript II (Invitrogen). The primers were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CTT CAT TGA CCT CAA CTA CAT G-3′ and 5′-AAA GTT GTC ATG GAT GAC CTT G-3′; ErbB1 [20], 5′-GAA CAA CAC TCT GGT CTG GAA G-3′ and 5′-ATG TTG TCC TTG TGT TCT CGG-3′; ErbB2 [21], 5′-GGT GGT GAG CTG ACA CTG G-3′ and 5′-CAC CAT CAA ACA CAT CGG AG-3′; ErbB3 [21], 5′-CAT CAG AGG GCC ATG TGA C-3′ and 5′-TTC CTC TAA CCC TGG TGG G-3′, and ErbB4 [22], 5′-GAA ATG TCC AGA TGG CCT ACA GGG-3′ and 5′-CTT TTT GAT GCT CTT TCT TCT GAC-3′.

Immunocytochemistry. Cells were fixed in 2.5 mM ethylene glycol succinate. After treatment with a blocking solution containing 0.1% Triton X-100 and 2% skimmed milk, cells were incubated with mouse anti-myosin heavy chain (MHC) monoclonal antibody (F109.3E1, Alexis, San Diego, CA) and rabbit anti-ErbB4 polyclonal antiserum (SC-283, Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibodies were visualized with anti-mouse IgG-Alexa488 or anti-rabbit IgG-Alexa546. Fluorescence signals were observed under a fluorescence microscope (IX70, Olympus Optical, Shinjuku-ku, Tokyo, Japan) and the image data were acquired with a SPOT CCD camera (Diagnostic Instruments, Sterling Heights, MI).

Effect of growth factors and ErbB receptor inhibitors on cardiac differentiation of embryoid bodies. Embryoid bodies were cultured with the growth factors, HRG-β1, EGF, hepatocyte growth factor (HGF) or keratinocyte growth factor (KGF) (Research & Diagnostic Systems, Minneapolis, MN), from day 4 to day 8. To determine the influence of ErbB receptor inhibitors, AG1478 or PD153035 (Calbiochem–Novabiochem, San Diego, CA) was administered to 3-, 4- or 6-day-old EBs. Generation of cardiomyocytes in EBs was detected by flow cytometry or by counting the number of beating EBs. To examine the generation of the mesodermal cell lineage, EBs were dissociated at day 5 and stained with R-phycoerythrin (PE)-conjugated anti-Flk1 antibody (BD Biosciences Pharmingen, San Diego, CA) and analyzed by flow cytometry.

Proliferation assay of isolated ES cell-derived cardiomyocytes. Nkx2.5/GFP(+) cells purified from 8-day-old EBs were attached to a gelatin-coated dish in the presence of serum. Medium was then replaced with serum-free medium and further cultured for 2 days with or without HRG-β1. Bromodeoxyuridine (BrdU) was added to the culture medium 24 h prior to cell harvest. Labeled cells were detected with a BrdUs taining kit (Zymed Laboratories, South San Francisco, CA).

Determination of apoptotic cells. Embryoid bodies were treated with 10 μM of the ErbB receptor inhibitors, AG1478 or PD53054, for 12 and 24 h. For analysis by flow cytometry, EB-derived single cells were fixed in 2% paraformaldehyde, followed by cell permeabilization with 0.1% Triton X-100 and 0.1% sodium citrate, pH 7.4. After washing in PBS, cells were incubated in TUNEL reaction mixture (In Situ Cell Death Detection Kit, Roche Diagnostics, Basel, Switzerland). For analysis by fluorescence microscopy, EBs were cryosectioned at 5–7 μm. The sections were incubated with the TUNEL reaction mixture and anti-MHC antibody.

Results

Expression of ErbB receptors in developing EBs

Under our in vitro differentiation conditions, the transcripts of cardiac transcription factor genes, such as Nkx2.5 and Mef2C, were detected and upregulated at day 5 and day 6, followed by the induction of contractile protein genes, such as MHCβ and α and myosin light chain 2a and 2v, at day 6 and day 7 [15]. ErbB3 transcripts were detected in undifferentiated ES cells as well as in developing EBs, while ErbB1, ErbB2, and ErbB4 transcripts were gradually increased during EB development (Fig. 1A). Of note, ErbB4 transcripts were upregulated around day 6 and day 7, coincident with the induction of the contractile protein genes. Taking advantage of Nkx2.5 GFP ES cells, we sorted EB-derived cells to separate cardiomyocyte- and non-cardiomyocyte-containing fractions [15]. ErbB4 was preferentially expressed in the GFP(+) fraction, while ErbB1 was expressed in the GFP(−) fraction (Fig. 1B). ErbB2 was abundantly expressed in both GFP(+) and GFP(−) fractions. We also performed immunostaining using a polyclonal antibody against ErbB4. Although the antibody stained nuclei non-specifically, we observed typical patterns of cell membrane staining in isolated GFP(+) cells as well as in MHC(+) cells in the unsorted fraction (Fig. 1C).

Effects of growth factors on generation and proliferation of ES cell-derived cardiac cells

We next examined the effects of ErbB receptor ligands and other growth factors on developing EBs. As shown in Fig. 2A, the application of HRG-β1 resulted in a significant increase in the percentage of GFP(+) cells. In contrast, treatment with EGF (100 ng/ml), KGF (50 ng/ml), or HGF (50 ng/ml) did not significantly increase the percentage of GFP(+) cells (data not shown). To further determine whether HRG-β1 affects the proliferation of GFP(+) cells, we applied HRG-β1 to GFP(+) cells purified from EBs (Fig. 2B). When cultured in the absence of serum for 3 days following isolation from EBs, approximately 25% of the cells incorporated BrdU. The BrdU-positive cells were not increased significantly by treatment of HRG-β1. These results indicate that the expansion of GFP(+) cells in EBs by HRG-β1 may not be due to enhanced proliferation of ES cell-derived cardiomyocytes.

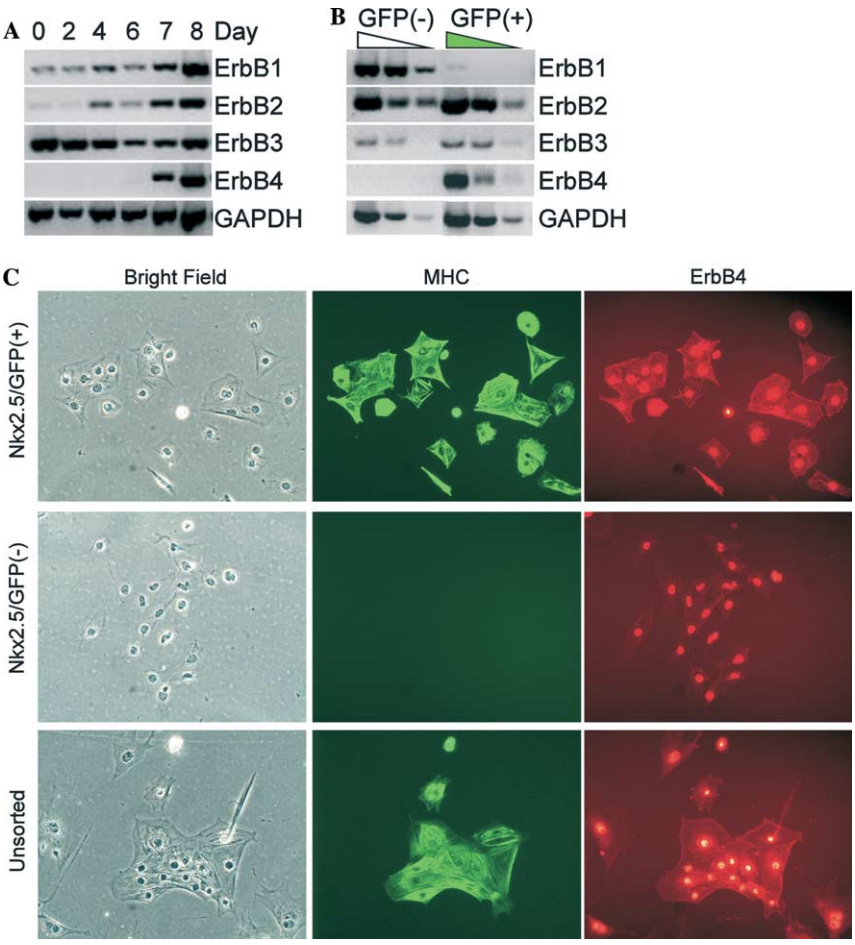

Fig. 1. Expression of ErbB receptors in developing embryoid bodies (EBs) and ES cell-derived cardiomyocytes. (A) Developmental expression of ErbB genes in EBs. Total RNA was extracted from undifferentiated ES cells (d0) and developing EBs at days 2, 4, 6, 7, and 8. (B) Preferential expression of ErbB4 in the Nkx2.5/GFP(+) cell fraction. Nkx2.5/GFP(+) and Nkx2.5/GFP(−) cell fractions were obtained by sorting day 8-old EBs using a cell sorter. (C) Immunostaining for ErbB4 in ES cell-derived cardiomyocytes. GFP(+)- or GFP(−)-sorted cells, or unsorted cells derived from Nkx2.5GFP EBs were stained with an anti-ErbB4 (red) and an anti-MHC (green) antibodies. Cell membrane staining was considered positive for ErbB4.

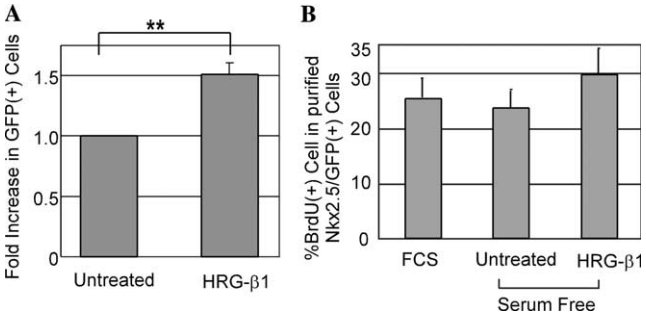

Fig. 2. Effect of growth factors on cardiac development of EBs. (A) Effect of growth factors on generation of Nkx2.5/GFP(+) cardiomyocytes in EBs. Embryoid bodies were cultured with HRG-β1 at 100 ng/ml for 4 days (from day 4 to day 8). Nkx2.5/GFP(+) cells were analyzed by flow cytometry at day 8. Data are expressed as the fold increase in GFP(+) cells between treated and untreated EBs. Values are means ± SE from five different experiments (** $p < 0.05$). (B) Effect of HRG-β1 on proliferation of isolated Nkx2.5/GFP(+) cardiomyocytes. Nkx2.5/GFP(+) cells sorted from 8-day-old EBs were cultured for 2 days in the absence of serum, with or without HRG-β1 at 100 ng/ml. Cells were labeled with BrdU for 24 h prior to harvesting. Data are expressed as means ± SE from five different experiments.

Influence of ErbB receptor inhibitors on development of ES cell-derived cardiomyocytes

To determine whether inhibition of ErbB receptor-mediated signaling affects development of cardiomyocytes, we measured the appearance of beating EBs in cultures treated with ErbB inhibitors, AG1478 or PD153035 [23], from day 3 to day 8. More than 90% of EBs contracted spontaneously and 5% of total EB-derived cells were cardiomyocytes at day 8 in the normal condition [15]. In contrast, a drastic reduction in the percentage of beating EBs (Fig. 3A) as well as in percentage of GFP(+) cardiomyocytes (Fig. 3B) was observed following treatment with AG1478 or PD153035. On the other hand, the percentage of Flk1(+) cells was not affected by treatment with AG1478 (Fig. 3C), indicating that the inhibitor did not block the generation of the mesodermal cell lineage.

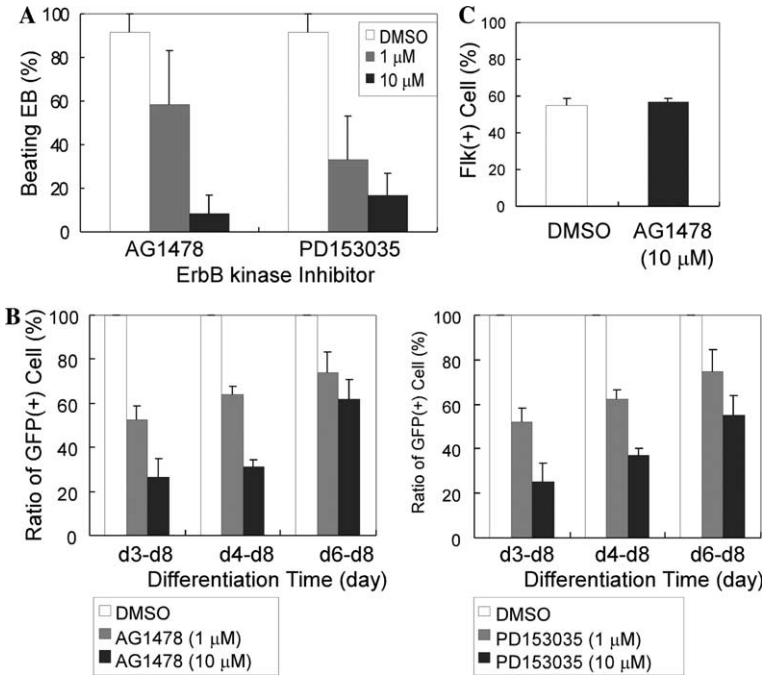

Fig. 3. Inhibition of cardiac development of EBs by ErbB inhibitors. (A) Influence of ErbB inhibitors on appearance of spontaneous beating EBs. Embryoid bodies were cultured in a suspension until day 6 and then attached in a 24-well gelatin-coated plate. The number of spontaneous beating EBs was counted at day 8. ErbB inhibitors were administered from day 4 to day 8. (B) Influence of ErbB inhibitors on generation of Nkx2.5/GFP(+) cells. Embryoid bodies treated with the inhibitor were analyzed by flow cytometry. Data are presented as percentage ratio of GFP(+) cells compared with untreated control. Data are means ± SE from five different samples. (C) Influence of the ErbB inhibitor on generation of Flk1(+) mesodermal cells. Embryoid bodies were cultured in a suspension until day 5 and Flk1(+) cells were analyzed by flow cytometry. The inhibitor was administered from day 3 to day 5.

Influence of ErbB receptor inhibitors on survival of ES cell-derived cardiomyocytes

To explore the mechanism by which ErbB inhibitors impair the generation of cardiomyocytes, we measured apoptotic cell death induced by treatment with AG1478. Apoptotic cells were not observed in the clusters of differentiated cardiomyocytes, which had been differentiated in the untreated control condition (Fig. 4A, upper panel). In contrast, application of AG1478 to EBs resulted in induction of apoptosis in the cardiomyocytes (Fig. 4A, lower panel). Induction of TUNEL(+) cells in EBs was also measured by flow cytometry (Fig. 4B). Untreated control EBs contain about 12–14% TUNEL(+) cells, while AG1478-treated EBs contain about 36% TUNEL(+) cells. These results indicate that ErbB-mediated signaling plays an important role in cell survival of ES cell-derived cells, including cardiomyocytes.

Discussion

NRG1-ErbB signaling in induction of cardiomyocytes in EBs

We found that the expression of ErbB4 coincided with the induction of cardiac specific markers at day 6 and day 7 and that ErbB4 was preferentially expressed on the cell surface of differentiated cardiomyocytes. This implies that ErbB4 could be used as a marker of differentiated cardiomyocytes in EBs. However, the inhibitory effect of the ErbB inhibitors was observed before induction of ErbB4. Thus, putative cardiogenic progenitors, which do not yet express cardiac specific markers or ErbB4, may be under the control of an ErbB2 and/or ErbB3-mediated signaling pathway. Since the induction of Flk1(+) cells was not perturbed by treatment with the ErbB inhibitor, the ErbB-mediated pathway may play a role after mesodermal induction and before cardiac differentiation. To date, cardiogenic mesodermal progenitors, which correspond to the intermediates between mesodermal cells and committed cardiomyocytes, have not yet been identified. Thus, it is of interest to dissect the differentiation steps during cardiac induction in EBs using various inhibitors with different target spectrums.

NRG1-ErbB signaling in survival of cardiomyocytes in EBs

In contrast to its reported mitotic activity on neonatal cardiomyocytes, NRG1 had only limited effects on proliferation of isolated ES cell-derived cardiomyocytes. Thus, mitotic activity of NRG1 may not significantly contribute to expand cardiomyocytes in our system,

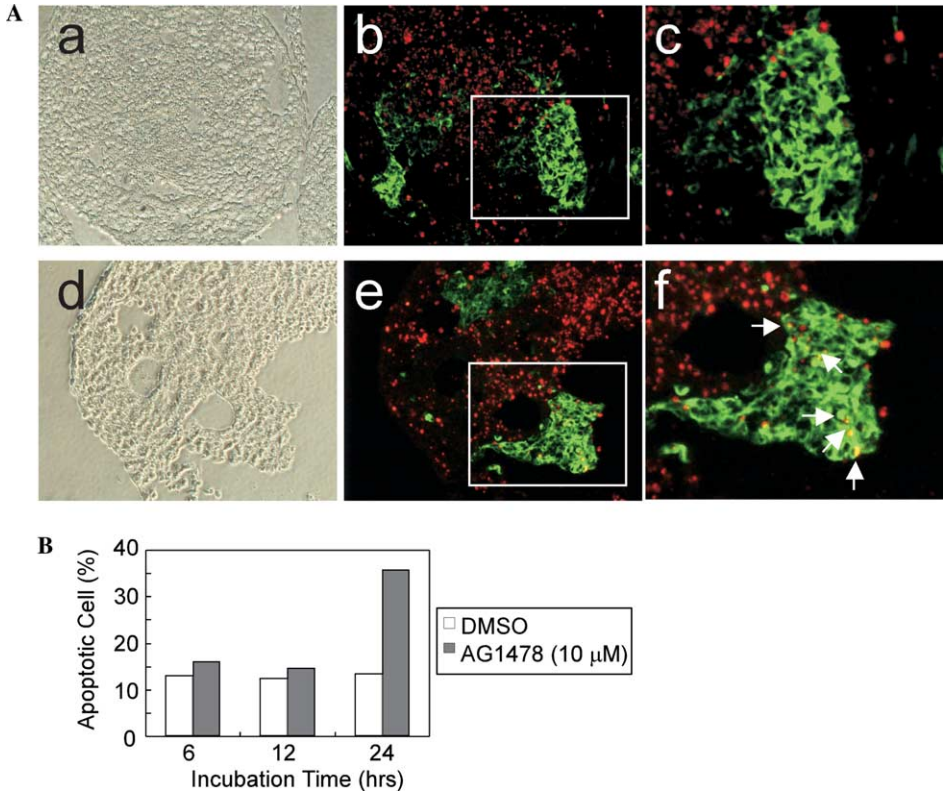

Fig. 4. Apoptosis induced by AG1478 in developing EBs. (A) In situ cell death in day 8 EBs. AG1478 was administered 27 h before the assay. Cardiac cells were visualized with an anti-MHC antibody (green color). Apoptotic cells were indicated as red color. (B) Flow cytometric analysis of apoptotic cells induced by AG1478 in EBs. Embryoid bodies were treated with AG1478 12 or 24 h before the analysis. Single cells were analyzed for TUNEL(+) cells by flow cytometry. Two independent experiments were performed.

where the purified Nkx2.5/GFP(+) cells do not proliferate a great deal, even in the presence of serum. Neuregulin-ErbB signaling may be more important for survival of cardiomyocytes in EBs. Although programmed cell death plays an important role in morphogenesis in heart development [24], differentiated cardiomyocytes usually escape from cell death in EBs (see Fig. 4A). Blocking ErbB-mediated signaling by an ErbB inhibitor results in induction of apoptosis in ES cell-derived cells, including cardiomyocytes, suggesting that ErbB-mediated signaling is important for cell survival during EB development. Since ErbB4 is preferentially expressed in differentiated cardiomyocytes, it is possible that ErbB4-NRG signaling protects ES cell-derived cardiomyocytes from cell death in developing EBs.

In summary, we have obtained the first evidence that ErbB4 is preferentially expressed in ES cell-derived cardiomyocytes at the protein level. In addition, NRG-ErbB-mediated signaling was found to be important for promoting differentiation and survival of ES cell-derived cardiomyocytes. To date, there is no proper method to induce or expand cardiomyocytes from ES cells. Our finding would provide useful information to control ES cell differentiation and to utilize ES cell-derived cardiomyocytes as a source of cells for cell transplantation therapy.

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References

[1] K.L. Carraway 3rd, Bioessays 18 (1996) 263–266.
[2] K.F. Lee, H. Simon, H. Chen, B. Bates, M.C. Hung, C. Hauser, Nature 378 (1995) 394–398.
[3] M. Gassmann, F. Casagranda, D. Orioli, H. Simon, C. Lai, R. Klein, G. Lemke, Nature 378 (1995) 390–394.
[4] S.L. Erickson, K.S. O'Shea, N. Ghaboosi, L. Loverro, G. Frantz, M. Bauer, L.H. Lu, M.W. Moore, Development 124 (1997) 4999–5011.
[5] D. Meyer, C. Birchmeier, Nature 378 (1995) 386–390.
[6] Y.Y. Zhao, D.R. Sawyer, R.R. Baliga, D.J. Opel, X. Han, M.A. Marchionni, R.A. Kelly, J. Biol. Chem. 273 (1998) 10261–10269.
[7] B.D. Ford, J.A. Loeb, G.D. Fischbach, Dev. Biol. 214 (1999) 139–150.
[8] I. Kehat, D. Kenyagin-Karsenti, M. Snir, H. Segev, M. Amit, A. Gepstein, E. Livne, O. Binah, J. Itskovitz-Eldor, L. Gepstein, J. Clin. Invest. 108 (2001) 407–414.

[9] C. Xu, S. Police, N. Rao, M.K. Carpenter, Circ. Res. 91 (2002) 501–508.
[10] J. Hescheler, B.K. Fleischmann, J. Clin. Invest. 108 (2001) 363–364.
[11] J.Y. Min, Y. Yang, K.L. Converso, L. Liu, Q. Huang, J.P. Morgan, Y.F. Xiao, J. Appl. Physiol. 92 (2002) 288–296.
[12] K. Monzen, Y. Hiroi, S. Kudoh, H. Akazawa, T. Oka, E. Takimoto, D. Hayashi, T. Hosoda, M. Kawabata, K. Miyazono, S. Ishii, Y. Yazaki, R. Nagai, I. Komuro, J. Cell Biol. 153 (2001) 687–698.
[13] P. Pandur, M. Lasche, L.M. Eisenberg, M. Kuhl, Nature 418 (2002) 636–641.
[14] M. Jamali, C. Karamboulas, P.J. Rogerson, I.S. Skerjanc, FEBS Lett. 509 (2001) 126–130.
[15] K. Hidaka, J.-K. Lee, H.S. Kim, C.H. Ihm, A. Iio, M. Ogawa, S.-i. Nishikawa, I. Kodama, T. Morisaki, FASEB J. 17 (2003) 740–742.
[16] I. Lyons, L.M. Parsons, L. Hartley, R. Li, J.E. Andrews, L. Robb, R.P. Harvey, Genes Dev. 9 (1995) 1654–1666.
[17] M. Tanaka, Z. Chen, S. Bartunkova, N. Yamasaki, S. Izumo, Development 126 (1999) 1269–1280.
[18] T.J. Lints, L.M. Parsons, L. Hartley, I. Lyons, R.P. Harvey, Development 119 (1993) 419–431.
[19] I. Komuro, S. Izumo, Proc. Natl. Acad. Sci. USA 90 (1993) 8145–8149.
[20] L. Shum, Y. Sakakura, P. Bringas Jr., W. Luo, M.L. Snead, M. Mayo, C. Crohin, S. Millar, Z. Werb, S. Buckley, et al., Development 118 (1993) 903–917.
[21] L.M. Moscoso, G.C. Chu, M. Gautam, P.G. Noakes, J.P. Merlie, J.R. Sanes, Dev. Biol. 172 (1995) 158–169.
[22] B.C. Paria, K. Elenius, M. Klagsbrun, S.K. Dey, Development 126 (1999) 1997–2005.
[23] M. Egeblad, O.H. Mortensen, L.C. van Kempen, M. Jaattela, Biochem. Biophys. Res. Commun. 281 (2001) 25–31.
[24] M. Watanabe, A. Choudhry, M. Berlan, A. Singal, E. Siwik, S. Mohr, S.A. Fisher, Development 125 (1998) 3809–3820.