A chemical approach to myocardial protection and regeneration

Marco Piccoli[1,#], Federica Cirillo[1,#], Guido Tettamanti[1] and Luigi Anastasia[1,2,*]

[1]Laboratory of Stem Cells for Tissue Engineering, IRCCS Policlinico San Donato (San Donato, Milan), Italy; [2]Department of Biomedical Sciences for Health, University of Milan (Milan), Italy.
[#] These authors equally contributed to this paper.

Corresponding Author: Luigi Anastasia, Laboratory of Stem Cells for Tissue Engineering, IRCCS Policlinico San Donato, 20097 San Donato M.se (Milan), Italy; Tel: +39 (02) 52774674; Fax: +39 (02) 52774666 - Email: luigi.anastasia@unimi.it

Introduction

Myocardial damage often results in chronic failure due to loss and insufficient regeneration of cardiomyocytes [1]. This has prompted efforts to devise cardiomyocyte-replacement therapies mainly by cell transplantation or by the promotion of endogenous regenerative processes [2]. Among others, stem cell based approaches and, more recently, cell reprogramming [3] have generated an enormous attention as they promised to have a direct therapeutic application.

In particular, the generation of induced pluripotent stem cells (iPSCs) from mouse embryonic fibroblasts in 2006 [4, 5] and, shortly after, from human adult fibroblasts [6-9], has opened up a new perspective, as pluripotent stem cells can now be obtained not only from embryos but also from almost any differentiated cell. Moreover, the possibility of generating embryonic-like stem cells from adult cells let scientists speculate that generating true patient-specific therapies may become feasible. However, the fact that cells had to be reprogrammed by genetic manipulation, i.e. creating transgenic cells with retro- or lentiviruses often carrying oncogenes like c-Myc, posed serious doubts about the safety of the method. Thus, a chemical approach for the generation of stem cells from adult cells has been invoked as a safer and more convenient alternative to the genetic approach [10]. Anyhow, while the reprogramming approach was being born, several adult stem cell-based clinical trials for heart regeneration were already on-going,

mainly using bone marrow stem cells [11]. Moreover, the possibility of isolating progenitor cells, also from heart specimens, had led some investigators to postulate that these cells could be a better choice than bone marrow cells, as cardiac stem cells are already committed toward the heart phenotype [12]. However, results have been generally poor and quite controversial. Nonetheless, a key concept has emerged, which is that injected stem cells activate the growth and differentiation of progenitor cells that are already present in the heart, by releasing signalling factors and/or exosomes carrying them [13]. Therefore, stimulating the endogenous regenerative processes, possibly with a drug, could overcome the limitations and drawbacks of a cell-based approach. Moreover, understanding the mechanisms regulating cardiomyocytes turnover could be instrumental not only to repair a damaged heart, but also to increase cell resistance under adverse circumstances as ischemia or diabetes.

In this work, these novel therapeutic approaches for heart regeneration will be critically reviewed, with particular attention to: (a) cell-reprogramming, using small molecules; (b) activation of cell response to hypoxic stress. This will allow showing the original contribution to the field of the Laboratory of Stem Cells of Tissue Engineering at IRCCS Policlinico San Donato.

Chemistry for Cell Reprogramming

The process of cell differentiation, from embryonic stem cells to terminally differentiated somatic cells, has been considered for years as a one-way multistep process. In fact, even in the case of self-regeneration upon tissue damage, adult mammals can only use pre-existing progenitor cells, which are activated to proliferate and differentiate to replace the lost cells. This has been shown to be true for most tissues, including the heart, where several types of resident progenitor cells have been reported, although the results in the field are very controversial [14]. Moreover, the regenerative potential of the human heart is very limited, and it has been recently clarified that most cardiomyocytes do not regenerate during lifespan [15]. Nonetheless, there are

extraordinary examples of heart regenerative capability in other animals. For instance, the zebrafish can fully regenerate its heart following amputation of up to 20% of the ventricle mainly through cardiomyocytes de-differentiation and proliferation [16]. Even the mouse has been shown to possess a higher heart regenerative potential than humans [17]. Nonetheless, if the mechanisms underlying these regenerative capabilities are elucidated, it could be envisioned to apply the insights gained to humans, perhaps by chemically activating the appropriate regenerative stimuli [18].

Several attempts have been made over the years to unleash the same de-differentiation process in mammalians, with the ultimate goal of generating stem cells from adult cells in vitro. These approaches include somatic cell nuclear transfer, fusion of adult cells to ES cells and induced pluripotent stem cells (iPSCs) [19]. However, all these strategies have ethical and technical issues that still keep them far from a possible clinical application [10]. Actually, the possibility of using small synthetic molecules to activate the reprogramming process seems to have intrinsic advantages over genetic manipulation, including the possibility of adjusting the drug dosage and of suspending the treatment at any time. To date, several chemical strategies to generate iPSCs have been reported in the literature. In particular, since the WNT pathway contributes to the maintenance of pluripotency in mouse and human ES cells [20], as well as the self-renewal of undifferentiated adult stem cells in multiple tissues [21], several small molecules targeting this signaling pathway have been employed to increase the reprogramming efficiency of adult cells. For example, CHIR99021, an inhibitor of the GSK-3β, has been shown to modulate the WNT signaling pathway, replacing c-Myc over-expression, and improving the efficiency of reprogramming [22, 23]. Another important target to enhance the formation of iPSCs is TGF-β, which is directly involved in the mesenchymal-epithelial transition (MET). MET is a reversible biological process that mediates the transition from spindle-shaped mesenchymal cells to polarized epithelial cells and represents a fundamental step in the morphological changes needed for fibroblast reprogramming [24, 25]. Indeed, the combined use

of two small molecules, SB431542, a TGF-β receptor inhibitor, and PD0325901, a MEK inhibitor, dramatically improved (>200 folds) the efficiency of iPSC generation from human fibroblasts [26]. Within this scenario, two years before iPSCs were first reported, the synthetic purine reversine was identified from a high-throughput screen of a combinatorial library, and it was shown to be able to revert mouse myoblasts C2C12 into a more immature state, similar to that of multipotent stromal cell [27]. In fact, reversine-treated myoblasts acquired a stem-cell-like phenotype, as they could be induced to differentiate into adipocytes and osteoblasts upon treatment with the appropriate differentiating media [27]. Since C2C12 are an immortal, aneuploid and tumorigenic cell line and, most importantly, they have been shown to easily transdifferentiate into osteoblasts and adipocytes, back in 2005 our research group decided to test the effects of reversine on mouse and human fibroblasts, as they are normal cells and an easily accessible cell-source for a potential therapeutic application. Moreover, these cells are easily expandable in vitro and maintain normal phenotype and genetic stability. Our initial study was directed to evaluate the effect of different doses of reversine on fibroblasts proliferation and expression of their tissue-specific markers [28]. We also co-cultured reversine-treated fibroblasts with myogenic C2C12 cells and evaluated their ability to differentiate into skeletal muscle cells. In the same study, we also revealed the ability of reversine-treated murine fibroblasts to differentiate in vivo, after direct cell transplantation into cardiotoxin-injured tibialis anterior (TA) muscle of wild-type syngeneic mice. Based on these results, reversine-treated adult cells have been shown to be able to differentiate into neural cells [29], and even cardiomyocytes [30]. However, since the beginning, it was clear that understanding the mechanism of reversine-induced reprogramming was crucial to design new and better molecules. Over the past decade, our group and others have reported a possible mechanism of action of the purine. In particular, reversine has been shown to be a dual inhibitor of MEK1 and non muscle myosin II heavy chain, inducing an alteration of the cell cycle and changes in histone acetylation status. More recently, reversine has been shown to be also an aurora kinase

inhibitor, thus its potential use also as an antitumoral drug has been shown to be effective [31]. Nonetheless, in a recent report, other aurora kinase inhibitors, analogous of reversine, have been shown to induce cell reprogramming by activation of AKT mediated phosphorylation and increase of GSK3β [32]. Therefore, further studies in this direction are currently undergoing in our laboratory.

Chemistry for cell differentiation

Finding a safe and efficient way of generating progenitor cells through the de-differentiation process is not the only field where a chemical approach could be vital. In fact, once progenitors cells are generated, it is crucial to have selective and high-yielding methods to pilot their differentiation toward the desired cell phenotype [10]. Several successful examples have already been reported, especially in the case of ES cells, and in particular, the possibility of differentiating stem/progenitor cells towards cardiomyocytes or cardiac progenitors, even with very low efficiency, has recently gathered great attention, promising new frontiers in the treatment of myocardial infarction (MI) and heart failure [33]. At this stage, small molecules may have a primary function in the development of new strategies for progenitor cells determination and differentiation. In this regard, an improvement of cardiac differentiation of pluripotent cells has been obtained by inhibition of the bone morphogenetic protein (BMP) signaling with small molecules. BMPs are involved in the regulation of several key processes underlying cardiovascular development and their temporal modulation is crucial for cardiomyogenesis [34]. For example, dorsomorphin, a molecule identified by a chemical screen in zebrafish, inhibits the BMP signaling by specifically targeting the BMP type I receptor [35]. Dorsomorphin treatment of mouse ESCs during the first day of differentiation improved (up to thirty folds) the formation of beating cardiomyocytes [36]. Another chemical inhibitor of the BMP signaling, DMH1, robustly promoted cardiomyogenesis in multiple human iPSC lines by a reproducible and faster (only one week) method of differentiation, supporting the importance of a chemically-defined strategy to obtain large numbers of cardiomyocytes [37].

Along this line, our group has worked on a new class of small molecules, the sulfonyl-hydrazones (SHZ), discovered by a high-throughput screening of synthetic compounds that might be a promising chemical inducer for in vitro and in vivo cardiomyogenic stimulation. SHZ acted as an activator of the NK2 transcription factor related (Nkx2.5), one of the earliest lineage-restricted genes to be expressed in cardiovascular progenitor cells [38], and it was able to induce the expression of cardiac mRNAs and proteins in murine ESCs [39]. Furthermore, SHZ-pre-treated human mobilized blood mononuclear cells displayed cardioregenerative activity as xenografts in injured immunocompromised rat heart [39].

Initially, in a collaborative study with Dr. M. Sampaolesi at the Katholieke Universiteit, Leuven, Belgium, we tested SHZ effects on iPSC-derived early and late cardiomyogenesis at different concentrations, monitoring eventual effects on cell survival and apoptosis. Moreover, we examined and quantified beating foci percentages and cardiomyocytes isolation rates in order to verify whether this small chemical compound could effectively improve cardiomyogenesis of iPSCs. Results showed that 5 μM SHZ treatment was able to increase early cardiac marker expression as Nkx2.5 and GATA binding protein 4 (GATA 4) and late cardiac marker expression as cardiac myosin heavy chain (MHC), connexin 43 (Cx43) and cardiac troponin I (cTni) in a dose-dependent manner, but high concentrations of the molecule induced an apoptosis-driven loss of viability during mid-late differentiation stages. In addition, SHZ treatment resulted in a significantly higher yield of cardiomyocytes isolated from differentiating embryoid bodies. The possible SHZ effects on beating activity were also analyzed by comparing both beating cardiac foci percentages and areas of differentiation between control and SHZ-treated iPSCs. As expected, SHZ treatment of iPSCs resulted in a significant increase in beating foci rate and area extension. To exclude the hypothesis that SHZ-dependent cardiomyogenic enhancement could rely on beneficial effects on cell proliferation, we evaluated growth curves of proliferating iPSCs and isolated cardiomyocytes in presence or absence of SHZ and we did not observe any significant variation in the proliferation rate.

Overall, our results indicated SHZ as a suitable molecule to increase in vitro iPSCs cardiac differentiation at low concentration [40]. Undoubtedly, it would be really desirable to find small molecules that will replace or increase the efficacy of known differentiating key factors, including growth factors, cytokines and conditioning media and these results supported the idea that a chemical approach to stem cell differentiation has started to became very effective and suitable, as many small molecules are generally not expensive, available in relatively large quantities and poorly immunogenic [41].

Another interesting perspective for the development of new therapeutic strategies to treat heart failure includes the use of a chemical approach to *in situ* activate resident cells to regenerate the tissue, without any *in vitro* amplification steps. In fact, cardiac progenitor cells possess a very limited turnover in vitro, thus their direct activation in the damaged tissue may represent a valuable alternative [10]. Along this line, in our laboratory, we isolated human cardiac stromal cells from auricles obtained during cardiac surgeries of IRCCS Policlinico San Donato. These cells have been treated with SHZ before the induction of cardiac differentiation and tested for cardiac specific marker expression. SHZ-treated cardiac stromal cells showed a 20-fold increased expression of cardiac troponin T, as compared to untreated cells (unpublished data). Even if preliminary, these results may represent an important starting point for the development of new therapeutic strategies to promote in situ myocardial repair/regeneration.

Activation of protection system

As stated in the introduction, another approach for fighting heart failure could be to increase myocytes resistance to stress. For example, ischemic conditions lead to “hypoxia” which causes functional impairments of cells and often structural tissue damage. A possible approach to reduce hypoxia consequences could be the activation of the hypoxia-inducible factor (HIF-1α), a transcription complex which responds to oxygen deprivation by stimulating the cell defence machinery, ultimately protecting tissues against the consequences of hypoxia [42]. Activation of HIF in mice, by genetic inhibition of its main regulator PHD2, caused preformed collateral

arteries that preserved limb perfusion and prevented tissue necrosis in ischemia [43]. It was also found that PHD2 inhibition amplifies the antioxidative response in the heart reducing the tissue damage caused by chemotherapeutic drugs [44]. Moreover, it was reported that inhibition of prolyl hydroxylases (PHDs) improved long-term ventricular function, remodeling, and vascularity after myocardial infarction in a rat model [45]. While we were developing new synthetic drugs to inhibit PHD2 with Dr. Mazzone, we found and reported a novel mechanism of activation of HIF-1α, not involving PHD2, and mediated by sialidase NEU3, the enzyme that releases sialic acid residues from sialoglycoconjugates (for instance from ganglioside GM3), and that we found to be triggered under hypoxia [46]. Moreover, NEU3 overexpression protects myocytes from hypoxia-induced cell death. NEU3 is a member of sialidase family, which are glycosidases that catalysed the removal of α-glycosidically liked sialic acid residues from carbohydrate group of glycoproteins and glycolipids [47]. NEU3 is known as the "ganglioside sialidase" because it removes sialic acid preferentially from ganglioside localized on the plasma membrane, and in particular its action is targeted on ganglioside GM3 [48]. In cancer, it has been demonstrated that an up-regulation of NEU3 is fundamental for the activation of EGFR pro-survival signalling pathway by reducing the content of ganglioside GM3 [49]. We moved on this direction, and we focused the attention to validate the possible involvement of NEU3 in cell response to hypoxic and ischemic stress in the cardiovascular field. In our initial study, murine skeletal muscle cells, C2C12, were used to study the role of NEU3 under hypoxic conditions. Interestingly, culturing C2C12 at 1% oxygen for 72 h caused an up-regulation of NEU3. Moreover, a overexpression of NEU3 induced a marked increase of EGFR signalling cascade, thus activating its down-stream pro-survival and anti-apoptotic signalling pathways, including AKT, p70S6K, and eventually up-regulating the hypoxia inducible factor (HIF-1α). Overall, these effects increased cell resistance to hypoxia, ultimately opposing apoptotic cell death. However, although NEU3 mechanism of action was clear, the physiological up-regulation of the enzyme under hypoxic condition was still unknown [46]. Actually, a recent study reported the binding of transcription factor SP1 and SP3 to NEU3 promoter region [50].

These factors are known to be activated under hypoxic conditions [51]. Indeed, C2C12 cells cultured under hypoxic conditions showed an up-regulation of SP1 and SP3, supporting their involvement in activating NEU3 gene transcription under low oxygen levels [46].

Overall, these initial studies showed that NEU3 is involved in cell response to hypoxia through the down-regulation of ganglioside GM3 and the up-regulation of HIF-1α. For this reason, finding a way to mimic the action of NEU3 through the use of specific molecules could be a novel approach that may result in the development of new therapies. In this regard, our laboratory is undertaking two parallel lines of study: (a) to mimic NEU3 activity by chemically impairing GM3 biosynthesis with new sialyltransferase inhibitors; (b) to increase HIF-1α activation by chemical inhibiting key prolyl hydroxylases. In particular, GM3 synthase (ST3Gal-V) belongs to the sialyltransferase family, and is a unique enzyme among all sialyltransferases, since it is specifically involved in ganglioside GM3 formation [52]. In our laboratory, several GM3-synthase inhibitors have been synthesized and tested, revealing the feasibility of this approach (data submitted for publication). For the second approach, it is known that HIF-1α is tightly regulated by a family of enzymes called prolyl hydroxylases (PHDs) [53]. PHD enzymes introduce a hydroxyl group into specific prolyl residues on the HIF-α molecule. Inhibition of the PHDs has potential for the treatment of anaemia, ischemia-related diseases, and other diseases [54, 55] by enabling a range of cellular and systemic responses that enhance oxygen delivery or reduce oxygen demand. For this reason our laboratory is screening several commercial and newly synthesized PHDs inhibitors to test if they could activate cell-response to hypoxia.

Overall, either NEU3 activation or PHD2 inhibition have the ultimate goal of activating cell response to stress, thus increasing their resistance to adverse conditions like ischemia or diabetes.

In conclusion, the research activities of the Laboratory of Stem Cells for Tissue Engineering at IRCCS Policlinico San Donato are focused on the development of a chemical-pharmacological approach to heart failure. This could be achieved by activating stem cells in the heart, and/or by increasing cell resistance to ischemia (Figure 1 and 2). This new and original approach, which can be developed by the strict collaboration of synthetic organic chemists, biochemists, and cardiac surgeons, may lead to the generation of new drugs for the treatment of heart failure without the need of cell therapy or organ replacement.

Figure 1

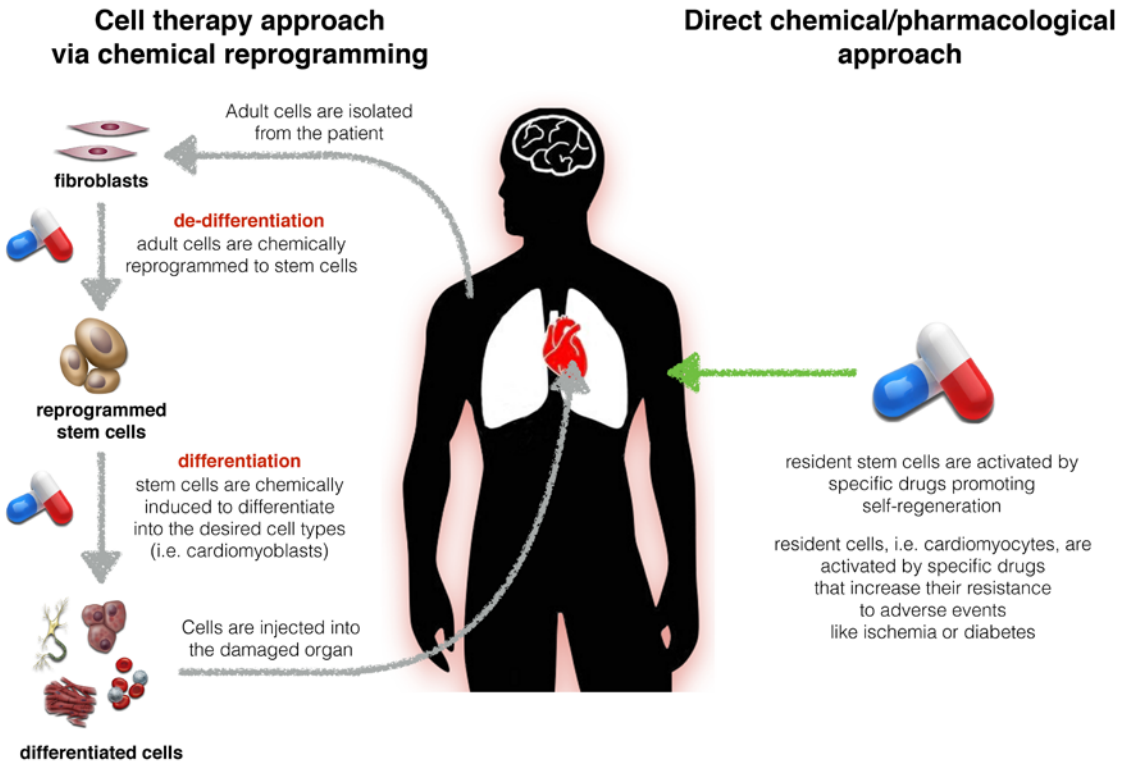

Figure 1: Schematic representation of a possible cell therapy approach via chemical reprogramming of adult cells isolated from patients and of the direct chemical/pharmacological approach for the in situ activation of resident cells promoting self-regeneration of tissues.

Figure 2

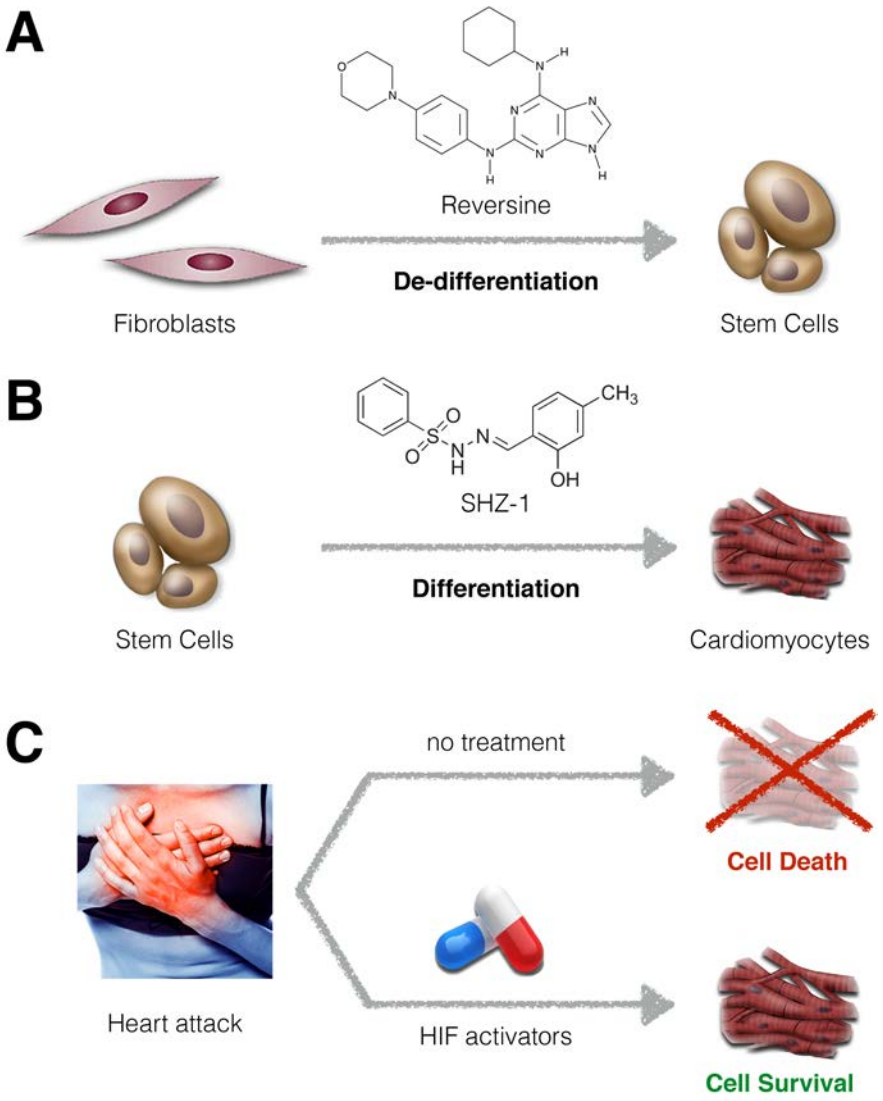

Figure 2: Examples of chemical approaches to stem cell reprogramming in cardiovascular diseases.

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