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Reprogramming adipose tissue derived mesenchymal stem cells into insulin-producing cells

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Abstract

β-cell replacement is currently considered the most promising treatment for type 1 diabetes. However it is strongly limited by the scarcity of transplantable material, i.e. islets of Langerhans from cadaveric donors. In this context, stem cells are an alternative source due to their potential to duplicate and differentiate into certain lineages under specific culture conditions. Both embryonic and adult stem cells can differentiate into insulin-producing cells, however existing protocols still require substantial improvements. Concerning embryonic stem cells, there are also certain ethical limitations that constrain its possible clinical use. Adult stem cells, on the other hand, do not present these problems. In addition, as seen with adipose tissue-derived mesenchymal stem cells, they can be easily isolated and transplanted again, once reprogrammed, to the donor without the need of immunosuppresors. Theoretically, the differentiation of mesenchymal stem cells, either from adipose tissue or bone marrow, to insulin-producing cells can be achieved by various strategies, which can be grouped, according to the existing information, into coaxial and directional protocols.

Keywords: adipose mesenchymal stem cells, pancreatic β-cell, cell reprogramming, diabetes.

Introduction

Diabetes mellitus is a severe metabolic disorder caused either by insulin absence due to the autoimmune destruction of pancreatic β-cells (as in type 1 diabetes) or by a complex syndrome that includes insulin resistance and a progressive decrease in insulin production, culminating in the apoptosis of pancreatic β-cells (as in type 2 diabetes). β-cell replacement could be an alternative to daily insulin injections for type 1 diabetic patients and in advanced stages of type 2 diabetes. In this context, islet transplantation has supposed a hopeful strategy.[1] However, there are major obstacles that need to be surpassed in order to fully develop this technique. These include an adequate immunosuppresor regime and an alternative cell source to compensate the disproportion between the number of donors compared to the recipients.

Stem cell-derived insulin-producing cells represent a promising alternative to the scarcity of islets for transplantation. Stem cells have the ability to self-renew and to differentiate in vitro into many cell types, conferring them an enormous potential in future clinical applications. In addition, both embryonic (ESCs) and adult stem cells (ASCs) possess the ability to differentiate into insulin-producing cells. However, the use of human ESCs for therapeutic purposes opens a legal and ethical debate that limits their use in many countries. These barriers, on the other hand, do not exist when using ASCs. These cells are found in many organs and are mainly involved in maintaining cell homeostasis and in regeneration after severe injuries. Initially, it was believed that ASCs were only capable of differentiating into cell types of the organ they were located. However, recent studies suggest that these cells may overcome the limits of the parental niche and give rise to other cell types of different origin.[2]

Currently, multipotent ASCs have been identified in several organs and tissues such as bone marrow, blood, muscle, liver, brain, gut, pancreas, retina, adipose tissue and skin, among others. Of these, stem cells derived from the pancreas, liver, nervous system and bone marrow have been differentiated towards insulin-producing cells.[3-6] In the majority of the cases, these differentiation processes

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Abbreviations:

AMSCs: adipose mesenchymal stem cells; ASCs: adult stem cells;
ESCs: embryonic stem cells; MSCs: mesenchymal stem cells.

are produced by a specific cell type present in these organs, the mesenchymal stem cells (MSCs).[3, 7] The main advantages of ASCs and in particular MSCs are their easy in vitro culture, their plasticity to differentiate into many cell types and the possibility of self-transplantation once they are reprogrammed to the desired cell product.[7]

Bone marrow-derived MSCs is the most well known source of these cell types, although mesenchymal stem cells derived from adipose tissue are currently considered a promising alternative. Both populations are very similar phenotypically[8] and can adopt endocrine pancreatic fates in vitro.[9] However, the adipose tissue is easily accessible and abundant in adults compared to bone marrow biopsies, which are painful for patients and give lower amounts of biomaterial. Due to these advantages, adipose mesenchymal stem cells (AMSCs) from lipoaspirates are being strongly considered for future cell therapy protocols.

The strategies to differentiate MSCs are based on modifications in the culture medium through the use of various compounds (coaxial methods). Also, in some cases directional methods are used to complement the coaxial strategies. These include transfection with specific DNA constructs, cell fusion events and the use of reprogramming protein extracts. This directional methodology has been successfully used to reprogram differentiated as well as non-differentiated cells into various fates. This manuscript describes the most important methods used to reprogram MSCs, understanding that the final cell product (i.e. an insulin-producing cell) appears as a result of a transdifferentiation process (figure 1).

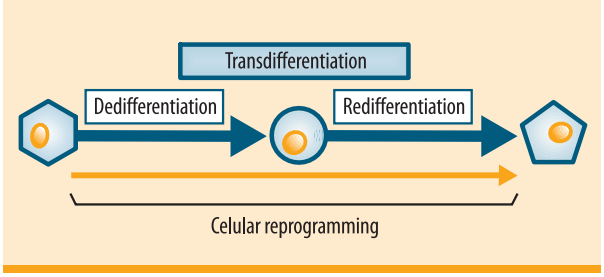

Figure 1. Scheme presenting the concept of cell reprogramming in the transdifferentiation process. Currently, part of the scientific community assumes that transdifferentiation, at least when using coaxial strategies, requires a dedifferentiation process to redifferentiate thereafter to a specific cell fate. Cell reprogramming may be able to avoid this process; however this remains to be confirmed (dotted line)

The rationale behind this transdifferentiation process is that all cells bear the same genetic material, since they derive from the inner cell mass of the blastocyst. These cells, which are genetically identical, progress towards their cell fate due to specific gene programs. Ideally, reprogramming strategies could change the gene expression profile of a certain cell type to another one closest to the cell of interest. In theory, the resulting cell should adopt a new phenotype and perform new functions. Due to the common genetic information present in all the nuclei of an adult organism, all cells are susceptible to be reprogrammed under the appropriate conditions. However, experience indicates that this is not as easy as stated, most likely because scientists are facing new challenges and deciphering new enigmas that Cell Biology has still not discovered.

Coaxial reprogramming methods

Coaxial methods are widely used and consist in the addition of different growth factors, cytokines and hormones to the MSC cultures. Theoretically, after the factors bind to membrane receptors, signalling pathways are activated, resulting in the modulation of specific genetic programs and ultimately in phenotypic changes that resemble the cell of interest. This methodology can be divided into two phases: differentiation or commitment to obtain cell precursors or progenitors, and maturation to give rise to the functional final cell type. Precursors can be maintained in vitro stimulating their proliferative potential in order to produce sufficient biomass. Obviously, in many designed protocols, these two phases form part of a continuous process, which makes it in some cases very difficult to distinguish them (figure 2). In any case, the major challenge is to identify which combination of factors must be added to the culture medium, their appropriate concentration, and the adequate exposure time required to obtain the desired cell type.

In this context, developmental studies of the various organs have given promising results with cultured ESCs towards insulin-producing cells.[10] However, the transfer of these concepts to ASCs or to MSCs does not seem obvious, resulting in differentiation protocols with low efficiencies. This occurs in part due to the fact that many factors used are not completely specific, exerting pleiotropic actions in different cell processes that include differentiation, proliferation and apoptosis. Thus, this results in a heterogeneous population of cells, which

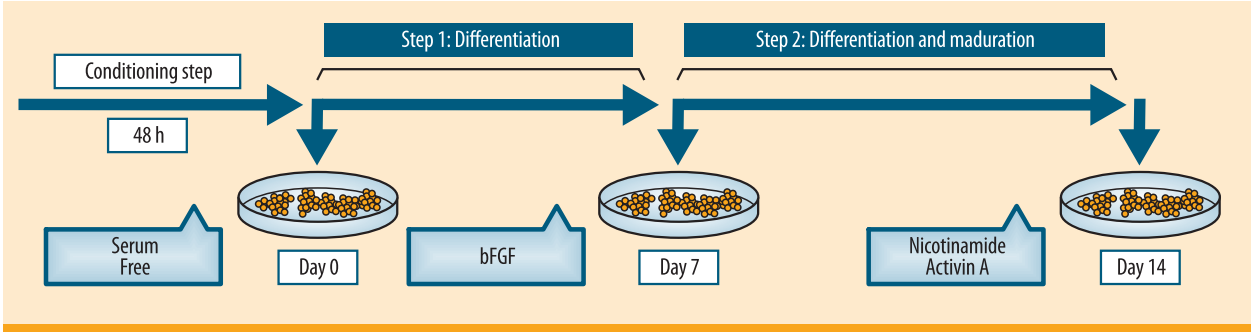

Figure 2. Scheme of a coaxial differentiation protocol to obtain in vitro insulin-producing cells from AMSCs or bone marrow MSCs. Briefly, cell division is ceased by serum withdrawal during 24-48 h. Under these conditions, differentiation (commitment) and maturation (acquisition of a cell fate) can be achieved by sequentially adding certain factors and compounds to the culture medium. bFGF: basic fibroblast growth factor

enormously conditions their therapeutic potential. In addition, many differentiation protocols towards insulin-producing cells assume that the expression of a certain gene battery, detected by RT-PCR, is sufficient proof that the resulting cell is a pancreatic β-cell. This is completely wrong, as many markers are also expressed in other cell types, i.e. insulin is not only expressed in endoderm-derived pancreas, but also in ectoderm-derived hypothalamic neurons and extraembryonic endoderm. From this simple rationale, it can be concluded that many protocols derive heterogeneous populations that may be similar to β-cells, but are not true β-cells.[11] Complementary proteomic and functional tests are necessary to define the final cell products.

Directional reprogramming methods

Reprogramming by cell fusion events

Cell fusion consists in the union of cells with different origins, i.e. adult cells with ESCs or adult cells with multipotent ASCs, giving rise to hybrids that may display a morphology, duplication rate and cell surface molecule expression pattern typical of the pluripotent cell partner.[12] For several authors, this process is the method with which bone marrow MSCs differentiate into insulin-producing cells. However, other groups have defended a transdifferentiation mechanism to explain these phenotypic changes.[13-15]

Reprogramming by gene transfer

Transdifferentiation can be performed in somatic cells by the use of retroviral vectors, obtaining a stable genetically modified cell. This strategy introduces DNA constructs that codify key pancreatic β-cell specific transcription factors. The main advantage of this group of protocols is the possibility to study the differentiation potential of specific transcription factors. However, the expression of many pancreatic transcription factors in other cell types could derive to a mixed population with a variety of phenotypes. A second problem concerns to the fact that many transfected genes are under the control of potent promoters that produce high supraphysiological levels of the codified protein.

Nevertheless, this strategy has been used in human bone marrow MSCs transfected with DNA constructions that codify key β-cell transcription factors such as Hlxb9, Foxa2 and Pdx1.[16-18] The introduction of these transcription factors together with coaxial methods (incubation in the presence of islet-conditioned media) can give rise to insulin-producing cells.[18] Even though, in some cases the results seem promising, genetic manipulations cannot be used in a human therapeutic context, posing many ethical restrictions.

Reprogramming using protein extracts

An interesting alternative to cell fusion and gene transfer is the use of reprogramming protein extracts. It has been demonstrated that the introduction of protein extracts in some adult pluripotent cells can produce phenotypic changes.[19] This procedure requires the permeabilization of the plasmatic membrane in order to introduce the protein extracts in the cell. Streptolysin O can be used for this purpose, since this compound is a bacteria toxin that binds to plasmatic membranes and opens pores, allowing the bidirectional flow of ions, metabolites and macromolecules.

In this context, human fibroblasts have been reprogrammed with T lymphocyte extracts using this tech-

nique.[20] In addition, cardiomyocyte extracts were used to reprogram AMSCs, resulting in cells with a cardiomyocyte-like phenotype.[21] Interestingly, insulin-producing cells were obtained by reprogramming fibroblasts with protein extracts from the insulinoma cell line INS-1.[22] In this assay, 5-30% of the cells were insulin$^+$ and the yield of reproducibility of the assay was approximately 23%. However, the resulting phenotype of the reprogrammed fibroblasts was transient and no functional probes were performed in order to demonstrate the efficiency of the protocol.

Altogether, this technology could have many applications in reprogramming MSCs, although several questions need to be previously resolved, such as the sensitivity of the mesenchymal cell to the side-toxic effects of streptolysin O or the ability of the cell to recover after the depletion of intracellular constituents during the permeabilization process. ATP regenerating systems are frequently used to restore cell function after streptolysin treatment; however in some cases this treatment is not sufficient. Therefore, the use of complementary coaxial strategies in order to rescue treated cells and act as a support during the reprogramming process has to be strongly considered.

Methods for protein transduction

As previously mentioned, the use of streptolysin O is limited due to its toxicity. Our personal experience indicates that certain cell types are especially sensitive to this toxin, thereby constraining its general application in reprogramming protocols and explaining in others the broad variability observed between experiments. Alternatively to streptolysin O, certain carrier peptides possess the ability of translocating extracellular macromolecules inside living cells. Carrier peptides are classified as protein transduction domains, if they require covalent binding to the cargo protein, or cell penetrating peptides, if the interaction between carrier and cargo is not covalent.[23]

Even though both types have the ability of introducing proteins and many extracellular macromolecules to the interior of the cell, they differ in the method of the process. Transduction domains require the attachment of the domain to the cargo protein, generally through the use of recombinant DNA, along with a subsequent denaturalization of the resulting protein before its transfer into the target cells. It is important to verify that the modified molecule with the new domain can function as the corresponding counterpart. The protein usually enters by endocytosis (retrograde transport). On the other hand, penetrating peptides do not require covalent modifications, maintaining the tridimensional structure of the cargo protein throughout the whole transfer process, which can be achieved either by micropore or inverted micelle formation.

Several carrier peptides have been recently characterized, such as the Drosophila homeodomain transcription factor Antennapedia, the VP22 protein of the Herpes simplex virus type I and the transactivating protein TAT from the human immunodeficiency virus. Interestingly, it has been observed that once the cargo has reached the intracellular domain, the proteins do not remain in the cytosol, possibly migrating to the nucleus (figure 3).[24] This opens an interesting possibility of introducing key transcription factors in order to sequentially direct the reprogramming process.

This technology has also opened attractive possibilities in the investigation of many diseases, including diabetes.[25] One therapeutic possibility includes the design of targeted peptides for pharmacological purposes. Unfortunately, these peptides cannot enter the cell due to the plasma membrane of the target cells, which acts as a barrier. In this context, diabetic patients could benefit from new immunosuppressors or antiapoptotic proteins that can slow down the development of the disease. MSC reprogramming into insulin-producing cells is another ap-

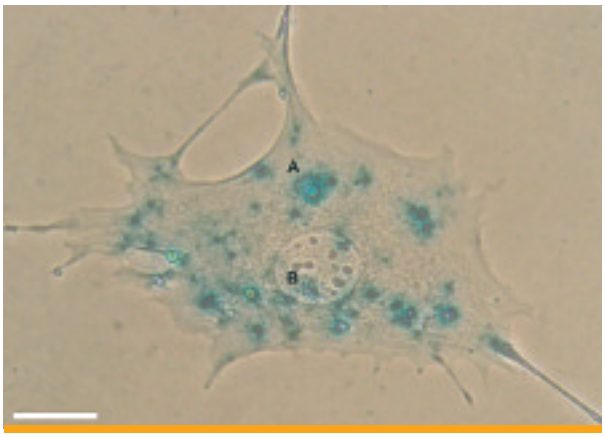

Figure 3. Transmission image of a human mesenchymal stem cell. The blue colour corresponds to β-galactosidase in cytoplasm (A) and nucleus (B) that has been introduced into the cell by a carrier peptide system. Bar: 5 µm

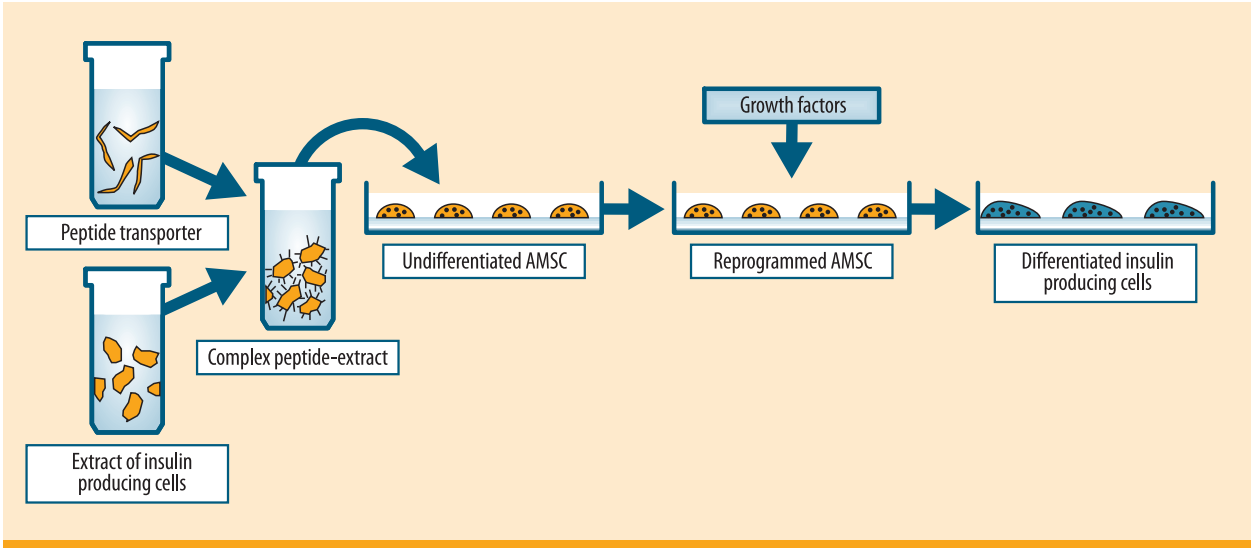

Figure 4. Proposed protocol to differentiate AMSCs to insulin-producing cells using reprogramming extracts. Cargo proteins extracts are incubated with penetrating peptides and the resulting complexes are added to the AMSC cultured clones. The differentiation process can be enhanced by adding specific growth factors to the culture medium

plication field using specific cargo proteins.[26] Key transcription factors involved in islet development, metabolism control or insulin gene expression, such as Pdx1[27] and Ngn3,[28] have been used in cultured islets and pancreatic explants. This has resulted in an increased commitment in endocrine differentiation and insulin gene expression.

Conclusions

Several strategies capable of reprogramming AMSCs into insulin-producing cells have been shown. These strategies can be used in combination with coaxial methods in order to develop a more complete protocol (figure 4). Several key points must be taken into account: the nature of the cell to be reprogrammed, the final cell type to obtain and the requirements to fulfil in a therapeutic context. In the case of insulin-secreting cells, the use of protein extracts from mature β-cells or β-cell lines, as well as extracts enriched in specific transcription factors, could be interesting reprogramming tools that deserve to be explored. In addition, carrier peptides avoid the use of retroviral vectors, which cannot be used in human cells for ethical reasons, or the toxic side-effects of streptolysin O.

On the other hand, AMSCs represent an attractive choice since they can be easily isolated in sufficient amounts from adult individuals, facilitating autotransplants once cells are reprogrammed. However, AMSCs are initially composed of a heterogeneous population. Clonal selection is required to obtain populations with a homogeneous differentiation potential. Epigenetic differences seem to be responsible for this variability. Also, AMSCs present a hypermethylation in non-adipogenic compared to adipogenic promoters.[29,30] This suggests that AMSCs are epigenetically committed and the use of demethylating agents and chromatin remodelling compounds could be complementarily used with current protocols.

In conclusion, the use of reprogramming technology in ASCs and in particular AMSCs could be an interesting option. Nevertheless, several questions remain to be answered before its application in a therapeutic context. Therefore, it is mandatory to confirm not only the efficiency of the differentiation process, but also the modifications of other cellular functions, including changes in the cell cycle that could result in an accelerated apoptosis or, even worse, uncontrolled cell proliferation.

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Key points

- Mesenchymal stem cells derived from adipose tissue are a promising source of cells capable of differentiating into insulin-producing cells.
- The differentiation of mesenchymal stem cells to insulin-producing cells can be achieved by two strategies using coaxial and directional protocols.
- Coaxial methods consist in the addition of different compounds to the MSC cultures. Directional methods include gene transfer, cell fusion or the use of reprogramming proteins that are placed into the cell.

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