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Cardiac Specification of Embryonic Stem Cells

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Abstract Over the past decade, cell transplantation has been recognized as a mean of repairing infarcted myocardium. Both adult stem cells and differentiated cells have yielded encouraging results with regard to engraftment into postinfarction scars. However, these cells now feature serious restrictions. As an alternative, embryonic stem (ES) cells are particularly attractive, because of their plasticity and the subsequent possibility to drive them towards a cardiomyogenic phenotype after exposure to appropriate growth factors. An additional theoretical advantage of ES cells is their expected immune privilege. In this article, we summarize the findings obtained in cell therapy using ES cells and discuss the molecular mechanisms of cardiac specification of the cells. J. Cell. Biochem. 93: 681–687, 2004. © 2004 Wiley-Liss, Inc.

Key words: heart failure; stem cells; cardiac differentiation; immune properties of stem cells

Cardiac failure emerges as a predominant disease in developed countries. Most of chronic myocardial diseases develop after cardiac stroke, hypertension, and cardiomyopathies. The clinical picture of these pathologies is non specific and includes an irreversible loss of cardiomyocytes and an increased interstitial fibrosis [Braunwald and Bristow, 2000]. In contrast to urodel amphibians or the MRL mouse strain, mammalian cardiomyocytes suffer from a limited mitotic activity [Beltrami et al., 2001]. They are thus unlikely to be capable to regenerate an injured myocardium.

Although angioplasty or thrombolytic agents relieve the origin of the stroke, it does not prevent the deleterious effect of ischemia/reperfusion which leads to dramatic cell mortality and in turn a decrease in contractility. Then, pharmacological therapeutic treatment of heart failure remains problematic. Often the endpoint remedy is heart transplantation, but such a surgery is demanding, treatment of anti-graft rejection leads to deleterious side effects and, above all, the number of donors is limited. The concept of cell therapy is thus tempting and regenerative medicine is becoming a very exciting challenge.

CELL BASED THERAPEUTIC APPROACHES

The concept of experimental regenerative medicine has emerged a decade ago. Transplantation of different cell types including myoblasts, cardioblasts, fetal, or neonatal cardiomyocytes has revealed that the stage of cell differentiation is crucial for successful engraftment. Fetal cells provided the best results since they couple with the host cells [Rubart et al., 2003]. From a therapeutic point of view, availability of autologous myoblasts has paved the way for early clinical trials [Menasche et al., 2001]. Differentiated muscle cells suffer, however, from several restrictions. In particular, myoblasts remain committed toward a muscle phenotype and cardiac area colonized by the cardiomyogenic implanted cells is limited due to a low dividing capacity [Beltrami et al., 2001]. Moreover, long-term survival and electrical coupling of such grafted cells within the myocardium is improbable [Leobon et al., 2003] which may potentially generate troubles in cardiac rhythm.

For the last years, studies have shown that many organs possess adult stem cells. Such multipotent cells can be isolated from the bone marrow including the hematopoietic stem cells and mesenchymal cells. Both cell types have been tested as a potential source for cardiac repair. Cells enriched in hematopoietic

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progenitors ("side population") engrafted into ischemic hearts without significant functional consequences [Jackson et al., 2001]. A heterogeneous population of bone marrow stem cells transplanted into infarcted heart was also suggested to differentiate in smooth muscle cells, cardiomyocytes, and endothelial cells. Although functional benefit of cells was rather modest in small rodents [Orlic et al., 2001], clinical trials have been launched as early as 2002 [Assmus et al., 2002]. More recently, transdifferentiation of hematopoietic stem cells has been suggested to be a rare event [Reinecke and Murry, 2002] or even not to occur at all [Balsam et al., 2004; Murry et al., 2004]. This should call for caution for the continuity of clinical trials.

Mesenchymal stem cells (MSC) feature the potentiality to differentiate along various mesenchymal lineages including bone, muscle, and possibly heart both in vitro [Prockop, 1997] and in vivo [Liechty et al., 2000]. Transplanted into the myocardium, they transdifferentiated in cardiac-like cells [Wang et al., 2000]. However, 5-azacytidine, a drug which affects the synthesis of nucleic acids by causing DNA demethylation was required to induce differentiation of these cells toward the mesodermal cell lineage including skeletal muscle and cardiomyogenic phenotype [Makino et al., 1999]. As long as this drug has to be used, this attractive cell population cannot be envisioned to be used in clinics. Other safer protocols require to be investigated to induce cardiac differentiation of these cells.

Altogether, adult stem cells have a much lesser plasticity than expected a few years ago. From a clinical point of view, they are no longer so promising at least to regenerate a myocardium.

More recently, several laboratories have highlighted in the myocardium stem or progenitor cells with different phenotypic characteristics [Beltrami et al., 2003; Oh et al., 2003]. A cell population expresses the stem cell antigen 1 (Sca1) but not cardiac markers such as Nkx2.5 [Oh et al., 2003]. The other population expresses Sca-1, c-Kit as well as the cardiac transcription factors GATA 4 and Mef2c [Beltrami et al., 2003]. However, these cells are not mobilized upon cardiac stroke. Their "raison d'être" remains therefore questionable. They may participate in local repair after restricted cell death. More investigation will probably bring the clue as to the benefit of such resident cells for the myocardial function.

Embryonic stem (ES) cells may provide an alternative source of regenerative cells. These pluripotent cells are derived from the blastocyst of the embryo and are capable to give rise to different progeny representative of the three embryonic layers endoderm, mesoderm, and ectoderm. In 1981, Evans and Kaufman [1981] derived the first pluripotent ES cells from the inner cell mass of a mouse blastocyst. In 1995 and then in 1998, a non human primate and later on a human ES cell lines were generated [Thomson et al., 1995, 1998]. Since then, a hundred of human ES cells lines have been derived around the world. The ES cells share with the germinal and carcinoma cells the property of self renewal. They do not undergo senescence and do not show contact inhibition when cultured in vitro. These cells have almost unlimited regenerative capacity and can generate any tissue but placenta. The colonization of chimeras from injection of a few cells and efficient generation of equipotent subclone demonstrate symmetric self renewal of the cells. Like mouse ES cells, human ES cells have been shown to differentiate toward the cardiac lineage although with a much lesser propensity [Kehat et al., 2001; He et al., 2003; Mummery et al., 2003]. Intensive investigation is under way to improve differentiation of these cells towards specific cell lineages.

In contrast to autologous adult stem cells, ES cells face the immunologic barrier. Recent data, however, challenge this problem. Indeed, human ES cells do not express any MHC-II antigens and only a very low level of MHC-I even following differentiation. Furthermore, they are not targets for natural killer cells [Drukker et al., 2002]. Although ES cell-derived cardiomyocytes might become immunogenic after interferon gamma treatment of the host [Drukker et al., 2002], it has been reported that in the presence of the thymus, stable engraftment of rat ES cell like cells to a fully MHC-mismatched rat can be successful without any sort of immunosuppressor. In fact the cells induced a mixed chimerism without graft versus host reaction. The stem cells also down-regulate the immune response of the host [Frandrich et al., 2002]. Injection of such cells without any co-stimulatory molecules does not trigger mobilization of T cells from the host. The pool size of alloreactive T cells to allogenic ES cell-derived cells is thus likely to be modest [Strom et al., 2001]. Furthermore, assuming that rejection

mediated by T-lymphocytes requires the concomitant presence of recognition and danger signals [Matzinger, 2002], the possible tolerance to ES cells could be expected from the temporal dissociation between these two signals. The danger signal triggered by tissue injury is present at the time of transplantation but the recognition signal is then expected to be blunted. Conversely, when, at a later stage, the recognition signal is expressed by the differentiated ES cells, the danger signal should no longer be present because of tissue healing.

We [Behfar et al., 2002; Hodgson et al., 2004] and others [Xiao et al., 2004] have successfully transplanted murine ES cells into rats without any sort of immunosuppression. Although intriguing and challenging dogma in immunology, this requires a detailed investigation to comprehend the mechanism of cell tolerance.

Altogether, these data make ES cells a very attractive cell population to be used for clinical purposes in the near future.

LINEAGE SPECIFIC COMMITMENT OF ES CELLS: A LESSON FROM EMBRYOGENESIS

Cardiac cell specification occurs before primitive streak formation. During embryogenesis, ES cells in the inner cell mass of the blastocyst respond to morphogens to give rise to all tissues of the embryo but the placenta. The cardiogenic morphogens belong to the transforming growth factor β superfamily (TGF) that includes TGFβ, nodal, and bone morphogenetic proteins (BMPs) and to the fibroblast growth factor (FGF) family. The agonists binding TGFβ receptors mobilize the transcription factors smads, direct activators of Nkx2.5. Stimulation of TGFβ receptors turns on activity of mitogen activated protein kinase kinase kinase (MAPKKK) TAK, acting via the MAP kinase, p38 on the transcription factors CREB and ATF2. ATF2 binds Smads to turn on expression of cardiac transcription factors. The FGFs activate MAP kinases p42/p44 (ERK, extracellular receptor regulated kinases) via the monomeric G protein, ras.

Many transcription factors play a key role in cardiac cell specification. These factors belong to different gene families, such as the homeoproteins Nkx, the MADS-box family (myocyte enhancer factor-2, Mef2), the zinc finger factors (GATA family) and the T-box family, Tbx. These factors are integrated in a transcriptional network (Fig. 1). The cardiac transcription factors are more specifically Nkx2.5, Mef2c, GATA4,5,6, myocardin, Tbx5, and Tbx20. These factors work in concert to turn on the transcriptional activity of cardiac genes promoters [Harvey, 2002].

Repressive signals of cardiac differentiation include the secreted glycoproteins Wnts inhibited by antagonists such as Crescent and Dkk1 (Dickkopf), these latter promoting cardiac differentiations. Wnt 11 is the only cardiogenic member of the Wnt family [Eisenberg and Eisenberg, 1999]. In contrast to other Wnts which activate the canonical pathway which implies β-catenin and transcription factors of the TCF/LEF family, Wnt11 activates a Ca^{2+}-dependent signaling pathway, including the Ca^{2+}-calmodulin dependent kinase II (CamKII), the protein kinase C (PKC), and the c-Jun kinase (JNK). Furthermore Wnt 11 is capable to prevent the signaling pathway of other Wnts, amplifying the cardiogenic signal.

Other factors such as Sonic and Indian Hedgehog (Shh et Ihh) favor cardiac differentiation, following activation of FGF receptors [Dell'Era et al., 2003]. More recently the receptor molecule Notch-1 and its ligand Serrate expressed within the same time window as Nkx2.5 in the procardiac area of the embryos have been implicated in the cardiac phenotypic decision. They may inhibit this process [Schroeder et al., 2003].

Inspired by these embryology data, murine ES cells were challenged with two cardiogenic morphogens BMP2 or TGFβ. The growth factors in the presence of leukemia inhibitory factor (LIF), a cytokine required to maintain the pluripotency of ES cells, induced within 24 h expression of a mesodermal marker, Brachyury as well as of cardiac specific transcription factors Nkx2.5, MEF2C, and GATA4 revealing the cardiac committed state of ES cells. Such a commitment process was translated into a differentiation state. Indeed, immunostaining of a sarcomeric structural protein sarcomeric actinin in ES-derived cardiomyocytes revealed a greater propensity of ES cells to differentiate toward the cardiac lineage after pretreatment with TGFβ or BMP2.

ES cells also spontaneously differentiate in cardiac cells when cultured in the presence of a conditioned medium prepared from long-term-culture of ES cells derived cardiac cells. This finding further supports the hypothesis that appropriate factors in the medium are sufficient

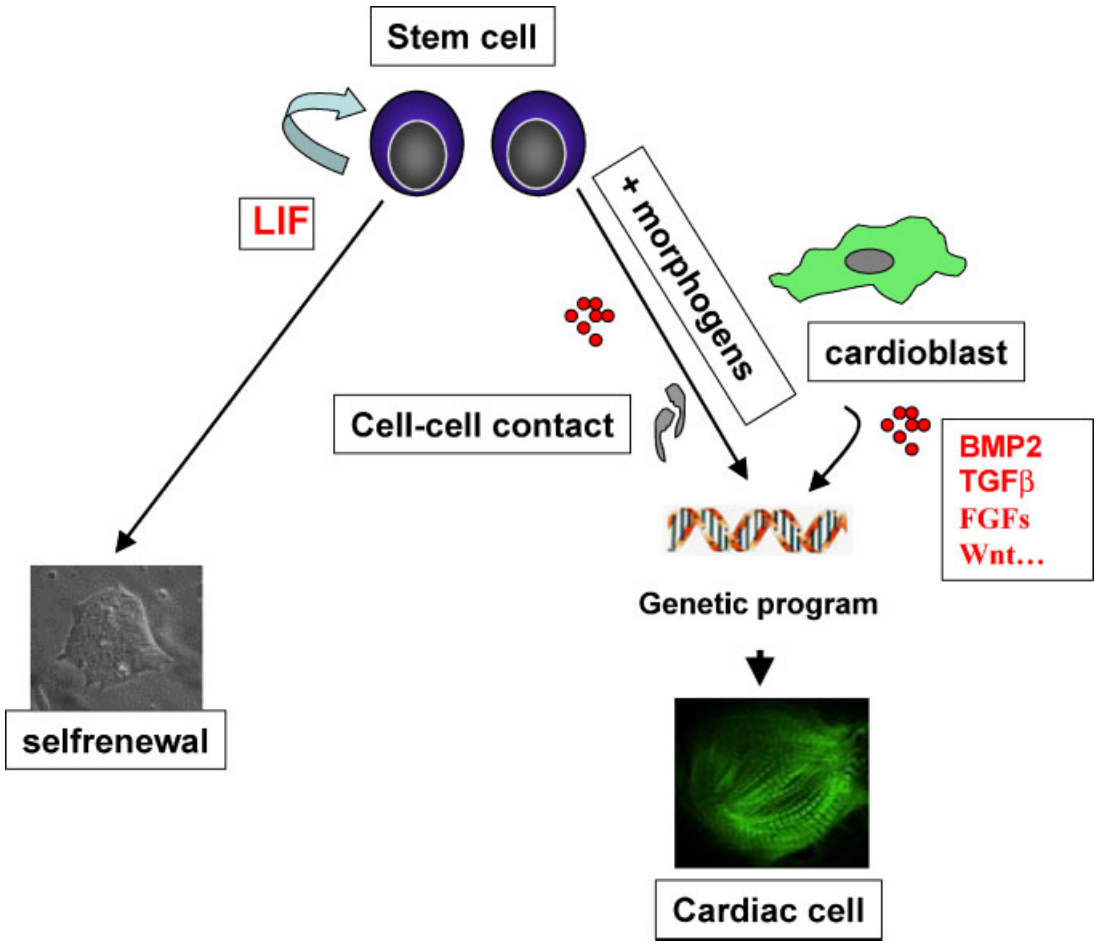

Fig. 1. Cell fate of the stem cells depends upon the presence of morphogens. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

to induce differentiation of ES cells toward the cardiac lineage [unpublished data].

ES cells expressing ECFP under the control of a cardiac promoter were transplanted into murine hearts. The cells differentiated in situ within 3 weeks into cardiomyocytes. ES cells genetically modified to express Noggin or a dominant negative mutant of the TGFβ receptor II to prevent the BMP or the TGFβ signaling pathway did not differentiate but hyperproliferated and developed cardiac teratomas. Engrafted into post-myocardial infarcted (PMI) rat hearts 4 weeks after coronary occlusion, ES cells expressing ECFP under the control of a cardiac promoter differentiated within 5 weeks into ECFP-expressing ventricular cells integrated with the surrounding myocardium and extensively repopulated the scar tissue, while still proliferating as revealed by positive anti-KI67 immunostaining. A significant improvement in myocardial function was observed in cell transplanted PMI rats [Behfar et al., 2002]. In line with our findings, endodermal factors have been recently reported to direct differentiation of both human [Mummery et al., 2003] and murine [our unpublished data] and [Rudy-Reil and Lough, 2004] ES cells toward a cardiac lineage.

Our findings showed that environmental factors are crucial for cardiac commitment of ES cells (Fig. 2). We also found that TGFβ upregulated the content of mRNAs encoding cyclin D1, and Cdk inhibitors $p21^{Cip1}$, $p18^{INK4c}$, and $p19^{INK4d}$ in ES cells which normally express very low levels of these G_1/S cell cycle partners [Savatier et al., 2002]. TGFβ allows the ES cells to acquire cell cycle characteristics similar to the ones of a somatic cell and thus to become dependent upon growth factors activating MAP kinase. This may provide an explanation for the lack of tumors in cell transplanted hearts.

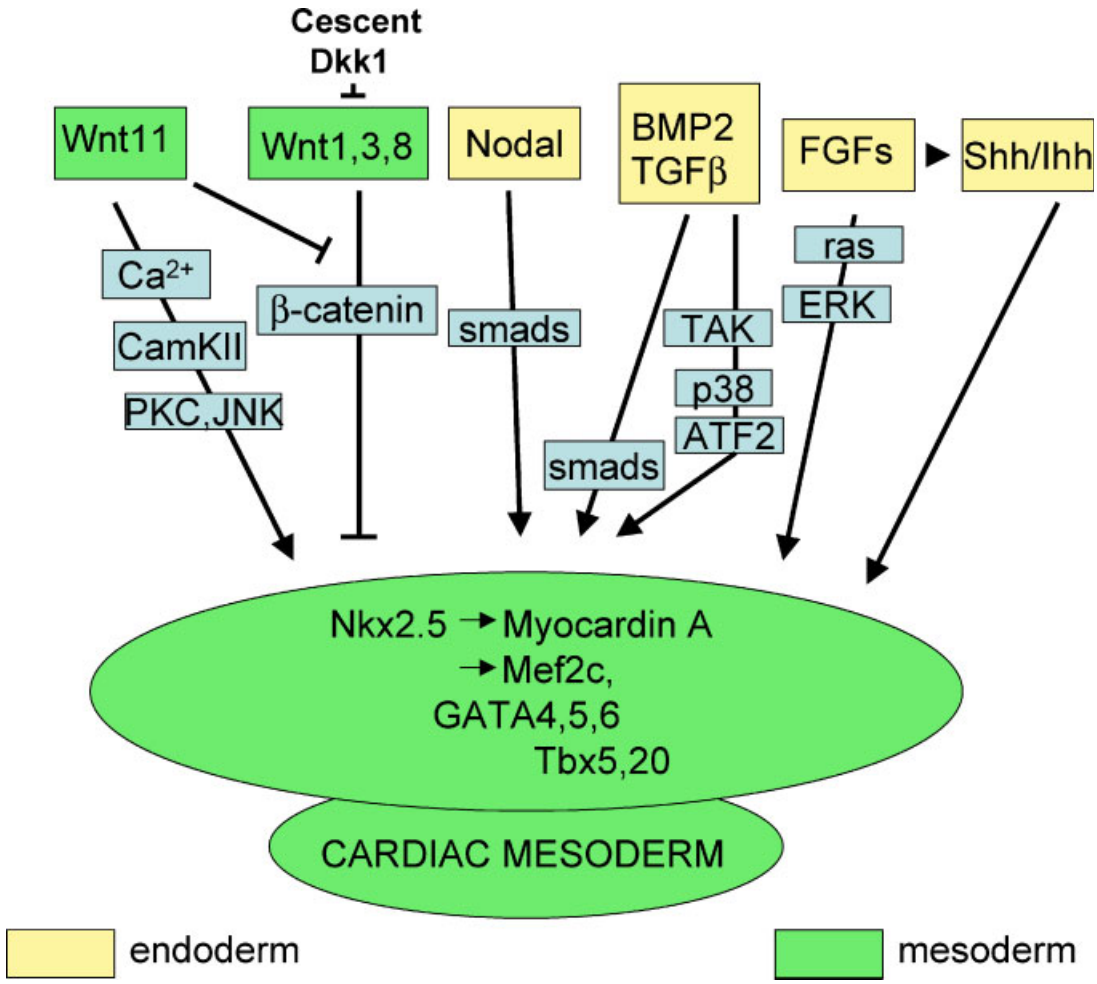

Fig. 2. Intracellular signaling pathways and transcriptional targets of cardiogenic morphogens. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

This observation is also in good agreement with the fact that graft of embryonic tissue also lose the capacity to form malignant teratocarcinomas early after differentiation of epiblast cells [Damajanov et al., 1971] when embryonic cells become under the control of ERKs.

FUTURE PROSPECT

To envision a cell therapy using ES cells, the molecular mechanisms underlying morphogen-induced cell commitment should be well understood. In fact, the question that remains to be answered is how the ES cell decides its fate when stimulated with dose-dependent and specific sets of morphogens. How many and what concentration of each morphogen is required to turn on a cell lineage specific genetic program which engages the cell toward a developmental pathway? How does the cell translate the concentration of the factor(s) into a gene transcriptional program?

Part of these issues has been extensively studied in *Drosophila* or *Xenopus* using Dpp/BMP or activin receptors. Cells can respond to threshold concentrations of morphogens. No evidence has been provided as to the existence of receptors of different affinities for their ligand that would have explained a response dependent upon a threshold concentration of agonists. Rather, the sensor of the response is likely to be the number of occupied receptors. The specificity of the response depending upon the number of occupied receptors might occur at the level of gene promoter through two mechanisms. Two separate genes may be turned on by two different concentrations of the transcription factors depending on the affinity of their promoter for the transcription factor. Alternatively but not exclusively, a repressor of the gene promoter

may be recruited and may bind to one but not to the other gene promoter [Mannervik et al., 1999; Ashe et al., 2000]. This hypothesis has to be tested in mammalian and more specifically in stem cells.

More recently microRNAs have been reported to be expressed specifically in mouse or human ES cells [Houbaviy et al., 2003; Suh et al., 2004]. Since these microRNAs are regulated in tissue-specific and developmental stage-specific manner [Lagos-Quintana et al., 2002] they may play a key role in regulating tissue specific gene expression and in turn in directing the fate of ES cells. Experimental modification of 3′ untranslated regions (UTR) of genes allows for predicting an increase or decrease in expression of the gene in stem cells [Kakoki et al., 2004]. Putative and physiological regulations of genes UTRs in vivo have to be elucidated. These concepts have to be thoroughly investigated in the stem cell context since they may represent novel ways to specify the phenotype of differentiating stem cells.

CONCLUDING REMARKS

Regeneration of tissue with stem cells clearly holds promise as a novel therapeutic approach to cure degenerative diseases. With all limitations of differentiated cells in mind, a sorted cell lineage-committed progenitor, proliferating in a growth factor regulated manner is likely to be the most appropriate therapeutic cell. To exploit its clinical potential, more research has to be devoted to understand the basic biology of the cell. More specifically, the molecular mechanisms underlying the switch between the undifferentiated state and the lineage committed progenitor including the changes in its cell cycle regulation have to be fully elucidated.

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