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Diabetes mellitus: new therapeutic approaches to treat an old disease

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ABSTRACT

Diabetes mellitus is a widespread disease whose frequency increases constantly and is expected to reach alarming levels by the year 2025. Introduction of insulin therapy represented a major breakthrough; however, a very strict regimen is required to maintain blood glucose levels within the normal range and to prevent or postpone chronic complications associated with this disease. Frequent hyper- and hypoglycemia seriously affect the quality of life of these patients. Reversion of this situation can only be achieved through whole organ (pancreas) transplant or pancreatic islet transplant, the former being a high-risk surgical procedure, while the latter is a much simpler and may be accomplished in only 20-40 min. The advantages and perspectives of islet cell transplantation will be discussed, in the light of tissue engineering and gene therapy. Ongoing research carried out in our laboratory, aimed at developing clinical cell and molecular therapy protocols for diabetes will also be focused.
Keywords: Diabetes mellitus, cell and molecular therapy, human pancreatic islets, degenerative diseases, recombinant biopharmaceuticals.

RESUMO

Diabetes mellitus: novas abordagens terapêuticas para uma doença antiga

O diabetes melito é uma doença de ocorrência mundial, com incidência crescente, com níveis alarmantes sendo esperados para o ano 2025. A introdução da terapia insulínica representou uma verdadeira revolução; entretanto, para manter os níveis sangüíneos de glicose dentro da faixa de normalidade e evitar ou adiar as complicações crônicas associadas com esta doença, é necessário um controle extremamente rígido. Hiper- e hipoglicemias freqüentes afetam seriamente a qualidade de vida destes pacientes. O transplante de pâncreas (órgão total) e o transplante de ilhotas permitem reverter esta situação, porém o primeiro se constitui num procedimento cirúrgico de alto risco, enquanto o segundo pode ser executado num procedimento mais simples que dura 20-40min. As vantagens e perspectivas do transplante celular de ilhotas serão discutidas, à luz da engenharia de tecidos e da terapia gênica. Pesquisas em andamento em nosso laboratório, visando o desenvolvimento de protocolos clínicos de terapia celular e molecular para o diabetes serão também focalizadas.
Palavras-chave: Diabetes mellitus, terapia celular e molecular, ilhotas pancreáticas humanas; doenças degenerativas, biofármacos recombinantes.

INTRODUCTION

Diabetes mellitus is a disease characterized by a slow course, but with very pervasive pleiotropic effects, as a consequence of the high blood glucose levels (hyperglycemia) caused by absolute or relative impairment of insulin secretion from the pancreatic β cell and/or resistance to insulin-mediated glucose availability in muscle, liver, and adipose tissues.

Two main types of diabetes are identified, namely: a) type I diabetes (T1DM), characterized by an autoimmune-mediated destruction of the β cells responsible for insulin synthesis and secretion in the pancreatic islets of Langerhans, affecting children, youngsters and even adults; b) type II diabetes (T2DM), which affects mostly middle-aged and older people, is caused by insulin resistance of the target tissues, which, at later stages, results in β-cells that are no longer able to compensate for insulin resistance by adequately increasing insulin production. Consequently, impaired glucose tolerance ensues and may evolve into overt diabetes. Insulin resistance, impaired glucose tolerance and overt diabetes are associated with increased risk of cardiovascular disease. Since these conditions are also accompanied by oxidative stress, this pathogenic mechanism is currently considered to be involved in the link between insulin resistance and dysfunction of β-cells and the endothelium, which are both related to overt diabetes and cardiovascular diseases (Ceriello & Motz, 2004). The high incidence of this disease in the world (around 170 million affected people) has been steadily growing in the past few years, and an alarming 300 million affected people are projected by the year 2025 (WHO 2000). This increase is likely to occur mostly in underdeveloped countries, due to population increase, aging, obesity, dietary factors and sedentary habits (Rodriguez et al., 2002). The number of diabetic patients in Brazil is estimated by the IDF (International Diabetes Federation) at more than 10 million T2DM and almost 200,000 T1DM patients. Around 2% of the T1DM patients present brittle diabetes, with life-threatening episodes of hypoglycemia and coma.

The association of hyperglycemia with the pancreas was first made in 1889 by von Mering and Minkowski, but

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the final proof only came with the elegant work of Banting and Best (Banting & Best, 1990; Banting et al., 1991). Hyperglycemia leads to important changes in the body metabolism, partly due to abnormal glycation (a non-enzymatic reaction that occurs in two steps, with a ketoamine as the final product) of body proteins, including the circulating oxygen carrier hemoglobin, leading to severe circulatory problems, increased cardiovascular risk, renal failure and retinopathy.

Insulin administration is the standard treatment for T1DM and also for some T2DM diabetic patients. However, in order to maintain the glycemic levels within a normal (80-100 mg/dL) range, great effort is demanded from the patients, who normally have a hard time bearing the constant pricking of their fingers to monitor blood-sugar levels and of their skin to inject multiple daily insulin doses.

More recently, some alternatives to insulin therapy have become available, namely: whole organ (pancreas) transplantation and islet cell transplantation. The former represents a major surgical intervention, carried out under general anesthesia, which usually involves an 8-10h procedure and at least two weeks of post-operative care. On the other hand, islet cell transplantation is similar to a blood transfusion, involving a 20-40min procedure, which can be carried out under mild sedation, the patient being discharged after 1–2 days of hospitalization. However, since these procedures, with rare exceptions, involve allotransplantation, both of them require immunosuppression therapy of the patient, who will exchange insulin injections for oral or injected immunosuppressive drugs.

These alternatives would be very attractive if it were not for the fact that the immunosuppressive drugs available are not free of secondary effects. In the past, the most widely used drugs were glucocorticoids (cortisone), which themselves are diabetogenic. Luckily, great progress has recently been made in the development of new and powerful immunosuppressive drugs with fewer side effects. In addition, last-generation anti-TNF (tumor necrosis factor) monoclonal antibodies are being evaluated. Furthermore, some hope comes from the new field of Nanotechnology, which has led to attempts to shield human islets from the immune response by encapsulating them in nanospheres of biocompatible materials specially designed for this purpose (Mares-Guia, 2001; Mares-Guia & Ricordi, 2001; Fraker et al., 2003; Mares-Guia & Ricordi, 2004). We also have been dedicating a great deal of effort towards this goal (Oliveira et al., 2004; ACC Vale & MC Sogayar, unpublished results).

Worldwide organ shortage is a major obstacle to both pancreas and islet transplantation, restricting their application to a very small percentage of potential recipients. One way to circumvent this problem would be the *ex-vivo* expansion of the islet cells.

Highly-differentiated insulin-producing β cells would not be expected to display significant proliferative potential and thus, for many years, no emphasis was placed on islet cell culturing. More recently, we (Maria-Engler et al., 2004; Labriola et al., 2005a), and others (Dor et al., 2004), have shown that these islet cells not only proliferate quite vigorously, for at least three months, but they also retain the capacity to synthesize and secrete insulin during the first few weeks in culture.

Attempts to characterize the nature and function of these islet cell cultures have been made, with the aim of evaluating their possible use, in the future, in clinical transplants.

Isolation and purification of human islets

In Brazil, a centralized system is responsible for pancreas procurement from organ donors. A strict set of rules dictates the destination of each organ, with Federal and local Transplant Centers directing the organization and distribution, according to previously established criteria.

The surgical team is specially trained to deal with this very delicate organ, which can undergo acute pancreatitis when subjected to manipulation. Therefore, all procedures are carried out in the cold, with extreme care.

Pancreas storage and transportation are carried out according to the two-layer method, which involves submerging the organ in a biphasic mixture of University of Wisconsin/Belzer transplant solution and oxygenated PFC (Perfluorocarbon), which has been shown to preserve islet structure, survival and function (Lakey et al., 2003; Matsumoto & Kuroda, 2002; Matsumoto et al., 2002). The organ is transported in ice from the hospital to the laboratory for processing. This period of cold ischemia should be kept to a minimum, since it inversely correlates with islet quality and yield.

In our Cell and Molecular Biology Laboratory (Human Pancreatic Islet Unit and NUCEL-Núcleo de Terapia Celular e Molecular - http://www.nucel.prp.usp.br), the pancreas is first perfused with a special blend of collagenase enzymes (Liberase, Roche Diagnostic, Indianapolis, IN) through the Wirsung duct, which spans the whole organ, in order to dissociate the tissue. It is believed that the pancreas actually comprises two different organs, namely: the exocrine pancreas, composed of the acinar tissue, which is responsible for synthesis and secretion of numerous digestive enzymes, and the endocrine tissue, represented by the islets of Langerhans, which constitute only 1-2% of the pancreatic tissue, but are responsible for synthesis and secretion of a number of hormones, such as insulin, glucagon, somatostatin and pancreatic polypeptide, that control carbohydrate metabolism and other physiological processes.

After collagenase perfusion, the pancreas is sliced and further digested in a special chamber (Ricordi´s chamber), generating a crude pancreas digest containing free-floating and tissue-embedded islets together with exocrine tissue and enzymes. The next step involves separating the free-floating islets from the rest of the

digested tissue, by centrifugation through a Ficoll density gradient, which is accomplished by an automated method (Ricordi *et al.*, 1988; Ricordi, 1992), generating a purified islet preparation (Figure 1).

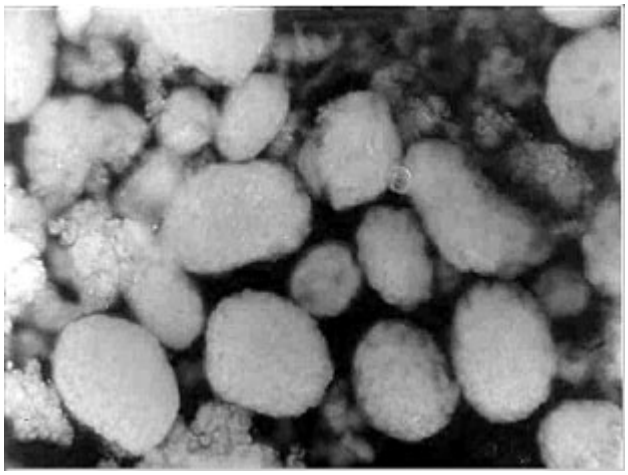

FIGURE 1 - Purified human islet preparation under dark-field microscopy.

The islet-rich fractions are collected and after rapidly washing off the toxic Ficoll, by centrifugation, the purified islets (Figure 2) are seeded into flasks with untreated surface for the first 24h and then into two different kinds of plastic flasks, namely: specially treated tissue culture flasks, to generate adherent cultures, and flasks with untreated surface, to produce islet suspension cultures.

FIGURE 2 - Pellet of purified human islets.

A number of quality control assays are routinely performed for each islet preparation. Cell viability is assessed by examining the material under a fluorescence microscope after double-staining it with acridine orange (that stains live cells in green) and propidium iodide (that stains dead cells in red). β-cell function is evaluated by insulin secretion upon glucose stimulation; cells are considered to have a good physiological response when the amount of insulin secreted in stimulated cultures is more than twice that in non stimulated cultures. Animal models are used to evaluate the quality of human islet preparations by reversion of streptozotocin-induced diabetes (Oliveira, 2004). For clinical transplantation, additional assays are used, namely: complete bacteriological and endotoxin tests and donor-receptor cross-matching.

Clinical islet transplantation

As the first team in Brazil to introduce islet transplantation as an alternative treatment for type I diabetic patients (Eliaschewitz et al., 2004), we generated a detailed protocol and submitted it to the appropriate authorities (local and federal Ethics Committees) for approval. The meticulous choice of patient inclusion and exclusion criteria was based on previously published reports (Lakey et al., 2003; Shapiro et al., 2000). Selection involves follow-up of patients for at least four months and further approval by two endocrinologists, to ensure the labile nature of the disease and the patients' eligibility based on clinical parameters.

Upon signature of the informed consent, the patient is directed to the waiting list until an appropriate donor appears.

When a high quality islet preparation becomes available, the indicated patient is hospitalized for preparation and immunosuppression and subjected to mild anesthesia. The islets are transferred to a transfusion bag (Figure 3). Highly trained radiologists introduce a needle into the liver portal vein, guided by ultrasound, radioscopy and portography (Figure 4). A catheter is then introduced into the needle and the islet suspension is introduced into the liver through this catheter, under gravity. After this procedure, which takes from 20 to 40 minutes, the patient is maintained in the hospital for 1 or 2 days and then discharged. Insulin is administered after islet transplantation in order to avoid occasional islet death/ failure due to toxic high glucose levels during islet engraftment (Figure 5), but it is slowly weaned after a period of a few months.

FIGURE 3 - Purified human islets in a blood transfusion bag during islet infusion into a patient.

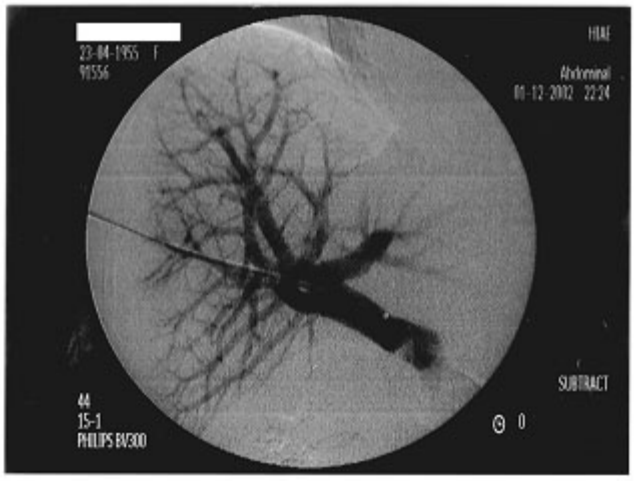

FIGURE 4 - Portography showing the needle introduced into one of the main branches of the portal vein, immediately before islet infusion.

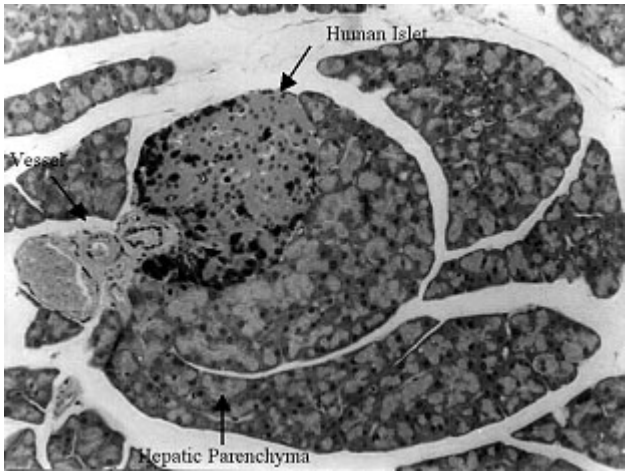

FIGURE 5 - Human islet engraftment into a rat liver biopsy.

The success of the intervention may be assessed by the decrease in insulin requirement and, also, by the levels of C peptide produced by the patient. Typically, the first islet infusion is likely to significantly benefit, but not to completely free the patient from the insulin requirement. Therefore, additional islet infusions may be necessary and should be administered after a minimum period of three weeks.

When the yield and/or quality is not appropriate for clinical transplantation, the islets are seeded to generate either suspension or adherent islet cultures, using appropriate culture media containing nutrients and complements such as serum and growth factors.

Stem cells and islet cell proliferation/differentiation

Tissue engineering is an emergent field in the therapy of degenerative diseases such as diabetes, Parkinson´s disease, Alzheimer, muscular dystrophy and cardiopathies, among others. The central players in bioengineering are precursor or specialized cells, which are capable of differentiating into the target-tissue, upon homing into proper carriers/scaffolds and being stimulated with peptide growth factors. The possibility of obtaining precursor cells from the patient to be treated, eliminating the risk of an immune-mediated rejection, and proliferation of these cells *ex-vivo*, constitutes a very attractive alternative to conventional therapy and to whole organ transplants. In this context, stem cells play a major role in view of their ability to differentiate into different types of tissue.

The existence of multipotent stem cells in adult tissues, which seemed elusive in the past, has become widely accepted (Ramiya et al., 2000; Weissman, 2000). Nestin was first described as a marker for brain stem cells (Dahlstand et al., 1992; Lendahl et al., 1990), but subsequently, this protein was also demonstrated in the pancreas (Trucco, 2005; Zulewski et al., 2001; Hunziker & Stein, 2000). We were able to show (Maria-Engler et al., 2004) that nestin-positive cells are present both in freshly isolated islets and in islet cultures, from their onset (in a low proportion) up to the time when these cultures senesce (a vast majority) and die (after 100 days). On the other hand, insulin-positive cells could be observed only in freshly isolated islets and in short-term (up to 3-4 weeks) cultures. However, by adjusting the medium composition, insulin production could be demonstrated in long-term cultures. Taken together, these data are highly suggestive that nestin-positive cells may give rise to insulin-positive cells and, moreover, that insulin-positive cells may generate other insulin-positive cells, pointing to new avenues to expand the mass of b-cell tissue for clinical transplantation (Maria-Engler et al., 2004).

Human mesenchymal cells from the umbilical cord vein and murine embryonic (ES) cells (Figure 6) are being used in our laboratory to elucidate the molecular basis for b-cell differentiation, using previously published (Broxmeyr et al., 1989; Broxmeyer et al., 1992; Zandstra et al., 2000; Cutler & Antin, 2001; Lumelsky et al., 2001) and modified differentiation protocols, followed by DNA microarray analysis (Lojudice et al., 2005).

Another approach to overcome the limited donor site supply is the use of gene therapy to engineer pancreatic islets (Kojima et al., 2003; Bretzel et al., 2004). In spite of

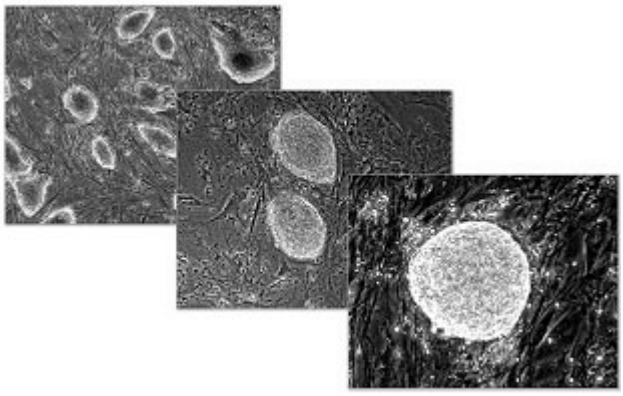

FIGURE 6 - Murine ES cells embryoid bodies.

the controversy surrrounding gene therapy, utilization of autologous cells or tissues represents a great advantage, eliminating the risk of rejection or transmission of infectious diseases. In the case of diabetes, potential targets for ectopic expression of the insulin/pro-insulin gene are gut, liver and muscle cells (Kojima et al., 2003; Riu et al., 2002).

Characterization of human islet and insulinoma cell cultures

Human islets are systematically maintained in adherent cultures for short (weeks) and long (months) periods of time. Using confocal microscopy and RT-PCR, we characterized several short and long-term cultures of different islet preparations (Maria-Engler et al., 2004). The fact that these cultures displayed a high proliferating potential prompted us to investigate the nature of these cells. We were able to demonstrate insulin-producing human islet cultures stained with dithizone, which reveals β cells by binding to the zinc ions present in the insulin granules.

Insulinomas are the commonest pancreatic endocrine neoplasms, comprising around 17% of all neuroendocrine tumors. Both benign (adenoma) and malignant (carcinoma) insulinomas cause hypoglycemia, as a consequence of insulin overproduction by the transformed β cells and/or defective insulin storage and secretion. Murine insulinoma cell lines, such as HIT-T15, INS-1E and RIN-m5F, have been generated (Santerre et al., 1981; Praz et al., 1983; Asfari et al., 1992) and used as model systems to study β-cell proliferation, differentiation and function. Unfortunately, previous attempts to prepare and stably propagate human β-cell lines have met with only limited success (Macfarlane et al., 1997; Macfarlane et al., 1999; de la Tour et al., 2001). Therefore, we collected fresh samples of human insulinoma tissue in collaboration with other investigators, and were able to generate cultures that can be maintained for long periods of time and which constitute the first available *in vitro* model of human insulinoma cells (Krogh, 2005).

Cultures of insulinomas derived from different patients have been maintained, stored in liquid nitrogen and used to investigate the molecular basis of human β-cell growth and differentiation, using the genomic (Krogh, 2005) and the proteomic (Labriola et al., 2005b) approaches.

Islet cell signaling and the extracellular matrix

Availability of normal human islet cell cultures prompted studies of mitogenic signaling by molecular, genomic and proteomic approaches.

Even though prolactin is a known mitogenic agent for β cells during pregnancy and the perinatal period (Fleenor et al., 2000), the mechanism of action of this hormone in human pancreatic islets is still not fully understood.

Recombinant human prolactin (rhPRL), produced in our laboratory with the baculovirus/insect-cell heterologous system (Pereira et al., 2001), was used to investigate the effects of this hormone on islet cell proliferation and function, in combination with extracellular matrix components, such as laminin (Labriola et al., 2005a). In addition to confirming the mitogenic action of PRL on human β cells, our results indicate that laminin has no significant effects on cell proliferation, but is highly effective in promoting glucose-induced insulin secretion, when used in combination with rhPRL. These results point to the possible use of PRL and laminin as supplements, to increase the β-cell mass by *ex-vivo* culture prior to transplantation.

Attempts to influence human β-cell differentiation, by culturing these cells on plates coated with Matrigel, a mixture of extracellular matrix components, revealed that, in fact, islet cells tend to adhere to each other, forming islet-like clusters, resulting in increased cumulative insulin secretion (Maria-Engler et al., 2004). In collaboration with other investigators, we are analyzing the proteoglycans composition of human pancreas and human islets, in order to mimic, in culture, the islet microenvironment (VA Fortuna, DM Cobayashi, HB Nader & MC Sogayar, unpublished results).

A proteomic approach has been taken to analyzing the protein profiles of islet cultures upon treatment with rhPRL, in the hope of discovering the molecular mechanisms involved in PRL signaling of human β cells. Bidimensional gel electrophoresis of protein extracts obtained from various islet cell preparations, in the absence and in the presence of rhPRL, allowed identification of several differentially expressed proteins (Labriola et al., 2005b). Peptide mass finger-printing and mass spectrometry is being used to identify these proteins and to gain insights into the molecular intracellular circuitry that leads to β-cell signaling .

The observation that injection of scorpion toxins from *Titius serrulatus* and *Titius bahiensis* caused an increased frequency of β cells in the pancreas, a phenomenon named nesidioblastosis (Novaes et al., 1990), prompted us to exploit the properties of these venoms and their possible proliferative effects on β cells. Results from our laboratory indicate that both murine β-cell lines and human islet cultures respond to crude venom extracts with increased cell proliferation (Luca et al., 2003; ACV Campos & MC Sogayar, unpublished results), opening new avenues for *ex-vivo* islet cell mass expansion prior to transplantation. The molecular nature of the active fraction is currently under intensive investigation in our laboratory (ACV Campos & MC Sogayar, unpublished results).

The future of islet cell transplantation

Several challenges lie ahead, among which are the: a) design of suitable culture conditions (mitogenic factors,

extracellular matrix components) to allow production of a sufficient mass of islet cells and their organization into an appropriate architecture, in order to ensure β-cell function; b) development and management of immunosuppressive drugs with minimal or no side effects; c) development of biocompatible materials for islet encapsulation, with specific properties related to resistance and permeability.

When these challenges are met, islet transplantation may replace whole organ pancreas transplants and may be extended not only to labile/brittle T1DM patients, but also to regular T1DM and T2DM patients.

Facing the challenges

As mentioned above, a major problem in diabetes is the control of β-cell growth and differentiation.

Researchers adopting the Cell Biology approach have already highlighted the role of the pancreatic microenvironment, islet architecture and extracellular matrix components in β-cell structure and function, emphasizing the importance of Tissue Engineering in islet transplantation. Perspectives for novel β-cell mitogens, represented by scorpion toxins, are encouraging efforts towards venom fractionation and characterization (Luca et al., 2003).

A genomic approach has been taken to probe into the genes involved in β-cell growth control and neoplasia (Krogh, 2005; Sa, 2005), and differentiation from precursor stem cells (Lojudice et al., 2005). Using suppressive subtractive hybridization and DNA microarrays, a number of genes have been isolated and characterized, some of which are well-known genes, while others are ESTs or full genes to which no known function has been attributed.

Proteomics is likely to reveal important components of the signaling pathways, involving PRL and other growth factors of human β cells. Therefore, we have adopted this strategy to address the issue of β-cell proliferation (Labriola et al, 2005b).

More definitive answers will also depend on a functional genomics approach, in which the gene is either ectopically overexpressed by transgenesis, or is silenced by small interfering RNAs (siRNAs) or antisense constructs. This type of research requires well-trained cell and molecular biologists and substantial financial support.

Concluding remarks

At the outset of this new century, the greatest challenge is to apply the basic knowledge generated by cell and molecular biologists to develop alternative therapies for degenerative and genetically-transmitted diseases. Tissue engineering integrates and connects professionals from a wide range of different areas, in the search for innovative and viable solutions. Translational Medicine promotes a two-way flow between the patient bed and the laboratory bench, with multidisciplinary approaches being likely to open new avenues in the treatment of these and other diseases. Public policies that ensure adequate support and training of human resources are crucial for scientific and technological progress in these areas.

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