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Combination of transcription factors, miRNAs and small molecules as a novel recipe for boosting cardiac differentiation

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ABSTRACT

After birth cardiomyocytes stop proliferating, they exit from the cell cycle and therefore in an adult heart the rate of dividing cells is really low. Since the cardiomyocytes turnover is almost inexistent in case of heart injury there is a loss of cardiac cells that are replaced by fibrotic tissue. The fibrosis leads to an anatomical and functional impairment. There have been investigated several techniques in order to induce cardiomyocytes division in order to repair the damage, here we are going to describe all the progress reached during these years. Then, mostly relying on previous knowledge we are going to suggest a strategy consisting in the transdifferentiation of fibroblasts into cardiomyocytes using a combination of transcription factors, miRNAs and the addition of small molecules in order to increase the reprogramming efficiency. To increase even more the differentiation we want to combine the mix with advanced delivery systems in order to direct the mix only in the area of interest and in a very precise way.

INTRODUCTION

The central role of the heart is highlighted also during the embryogenesis, since it's the first organ that is formed. The heart starts developing from mesodermal cell layer, and undergoes through a series of required passages, even one missing passage can jeopardize the embryo survival. From the cardiac mesodermal crescent there is the formation of the linear heart tube, then occur other steps necessary for the development of the more complex structure, like for instance looping morphogenesis, septation, chamber specification and cardiac valve formation. Moreover, before birth cardiomyocytes go through a massive proliferation to achieve a suitable size. Whereas, soon after birth cardiomyocytes exit the cell cycle and almost stop proliferating, and in this case the growth is achieved through increase cell size (hypertrophy) (Olson 2002; Scott, Crabbe et al. 2008; Mercola 2012; Porrello 2013). This narrow regenerative capacity in the adult heart is a problematic issue, because after a heart attack or other heart disease its function is compromised also because after a damage the structure and the composition of the heart change and undergo through excessive fibrosis (Mercola 2012).

Since heart diseases cause every year 17.3 million deaths the need to find a strategy to fully restore cardiac function is fundamental. So far drug treatments and invasive operations are the only cures available able to slow the progression of heart diseases, then heart transplant is the only long lasting solution. One of the most promising idea conceived in order to find a reliable treatment is cell therapy. Exploiting the networks of genes miRNAs involved in the cardiac embryology, the first

studies that have been conducted consisted on the reprogramming of embryonic stem cells and/or induced pluripotent stem cells using the principal transcription factors involved. These strategies have been also tested in vivo with little success, and they have been replaced for safer and more efficient techniques. These are based on the idea of reprogramming cardiac fibroblasts. These cells are the most present in an adult heart (about 60%) and in case of heart failure, damaged cardiomyocytes are substituted by fibroblasts (Srivastava and Ieda 2012). Using the factors and the miRNAs which have been confirmed to be essential in the ESCs and IPSCs reprogramming, it has been possible to obtain functioning cardiomyocytes both in vitro and in vivo. However, there are still issues that must be solved, among these the reprogramming efficiency must be improved.

AIM OF THE RESEARCH

In this research we are going to explain the progress and the techniques that have developed through these years in order to make possible the application of the cardiac regeneration as next main therapy. Furthermore we are going design a possible strategy to increase the reprogramming efficiency. To improve the efficiency of the fibroblast transdifferentiation into cardiomyocytes here we are going to suggest to combine two of the techniques used to obtain cardiomyocytes which involve the use of transcription factors and a mix of miRNAs all involved in cardiac development and differentiation. To further improve the transdifferentiation we also suggest to firstly improve the transfection of the transcription factors efficiency with advanced delivery systems and add to the strategy small molecules/drugs which are also able to increase cardiac differentiation. The main of all these improvements is to apply cardiac regeneration in patients and let it be the next therapy for cardiac damages.

MECHANISMS

It’s well known that regenerative capacity of adult cardiomyocytes is limited and the only clinically available therapy for heart failure is the transplantation, so the need to find new treatments to restore cardiac functionality is crucial. Several approaches have been investigated, including the replacement therapy using stem cells (Ieda and Fukuda 2012).

To repair an injured heart and repair the damaged cardiomyocytes Embryonic stem (ES) cells are used. ES cells derive from the inner cell mass of the blastocyst, are able to remain undifferentiated and to propagate ad infinitum (in vitro). ES cells give rise to all three embryonic germ layers, that is

ectoderm, mesoderm and endoderm. Moreover, if they receive the correct inputs they can differentiate in all the derivates of the three germ layers, thus they can also differentiate into cardiomyocytes. In order to induce cardiomyogenesis in ES cells, several proteins and genes involved in the cardiac differentiation at the embryonic stage have been studied (Ieda and Fukuda 2012; Ratcliffe, Glen et al. 2013). During the embryogenesis, the formation of the heart requires 6 specific stages, in which the heart develops from a simple tubular structure into a complex structure with atria and ventricles (Franco, Dominguez et al. 2002; Olson 2002). Cardiomyocytes originate from the mesodermal layer during gastrulation. This portion receives various cardiogenic signals, such as Gata4, Mef2c, Tbx5 and Hand 2, that induce the formation of the heart cavities and the heart itself. It has been tried to direct the ES differentiation towards a cardiac fate, using these cardiogenic signals, (Olson 2002; Olson 2006), however, even if the use of ESCs and cardiogenic signals seemed extremely promising, because the new proof of concept appeared to be correct and functioning, they have been substituted by induced pluripotent stem cells (iPSCs) since the application of ESCs is impeded due to ethical issues and immune rejection.

In 2006 Dr. Yamanaka and colleagues managed to reprogram mouse and human fibroblast into cells similar to ES, the iPSCs, using ES specific transcription factors Oct4, Sox2, Klf4 and c-Myc. IPSCs are more suitable for cardiac regeneration because they circumvent the ethical issue, since they don't derive from an embryo and since they could be obtained by the patient cells they also circumvent immunogenicity (Ieda and Fukuda 2012; Fu, Stone et al. 2013). Starting from iPSCs, cardiomyocytes are generated by transiently transfecting a mix of transcription factors, which are involved in the early heart development, i.e. Gata4, Mef2c and Tbx5. The GATA family has a role in the myocardial specification, whereas the functions of the other two are still not completely clear, it is believed that Gata4 makes the cardiac loci accessible by opening the chromatin structure, allowing the binding of Mef2c and Tbx5 to their target genes. Thus, they together in parallel and their interaction activates the transcription of cardiac genes and cardiac differentiation. Moreover, to highlight the importance of Mef2c and Tbx5 it has been shown during the embryogenesis that embryos without the expression of these two genes will develop cardia bifida, a pathological condition which leads to the formation of two hearts (Olson 2002; Olson 2006).

There are different strategies with integrating and non-integrating viruses used to insert these three TFs into iPSCs to generate cardiomyocytes. However, the reprogramming efficiency is low and the cardiomyogenic potential varies based on the starting cell type used for obtaining the iPSCs. Moreover, the percentage of iPSCs obtained is always around 0.1-1%, meaning that the number of obtainable cardiomyocytes is not enough to be therapeutically efficient (Ieda and Fukuda 2012; Fu,

Stone et al. 2013). Furthermore, the induced cardiomyocytes are functionally immature, i.e. they show cardiac markers but they are morphologically different from adult cardiomyocytes and they also show problems in the contraction (Nam, Song et al. 2013).

These results lead us to state that iPSCs are not applicable in the clinic. Besides the issues already described, it is difficult to purify induced cardiomyocytes with flow cytometric cell sorting and once injected in an infarcted heart, the induced cardiomyocytes are lost within several days. This means that there are problems in the survival and the engraftment of the cells (Ieda, Fu et al. 2010).

To circumvent the engraftment and survival problems, it has been postulated to deliver the three transcription factors directly to the fibroblasts that populate the injured portion. Though, also this option was not optimal, since the fibroblasts before differentiate into cardiomyocytes went through a pluripotent stage that could be potentially harmful (tumorigenic risk) (Fu, Stone et al. 2013). Since it is possible to convert a somatic cell into a pluripotent one a step forward has been made and fibroblasts have been directly reprogrammed into cardiomyocytes without passing through a pluripotent stage using different techniques (Ieda, Fu et al. 2010). This direct transdifferentiation potentially decreases the tumorigenic and engraftment risk, as it doesn't imply the insertion/injection of cells but the transformation of the fibroblasts present in the scar .

Transdifferentiation of fibroblasts into cardiomyocytes has been achieved in vitro using the same transcription factors used for the reprogramming of iPSCs, that is Gata4, Mef2c and Tbx5 (GMT). The induced cardiomyocytes are genetically and epigenetically similar to normal ones, but they show some differences, that is they resemble young cardiomyocytes instead of mature cardiomyocytes. From the starting population of fibroblasts, only 2.5% becomes fully reprogrammed, i.e. they express αMHC (cardiac-myocytes marker), cardiac troponin (structural cardiac marker) and they contract spontaneously (Williams 2010; Srivastava and Ieda 2012). Although initially this method has a low transdifferentiation efficiency, improvements were made. To the initial three transcription factors, Hand2, another fundamental transcription factor for the correct development of the heart and whose interactions with GMT is essential in maintaining the cardiac phenotype (Song, Nam et al. 2012; Nam, Song et al. 2013), was added to the mix. This addition has a higher percentage of induced cardiomyocytes from tail-tip fibroblast of about 9.2% (fourfold higher than with the initial mix of GMT). The GHMT mix also induced a more adult cardiac fibroblasts phenotype. 6.8% of these cells were positive for αMHC cardiac troponin, whereas GMT has induced the expression of those cardiac markers in only 1.4% of the cells. Furthermore, the GHMT mix works efficiency in vivo. The viral transfer of these transcription factors to fibroblasts in an infarcted heart improved cardiac function and decreased the fibrosis, but

still not good enough for a clinical application. However, the obtained results have gone beyond all expectations, since the improvements obtained were better of what was predicted. The induced cardiomyocytes looked more mature than the one obtained in vitro, and well integrated with the surroundings. Probably because the native environment with the extracellular matrix, growth factors, pre-existent contractility of the non-damaged cardiomyocytes help with a better transdifferentiation, however the exact mechanisms involved in these improvements are not yet fully understood (Song, Nam et al. 2012).

An alternative method which has been developed to obtain induced cardiomyocytes consists in the use of a mix of microRNAs (miRNAs). Since some miRNAs are also involved in the signalling network of cardiomyocytes, their implication in a future therapy in the cardiac regeneration could be beneficial, also because miRNAs can target multiple genes within different pathways and this means that the effect could be broader (Montgomery). MiRNAs are small non-coding RNA molecules, which negatively regulate gene translation, by binding to the 3'UTR region of their mRNAs. Several studies show the prominent role of miRNAs under normal conditions, but most of all under stress conditions (Kuppusamy, Sperber et al. 2013; Sluijter 2013).

The mix used for the transdifferentiation includes miRNAs involved in the development of cardiomyocytes: miRNAs 1, 133, 208 and 499 with the adding of JAK inhibitor I (Jayawardena, Egemnazarov et al. 2012). MiRNAs 1 and 133 are usually co-expressed and they cooperates in the specification on the cardiac lineage (Wystub, Besser et al. 2013). In ES cells they strongly repress endoderm gene expression and promote early mesodermal gene expression, and so probably they promote the development of the cell lineages deriving from it, like for instance cardiomyocytes. It has been demonstrated that miRNA 1 alone would be already enough to induce cardiac reprogramming. Meanwhile, miRNA 133 is able to stop the differentiation of myogenic precursors (Montgomery and van Rooij 2011; Jayawardena, Egemnazarov et al. 2012). Even if miRNA 1 alone is sufficient for the transdifferentiation, the addition of miRNA 133, 208 (highly present in cardiac tissue) and 499 (regulated by myosin) increase the expression of cardiac markers and so the maturation of those cells. Whereas, the further addition of JAK I inhibitor has increased the transdifferentiation efficiency of about 8-10 fold. JAK I inhibitor is a small molecule, which is able to suppress the pathways involved in pluripotency, however the exact mechanism of action is still unclear (Jayawardena, Egemnazarov et al. 2012).

The described recipe has also been tested in vivo, and it leads to the reprogramming of cardiac fibroblasts into cardiomyocytes in the scar and in the areas close to the damaged part of the heart. Also in this case the reprogramming efficiency in vivo is higher compared to the one obtained in vitro in the two dimensional petri culture dish (Jayawardena, Egemnazarov et al. 2012). Moreover,

also with this technique the cardiomyocytes were more mature compared to the ones obtained in 2D culture, they were well integrated with the surrounding beating cells, and they improved the myocardial functional and reduced the fibrosis in the scar area (Jayawardena, Egemnazarov et al. 2012).

Even though the huge progresses have been made, more efforts must be made in order to obtain the best technique that will be possible to apply also in the clinic. The first issues that must be solved is increasing the reprogramming efficiency and use vectors safe enough in order to make possible the clinical application. In this regard in the next session we are going to describe the approach that we conceived to improve efficiency and safety.

FUTURE PERSPECTIVES

Cardiac regeneration in vivo is the next therapy able to help patients affected by heart failure. However, to accomplish this goal many issues have to solved. First of all a strategy must be chosen. We listed some of the experimental strategies that have been developed in the past years. Although the first experiments were conducted in ESC first and then repeated in iPSCs cells, ESCs and /iPSCs could not be applied in the clinic mainly for ethical and safety issues but the obtained results paved the way to new approaches. As previously mentioned ESCs/iPSCs are related to several issues, that make them not suitable for a practical application. It has been observed that injected reprogrammed ESCs/iPSCs in infarcted hearts were lost after few days from the transplant. The number of cells obtained is low (0.1-1% efficiency) and so it is too difficult and too expensive to sort and isolate them without damaging them. Furthermore, the cardiomyocytes obtained from this technique are immature, meaning that the insertion in the heart could be potentially harmful since cells still pluripotent could be injected, which could lead to tumours/teratoma formation. Thus, the technique based on the insertion of cardiomyocytes derived from ESCs/iPSCs must be replaced with others which include the direct transformation of the population of fibroblasts present in the area of the cardiac damage and whose efficiency has been already tested in vivo.

Direct reprogramming fibroblast transdifferention into cardiomyocytes can occur via transcription factors (GHMT) or via a mix of miRNAs (miRNA 1, 133, 208, 499) and JAK I inhibitor. Although, both techniques show increased numbers of induced cardiomyocytes compared to the reprogramming of ESCs/iPSCs, the reprogramming efficiency remains an important issue that must be improved. In order to do so what we suggest is to optimize the insertion and expression of the transcription factors and the miRNAs plus JAK I inhibitor and combine the two techniques

together. In fact miRNAs and transcription factors work together during embryogenesis and they influence each other activity. We believe that the combination of the two will have an additive effect and will enhance efficiency. It has been already showed that from the combine use of GHT and miRNA1-miRNA133 on fibroblasts were obtained cardiomyocytes with sarcomeric structure and Calcium transients (Nam, Song et al. 2013).

To increase the reprogramming efficiency other miRNAs that have been noticed to promote cardiac differentiation could be added to the process. Furthermore to help even more could be useful the administration of some small molecules/drugs which have been tested in vitro to improve the reprogramming.

In order to increase the reprogramming efficiency to use also other miRNAs involved in cardiac development and differentiation in addition to those already use (miRNAs 1, 133, 208 and 499). For instance in vivo studies on the miRNA 17 – 92 cluster showed that miRNA 17 – 92 mutant fetus died after birth with ventricular sept defects, so they must play a crucial role in cardiac formation (Porrello 2013). Another candidate could be miRNA 199, whose expression is particularly abundant in the heart and during embryogenesis it increases (Gu and Chan 2012). Furthermore, miRNA 199 along with miRNA 590 were selected using a high-throughput screening in rat cardiac cardiomyocytes, and it has been observed that they promote cardiomyocytes proliferation and regeneration in infarcted mice (Eulalio, Mano et al. 2012; Porrello 2013).

Usually GHMT factors and miRNAs have been inserted in cells using retrovirus or lentivirus, but the number of reprogrammed cells obtained and the potentially safety problems related to the effects of insertional mutagenesis don't make possible the use of these type of viruses also in the clinic. In order to circumvent the related safety issue in the latest years a new technique has been generated. It is based in the use of Sendai virus (Hemagglutinating Virus of Japan - HVJ), which is a RNA-strand virus that doesn't integrate in the genome of the host, it is not pathogenic in human and it's effective. Thus, HVJ virus has become a powerful and required tool in gene therapy (Ieda, Fu et al. 2010; Nakanishi and Otsu 2012). We want to suggest to transfect GHMT factors and the mix of miRNAs together directly in the cardiac fibroblasts in the damaged area using and injecting the HVJ complexed with liposomes. Liposomes are structures made of concentric layers of lipids, whit an internal aqueous phase, which is able to retain and carry small molecules, small proteins, viruses and genes; thanks to the lipid phase is easy for this structure to be absorbed through the cells membrane. Through the years liposomes have gained more and more attention, since also liposomal applications have reached the clinical investigations (Torchilin 2005; Scott, Crabbe et al. 2008; Bowey, Tanguay et al. 2012; Levchenko, Hartner et al. 2012). Among these the complex HVJ-liposome has been tested in an animal model for cardiac infarction. The results obtained showed

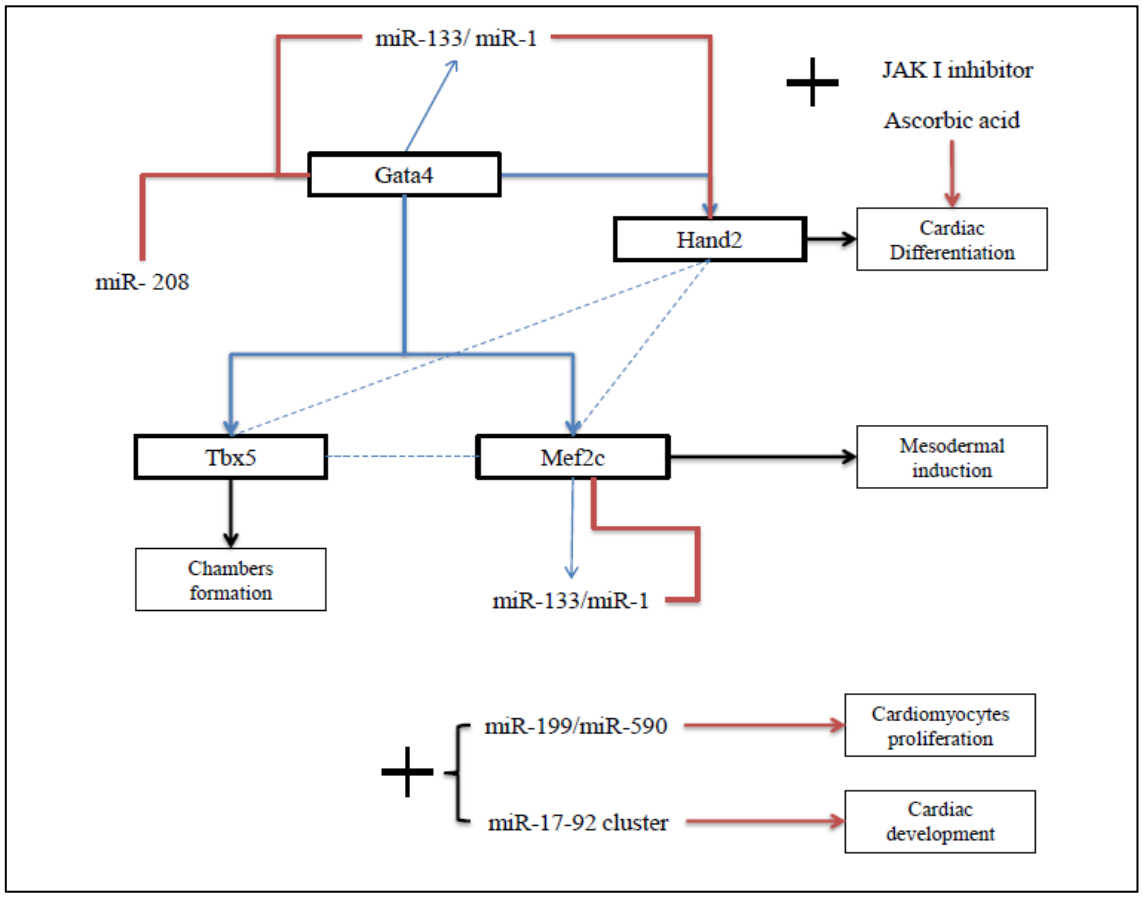
that this complex is highly efficient. Using two approaches (incubation in the pericardium and coronary infusion) the transgenic genes have been diffused in the damaged area, including cardiac fibroblasts (Nakanishi, Mizuguchi et al. 1999; Bowey, Tanguay et al. 2012; Nakanishi and Otsu 2012). However, one of the disadvantages of the use of liposomes as delivery system is the cell-targeting precision. To enhance the targeting of cardiac fibroblasts, therefore, it would be better the use of HVJ combined with immunoliposomes, that is liposomes which present antibodies on the surface (Bowey, Tanguay et al. 2012). In our case the antibody that could be used in order to target cardiac fibroblasts is anti-vimentin (Camelliti, Borg et al. 2005).

Several studies that have been conducted on cell reprogramming towards a cardiac fate involved also the application of some small molecules and/or drugs. For instance, 5-azacytidine, a compound analogue of cytidine which inhibits DNA methylation, it has been showed to induce and promote cardiac differentiation in ESCs. However its clinical application is unlikely since 5-azacytidine is a is used as a chemotherapeutic agent. Moreover it has effects on the epigenetic marks and this can lead to broader effects on different types of cells (Takahashi, Lord et al. 2003; Ohtani and Dimmeler 2011). Another compound which has been tested is ascorbic acid (vitamin C), and also this was able to enhances cardiac differentiation from ESCs. Particularly cardiac markers expression like Gata4, α-MHC and β-MHC was increased (Takahashi, Lord et al. 2003). Even if ascorbic acid was tested only in ESCs and so in vitro, we believed that it could be carried by the HVJ-immunoliposomes and its addition to the mix of TFs and miRNAs would benefit the outcome and therefore increase the yield of reprogrammed cardiac cells from cardiac fibroblasts.

Many progresses have been made in the order to improve the clinical applicability of cardiac regeneration and make it the next main therapy for treating cardiac failure. The most promising development is the direct reprogramming of the cardiac fibroblasts present in the damaged area towards a cardiac fate. The obtained cardiomyocytes showed cardiac markers, Calcium transients and once the technique was tested in vivo, the induced cardiomyocytes reduced the scar in the damaged area of the heart and improve the contractility. However, in vitro and in vivo studies showed that the main drawback is the reprogramming efficiency. Mindful of the experiments conducted on the ESCs and iPSCs we designed a new approach aimed to increase the efficiency of the reprogramming and so aimed to obtain a higher number of mature and functioning cardiomyocytes. We believe that the transfection of cardiac fibroblasts using the Sendai virus (HVJ) with the combination of transcription factors (GHMT), miRNAS (miRNA 1, 133, 208, 499, 199, 590 and cluster 17 – 92) and small molecules (JAK I inhibitor and ascorbic acid) all involved in cardiac differentiation would significantly increase the reprogramming efficiency. Moreover, in

order to apply this combination in the clinic (patients hearts) in a safe and effective manner the HVJ would be combined inside immunoliposomes direct against cardiac fibroblasts, which have been already successfully tested in vivo.

Here we show a schematic representation of the factors, miRNAs and small molecules carried by HVJ-liposome involved in our strategy aimed to increase the reprogramming efficiency. It's possible to see how actually factors and miRNAs interact and regulate each other in feedback mechanisms.



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