The use of gating technology in bioengineering insulin-secreting cells from embryonic stem cells

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Abstract

Embryonic stem cells display the ability to differentiate *in vitro* into a variety of cell types. This process is induced by embryoid body formation, addition of several soluble growth factors to the culture medium and other strategies. However, none of the used factors is capable to drive differentiation to only one specific cell type. The use of gating technology has allowed to partially overcome this problem. The rationale behind this technique is based on the transfection of stem cells with a transgene carrying expression cassettes for a cell type specific promoter, regulating expression of a selectable marker to select one cell lineage from other cell lineages. Using this system, we have obtained insulin-secreting cells by transfecting mouse embryonic stem cells with a DNA construct providing resistance to neomycin under the control of the regulatory regions of the human insulin gene. Furthermore, gating technology has been successfully used to isolate other cell types such as cardiomyocytes and neural precursors from undifferentiated embryonic stem cells. This review focuses on the possibilities offered by this technology in embryonic stem cell bioengineering, mainly to obtain insulin-secreting cells. Advantages and considerations of this selection system will be also discussed.

Abbreviations: ESC, Embryonic Stem Cells; EGFP, Enhanced Green Fluorescent Protein; FACS, Fluorescence-Activated Cell Sorting; bFGF, basic Fibroblast Growth Factor; LIF, Leukemia Inhibitory Factor.

Introduction

Embryonic stem cells (ESC) in theory have the ability to *in vitro* differentiate into any of the more than 200 cell types present in the human body, including the germinal lineage. This property together with the potential to indefinitely divide under defined culture conditions, make these cells a unique source for cell and tissue regeneration in different pathologies (Soria et al., 2000a). These may include diabetes, neuronal degenerative diseases, cardiac alterations and osteo-articular lesions. To this end, mouse and human ESC have been used in several *in vitro* differentiation

protocols to obtain specific cell lineages (Brook and Gardner, 1997; Odorico et al., 2001).

Mouse ESC can be maintained undifferentiated in culture medium containing fetal calf serum and the IL-6 family cytokine LIF (Leukemia Inhibitory Factor), which is instrumental in maintaining proliferation over differentiation (Smith, 1991). Mouse ESC can also be grown in the absence of LIF but on mitotically inactivated mouse embryo fibroblast feeder layers (Smith, 1991). However, optimal culture conditions for human ESC culture remain to be established. First culture protocols used irradiated mouse embryo fibroblasts either in the presence (Reubinoff et al., 2000) or in the absence of human LIF (Thomson et al., 1998).

The use of extracellular matrices, such as Matrigel or laminin, in medium conditioned by mouse embryo fibroblasts has also been used to expand human ESC (Xu et al., 2001). However, the use of cells or extracellular matrices from non-human xenogeneic origin carries the risk of a potential infection with animal pathogens. In this context, a recent report points to the use of human embryo or adult fibroblast feeders both supporting optimal growth conditions for human ESC (Richards et al., 2002).

When ESC are forced to grow in suspension and in the absence of LIF, they spontaneously form typical spherical aggregates named embryoid bodies (Smith, 1991). After 7–21 days of culture in suspension, embryoid bodies display spatially organized differentiation processes, evidenced by the presence of new cellular phenotypes. These cell types include cardiomyocytes, neurons, glia, adipocytes and insulin-containing cells (Soria et al., 2001; Weiss and Orkin, 1996). The molecular mechanisms operating in this particular stage are still unknown, but the variable access to nutrients and soluble factors, as well as the different micro environmental conditions within the embryoid body are probably instrumental in this process.

When embryoid bodies are transferred from suspension culture to adherent growth on plastic dishes, different phenotypes can be observed, such as contracting cardiomyocytes or dopaminergic neuronal-like cells (Odorico et al., 2001; Schuldiner et al., 2000; Klug et al., 1996). The addition of different soluble growth factors to the culture media increases the relative proportion of specific cell types (Schuldiner et al., 2000). However, no factor in itself is capable to completely drive this *in vitro* differentiation process towards a homogeneous cell population (Schuldiner et al., 2000). This phenomenon could compromise future therapeutical applications in cell replacement protocols, taking also into account that a sub-population of these cells might not be terminally differentiated and still maintains its proliferative capacity (Soria, 2001). The major problem is that most growth factors exert pleiotropic actions in cell cycle progression, affecting proliferation, differentiation and/or apoptosis (Soria, 2001). Therefore, the optimal mixture of factors, as well as its precise concentration and exposure time remains to be defined. However, the various differentiation methods using even combinatorial mixes of factors do not result in homogeneous cell populations. Due to those current limitations it is generally accepted that particular emphasis should be put on the establishment of technologies to ensure safety of

stem cell products that should be used as therapeutics in humans (*Stem cell report chapter 10*). Therefore and in this context, gating technology has been developed and used to overcome the problems inherent to cell-type selection.

Gating technology

The gating technology is a relatively simple genetic approach to produce essentially pure cultures of differentiated cells from ES cell.

This method utilizes two transcriptional units that are incorporated into a common vector backbone. The first unit makes use of a constitutively active promoter to drive expression of a marker gene (e.g. drug resistance protein) suitable for enrichment of cells that have incorporated the DNA-construct. The second transcriptional unit utilizes a promoter that is specifically and exclusively active in the desired cell type or lineage to drive expression of a second marker gene (e.g. drug resistance protein, GFP, cell surface marker) suitable for enrichment of the desired cells. In practice, the DNA is introduced into undifferentiated stem cells and successfully transduced cells are then selected by virtue of the activity encoded by the first transcriptional unit. Upon differentiation, the second transcriptional unit is activated only in the desired cell type (which is defined by the cell type specificity of the promoter utilized). The desired cells or lineages are then selected by eliminating all cells which did not express the cell type specific promoter (Klug et al., 1996; Li et al., 1998; Müller et al., 2000; Soria et al., 2000b).

Using this genetic selection principle, gating technology has previously been successfully applied to obtain different cell types, such as insulin-secreting cells, cardiomyocytes and neuronal-like cells (Klug et al., 1996; Li et al., 1998; Müller et al., 2000; Soria et al., 2000b).

The use of gating technology to obtain insulin-secreting cells

Restoration of β -cell function in order to control blood glucose levels is of prime importance in therapeutic strategies for treatment of type 1 and 2 diabetes. Insulin self-injection once or twice daily cannot exactly mimic hormone function, and thus provides only partial benefits for the patient who sometimes risks to

suffer hyperglycemic or hypoglycemic episodes, and subsequent complications (Soria et al., 2000a).

Alternatively, the use of gene therapy and ectopic insulin gene expression for treatment of diabetes still awaits crucial improvements (Efrat, 1998; Gros et al., 1998; Goldfine et al., 1997). The main obstacle these strategies have to face consists in the physiological modulations of the β -cell secretion pattern in the modified cells.

The difficulties in reconstructing β -cell function ectopically makes exogenous β -cells an ideal alternative choice to restore β -cell mass or β -cell function in either type 1 or type 2 diabetes. Transplantation of islets from human cadaveric donors already underwent major improvements for the treatment of type 1 diabetes (Shapiro et al., 2000). However, a big disproportion exists between the number of available donors and potential recipients. This lack of suitable donor cells has increased efforts to search for alternative sources of insulin-secreting cells, such as islet cell precursors (Bonner-Weir et al., 2000) and ESC (Soria et al., 2001).

In this regard, the use of gating technology has been instrumental to obtain insulin-secreting cells from ESC (Soria et al., 2000b). The strategy applied consisted of a double-selection protocol based on the resistance to two antibiotics: hygromycin and neomycin. The transfected DNA construct contained the hygromycin resistance gene under the control of the constitutive promoter of the phosphoglycerate kinase gene, thereby allowing the selection of single clonal cell lines.

The second selection strategy was based on the expression of the neomycin resistance gene under the control of the regulatory regions of the insulin gene. Thus, those cells expressing the insulin gene were functionally capable to express the neomycin resistance protein and survived when neomycin was added to the culture medium (Figure 1). The generation of insulin producing cells during the differentiation process could be monitored by using a construct coding for a fusion protein of the β -galactosidase enzyme and the neo^r marker. In this case the lineage specific promoter not only regulated expression of the neo^r gene but at the same time also the marker gene lacZ which could be easily visualized during the different stages of differentiation (Soria et al., 2001).

This selection system is particularly useful to isolate specific and highly purified cell types and is a prerequisite for using such cells in therapeutics. This indicates that gating technology is an essential part of

the bioengineering process, complementing the action of other agents, such as growth factors or biophysical determinants (Soria, 2001).

Using the insulin promoter to select insulin producing cells from differentiating ESC has several advantages. First, the insulin promoter shows strong transcriptional activity in β -cells which is important for generation of sufficient amounts of the reporter gene for optimal selection. Many other promoters of pancreas specific transcription factors may not have the sufficient expression level to select the desired cell type. Secondly, the insulin promoter will enrich for highly differentiated insulin producing cells which harbor most functions of a β -cell. These cells could be used directly for transplantation and would not have to undergo intensive maturation. The disadvantages in selecting terminally differentiated insulin producing cells consists in their limited capacity to proliferate, resulting in restricted cell numbers and limited survival due to apoptotic mechanisms.

One alternative to the generation of highly differentiated insulin producing cells would be the derivation of precursor cells committed to differentiate into beta cells. Gating technology also allows to work with constructs that are functionally active during intermediate stages of embryonic development (Jensen et al., 2000; Edlund, 2001), forcing in this way the derivation of endocrine pancreas precursor cells.

Such precursor cells would still be capable to proliferate and generate larger cell numbers. However, these cells will need to undergo further maturation in order to develop fully functional insulin releasing cells suitable for cell replacement therapies. To realize such a strategy different DNA regulatory elements could be used as potential candidates to derive β -cells or their pancreatic precursor cells.

In case pancreatic precursor cells will be used, a two step protocol could be feasible. In a first step precursor cells could be selected using gating technology. These cells could then be expanded and later matured into terminally differentiated insulin-producing cells. Such a final maturation process might be mandatory in many cases to obtain functional cell types that can be used for therapeutic transplantation. We are currently using various DNA constructs in combination with different maturation strategies in our laboratory, in order to test which one allows the best results in bioengineering insulin-producing cells from ESC (Figure 2).

Figure 1. Use of gating technology to derive β -cells. The expression of the neomycin resistance gene under the control of the regulatory regions of the insulin gene allows the selection of insulin-containing cells from other cell types (neurons, cardiomyocytes, etc.) obtained during the *in vitro* differentiation process.

Applications of gating technology to obtain other cell types

Gating technology has been first successfully used to obtain murine cardiomyocytes, which were capable to form intracardiac grafts, once transplanted into the hearts of dystrophic mice (Klug et al., 1996). In this case the neomycin resistance gene was under the control of the α -cardiac myosin heavy chain promoter. The selected neomycin resistant cells were >99% pure and they displayed a highly differentiated phenotype based on immunological, functional and structural analyses (Klug et al., 1996).

Another approach using gating technology applied EGFP-expression in cardiomyocytes. In these approaches, EGFP expression was under the control of the ventricular-specific myosin light chain-2v or the cardiac α -actin promoter (Müller et al., 2000; Kolossov et al., 1998). Upon differentiation only cardiomyocytes displaying a fluorescent phenotype due to the functional expression of EGFP were purified by cell sorting. This resulted in a 97% pure population of cardiomyocytes according to immunohistochemical and electrophysiological analyses (Müller et al., 2000).

Gating technology was also used to enable efficient selection of neuroepithelial progenitor cells (Li

et al., 1998). In those studies ESC were transfected with the fusion gene β -geo, conferring neomycin resistance and allowing β -galactosidase positive staining, under the control of the Sox2 gene. The product of the Sox2 gene is part of the SRY-related transcription factor family which is expressed in early neuronal progenitors (Pevny et al., 1998). ESC were transfected with the indicated construct, selected and expanded by adding basic fibroblast growth factor (bFGF) to the culture medium. 48–96 h after bFGF withdrawal, the undifferentiated Sox2⁺ precursors started to express characteristic neuronal markers, such as microtubule-associated proteins (MAP2 and tau), β -tubulin III, neurofilament heavy chain and synapsin I. The yield of this selection protocol consisted of 90% cells displaying a neuronal phenotype. Gating technology has also been applied to isolate neural stem cells from fetal human brain (Keyoung et al., 2001). In this protocol fetal forebrain was dissociated and the resulting cells were transfected with adenoviral vectors expressing green fluorescent protein under the control of nestin and musashi-1 promoters, two genes that are expressed in neuronal progenitors. The transfectants were recovered by fluorescence-activated cell sorting (FACS).

Altogether, these data indicate that gating technology is very efficient in obtaining pure populations of

Figure 2. Candidate DNA constructs used in gating technology to isolate insulin-producing cells: A) Construct based in the human insulin gene (INS) (Soria et al., 2000b). B) Scheme of potential constructs used to isolate differentiated insulin-containing cells (1) or insulin-producing cell precursors (2). Abbreviations used: β geo, fusion gene of β -galactosidase and neomycin resistance; e, exon; EGFP, enhanced green fluorescent protein; Hygro^R, hygromycin resistance; pGK, phosphoglycerate kinase promoter.

neuronal precursors and cardiomyocytes. The genetic modification is minimal and technically easy with no apparent interference with the proliferative capacity of the cells, subsequent differentiation potential and survival. This will allow the application of this technology to derive specific cell types or lineages from ESC, including cell precursors, or to isolate stem cells from fetal or adult tissues.

Considerations and future perspectives for the use of the gating technology

The gating technology is a promising strategy that has the proven capacity to generate highly pure populations of specific cell types. Although the application of this technique has been restricted to few cell types so far, the concept is generally applicable. Nonetheless, definitive working protocols need to be established for other cell types and several points should be considered to further improve and exploit the many advantages offered by this technology.

1. A cell type specific protein has to be known and a promoter has to be available. There are many examples of specific proteins expressed in terminally differentiated cell types. However, genetic markers for many types of precursors still need to be characterized. The obvious advantage of selecting progenitor lineages is the possibility to subsequently expand and to yield large numbers

of cells. On the contrary, terminally differentiated cells have a more limited life span and usually a restricted proliferation capacity (Soria, 2001). Therefore, the application of gating technology to the isolation of cell precursors implies the use of constructs derived from genes that are specifically expressed during development of the cell lineage to be selected. In many cases, the corresponding markers have not yet been identified or cloned and the regulatory gene regions have not yet been fully characterized. In other cases, the genes and regulatory regions might be available to design the appropriate selection constructs, but the precise subsequent protocols for maturation of the selected cell lineages remain to be established.

2. Expression of the gene driving the selection has to be specific and unique for the bioengineered cell type. This is a key consideration when applying gating technology. If the gene of choice to drive the selection process is also (albeit temporarily) expressed in other cell types, then the selection/enrichment of a specific cell type will be sub-optimal. Altogether, it seems that ectodermal and mesodermal-derived cells are easily obtained by gating technology, because very reliable marker genes are already available. As of today, endodermal derivatives, such as endocrine pancreatic cells, seem to be more difficult to select, mostly, because appropriate markers are less easy

to define and spontaneous differentiation rates are less pronounced.

3. Transgene regulation has to be similar to the endogenous gene. The influence of strong genetic regulatory elements, such as enhancers or silencers, proximal to the integration site of the reporter gene has to be taken into account. Positional effects as well as numbers of integrated copies of the selection cassette may have an influence on the outcome of the selection process. Finally DNA modifications, such as methylation or histone acetylation, may interfere with the access of regulatory transcription factors to the selection gene. All these phenomena are influencing the precise regulation of the reporter gene and explain certain variabilities sometimes observed in-between different cell clones isolated using the same construct under identical culture conditions. Therefore, it is mandatory to screen and characterize several clones to choose those which provide optimal results.
4. The protein product used for cell selection may display toxicity for the bioengineered cell. This point has to be taken in account specifically when using EGFP as a reporter gene and it may be necessary to verify the potential toxic effects of the persistent EGFP expression. Additionally it has to be considered if the reporter protein is inducing phenotypic changes that could affect physiologic cell function. In any case, gating technology based on EGFP expression has been successfully applied and toxic effects have not been described so far (Müller et al., 2000; Kolossov et al., 1998).

Aside from these considerations, gating technology has the clear potential to be broadly exploited by further improving on those individual aspects. This technology has been mainly applied to ESC so far, although it can also be used in other systems combined with viral vectors for cell tracking. This will allow *in vivo* identification and isolation of stem cells in fetal tissues or adult tissues, in transgenic animals or in human tissues (Keyoung et al., 2001).

The combination of different selection and detection systems is another great advantage of this technology. In this context, resistance to neomycin, hygromycin and mitomycin (Baumann et al., 2002) can be combined to achieve different goals during a cell selection protocol. In the same line, the use of different fluorescent proteins (green and yellow fluorescent proteins) can be exploited in order to sort several cell types derived from a same precursor by FACS.

In addition, the gating technology can be combined with inducible expression systems, in order to induce reporter gene expression during a specific time window of the *in vitro* differentiation protocol. This interesting strategy has been developed to obtain insulin secreting cells by using an insulinoma β -cell line (β TC), obtained from transgenic animals expressing the SV40 T antigen, under the control of the insulin promoter (Efrat et al., 1995). The uncontrolled proliferation of these transformed cells is under the control of the tetracycline regulated system (Gossen and Bujard, 1992).

Finally, biosafety genes can be added to the constructs in order to eliminate the cell implant in case of malfunction, once the bioengineered cells are transplanted. One of these safety genes is the herpes simplex virus thymidine kinase gene. Thymidine kinase is sensible to pharmacological agents such as ganciclovir or aciclovir, resulting in *in vivo* cell death as it has been demonstrated in gene therapy protocols (Culver and Blaese, 1994). Alternatively the inducible Cre recombinase system can be combined with the gating technology. For example, different elements of the selection construct can be flanked by loxP sites and thus excised after they become dispensable. This may allow the removal of the DNA construct after selection of the specific cell type (Fuhrmann-Benzakein et al., 2000).

Final conclusions

In conclusion, gating technology has a high potential for ESC bioengineering. Future improvements in applying the technology to multiple different cell lineages will probably result in its broad application. However, several key points have to be considered in order to efficiently apply this strategy to select one lineage or cell type from other lineages. Cell type specific expression of the gene driving the selection marker needs to be tightly controlled to prevent impurities in the final cell population.

Finally, the future development of *in vitro* maturation protocols and the identification of marker genes for different types of progenitor lineages will synergize with the concept of gating technology. Although this is not a trivial task, a better understanding of mechanisms driving and determining differentiation of cells will help to more efficiently generate specific types of cells from stem cells *in vitro*.

As of today, use of gating technology based on marker gene-driven selection, is the most efficient

way to obtain pure populations of cells and to efficiently eliminate unwanted cells from mixtures of differentiating cells.

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